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Acinus-provoked protein kinase C δ isoform activation is essential for apoptotic chromatin condensation

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Histone H2B phosphorylation tightly correlates with chromatin condensation during apoptosis. The caspase-cleaved acinus (apoptotic chromatin condensation inducer in the nucleus) provokes chromatin condensation in the nucleus, but the molecular mechanism accounting for this effect remains elusive. Here, we report that the active acinus p17 fragment initiates H2B phosphorylation and chromatin condensation by activating protein kinase C δ isoform (PKC-δ). We show that p17 binds to both Mst1 and PKC-δ, which is upregulated by apoptotic stimuli, enhancing their kinase activities. Acinus mutant susceptible to degradation elicits stronger chromatin condensation and higher H2B phosphorylation than wild-type acinus. Dominant-negative PKC-δ but not Mst1 robustly blocks acinus-initiated H2B phosphorylation. Surprisingly, depletion of Mst1 triggers caspase-3 activation, provoking H2B phosphorylation through activating PKC-δ. Further, acinus-elicited H2B phosphorylation and chromatin condensation are abrogated in PKC-δ-deficient mouse embryonic fibroblast cells and siRNA-knocked down PC12 cells. Thus, PKC-δ but not Mst1 acts as a physiological downstream kinase of acinus in promoting H2B phosphorylation and chromatin condensation.
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Chromatin condensation, a hallmark of apoptosis, is associated with histone H2B phosphorylation. Phosphorylation of H2B is usually negligible in both quiescent and growth states, but it is markedly stimulated when chromatin condensation and nucleosomal DNA fragmentation is initiated.[1] Allis and coworkers demonstrated that phosphorylation of H2B at serine 14 by caspase-cleaved Mst1 tightly correlates with cells undergoing programmed cell death.[2] Histone 2B serine 14 (H2BS14) phosphorylation has also been consistently accumulated at DNA double-strand breaks, indicative of its important role in apoptosis.[3] Chromatin condensation can be induced by a nuclear protein called acinus (apoptotic chromatin condensation inducer in the nucleus), which is cleaved during apoptosis.[4] Acinus is cleaved by caspases on both its N- and C-termini, producing a p17 active form (amino acids (a.a.) 987–1093 in –L isoform), which elicits chromatin condensation in the absence of caspase-3. Recently, we showed that Akt phosphorylates acinus on serine 422 and 573, resulting in its resistance to caspase cleavage in the nucleus and the inhibition of acinus-dependent chromatin condensation. Thus, Akt inhibits chromatin condensation during apoptosis by phosphorylating acinus in the nucleus, revealing a specific mechanism by which nuclear Akt promotes cell survival.[5] However, the molecular mechanism as to how p17 couples to chromatin condensation remains elusive.

MST1 (mammalian Ste20-like kinase 1) is ubiquitously expressed. MST1 contains a Ste20-related kinase catalytic domain in the amino-terminal segment (a.a. 30–270), followed by a noncatalytic tail that contains successively an autoinhibitory domain (a.a. 331–394), a dimerization domain (after a.a. 431), and a nuclear localization signal at the COOH terminus.[6–8] MST1 is predominantly cytoplasmic but cycles continuously through the nucleus. Mst1 is activated and cleaved by a broad range of stimuli in various cell types, which suggests that it is a common component in the diverse signaling pathways leading to apoptosis.[9] Overexpression of Mst1 in some cell types induces apoptotic features, which is blocked by kinase-dead Mst1 mutant.[10–14] JNK has been suggested as a central player for this apoptotic action, although opposite observations have also been reported.[15–17] Mst1 contains two caspase cutting sites and is cleaved by caspases to generate catalytically active enzymes of 36 and 40 kDa. Mst1 cleavage by caspase-3 results in the translocation of Mst1 into the nucleus, where it promotes chromatin condensation and nuclear fragmentation.[11,12,18] On the other hand, exogenously expressed Mst1 triggers caspase-3 activation. Thus, Mst1 may function both upstream and downstream of caspases.

Protein kinase C δ isoform (PKC-δ) is a mobile enzyme, and its intracellular distribution is altered upon enzyme activation. After activation, full-length PKC-δ translocates from the cytoplasm to the plasma membrane and mitochondria. Moreover, cytosine arabinoside (Ara-C) and etoposide induce nuclear translocation of PKC-δ.[19] PKC-δ is a substrate for caspase-3, which cleaves PKC-δ at the DMQD330N site in the

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Abbreviations: Acinus, apoptotic chromatin condensation inducer in the nucleus; DNA-PK, DNA-dependent protein kinase; Eto/STS, etoposide/staurosporine; H2BS14, histone 2B serine 14; MEF, mouse embryonic fibroblast; MST1, mammalian Ste20-like kinase 1; PKC-δ, protein kinase C δ isoform
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hinge region, generating a 40-kDa C-terminal fragment. Nevertheless, PKC-δ also acts upstream of caspase-3 to regulate its activation.[20,21] The feedback loop between PKC-δ and caspase-3 mediates their cleavage and amplifies apoptosis in response to apoptotic stimuli. PKC-δ facilitates apoptosis by phosphorylating multiple cellular proteins implicated in apoptosis at several subcellular locations. For instance, in the nucleus, PKC-δ phosphorylates lamin B, mediating its degradation.[22] PKC-δ can also phosphorylate and inactivate DNA-dependent protein kinase (DNA-PK), an important effector in DNA repair, attributing to cell death.[23] Moreover, PKC-δ has been proposed to phosphorylate histone H2B at serine 14 and mediate B-cell apoptosis.[24] Both Mst1 and PKC-δ reveal similar effect on caspase-3 activation and H2B phosphorylation, but whether they are both implicated in acinus-provoked chromatin condensation remains obscure.

Here we show that p17 strongly binds to both Mst1 and PKC-δ, and escalates H2B phosphorylation. However, only dominant-negative PKC-δ but not Mst1 blocks acinus-initiated H2B phosphorylation. Surprisingly, depletion of Mst1 does not diminish H2B phosphorylation; instead, it robustly enhances H2B phosphorylation at serine 14, probably through activating PKC-δ by triggering caspase-3 proteolytic degradation. Nevertheless, knockout of PKC-δ abrogates p17-initiated H2B phosphorylation and chromatin condensation regardless of Mst1 activation. Collectively, these findings demonstrate that PKC-δ but not Mst1 is essential for mediating H2B phosphorylation during apoptosis.

Results

The active acinus p17 form interacts with both Mst1 and PKC-δ apoptotic kinases. The caspase-3-cleaved active p17 fragment of acinus (a.a. 228–335) elicits chromatin condensation.[4] To search for its downstream effectors, we prepared GST-recombinant protein of p17 and incubated it with lysates from PC12 cells, treated with or without staurosporine. P17 bound proteins were resolved on SDS-PAGE. The proteins from PC12 cells selectively associated with p17 were characterized by proteomic analysis. Numerous proteins were identified: Hsp90 (heat shock cognate 90 kDa protein), PKC-δ, Hsp70, Mst1, Annexin A2, M2-pyruvate kinase, carbonyl reductase (Figure 1a). Amongst all p17-bound targets, Mst1 and PKC-δ have been mainly implicated in apoptosis. Immunoblotting analysis demonstrated that both Mst1 and PKC-δ selectively bound to p17 (Figure 1b). Therefore, acinus p17 binds to Mst1 and PKC-δ apoptotic kinases *in vitro*. A diagram of various acinus, PKC-δ, and Mst1 apoptotic fragments is depicted in Figure 1c.

Apoptotic stress stimulates acinus to interact with both Mst1 and PKC-δ. To examine whether apoptotic stress or growth factor regulates the association between acinus and Mst1 and PKC-δ, we cotransfected Myc-tagged Mst1 into HEK293 cells with various GST-acinus constructs, and treated the transfected cells with staurosporine or EGF. Compared to its C-terminal fragment (a.a. 228–583), both full-length acinus and p17 strongly bound to Mst1, which was decreased upon EGF stimulation. Strikingly, staurosporine evidently increased the interaction, indicating that apoptotic stimuli upregulate the interaction between acinus and Mst1 (Figure 2a, top panel). We conducted similar binding assay with GFP-PKC-δ. Compared to the robust binding by the C-terminal segment of acinus (a.a. 228–583) to PKC-δ, full-length acinus displayed a decreased affinity, which was not evidently affected by staurosporine or EGF. Interestingly, p17 interacted prominently with PKC-δ regardless of apoptotic stimulus or growth factor treatment (Figure 2b).

Our previous study reveals that acinus is essential for chromatin condensation in neuronal PC12 cells.[5] We wonder whether these interactions also occur with endogenous proteins in neuronal cells. Mst1 resides mostly in the cytoplasm, though it cycles between the nucleus and the cytoplasm.[12,18] PKC-δ occurs mainly in the cytoplasm, but it translocates to the nucleus upon apoptotic stimulation.[25,26] To further assess the interaction between acinus and Mst1 and PKC-δ, we performed subcellular fractionation of PC12 cells, pretreated with staurosporine or NGF. Unexpectedly, compared to control or NGF stimulation, staurosporine noticeably promoted endogenous Mst1 to interact with acinus in the cytoplasm. However, acinus just weakly associated with Mst1 in the nucleus. By contrast, staurosporine elicited robust interaction between acinus and PKC-δ in the nuclear fraction. In the cytoplasmic fraction, the faint association between acinus and PKC-δ under control and staurosporine conditions was abolished upon NGF stimulation (Figure 2c, left panels). Acinus occurred primarily in the nuclear fraction, but demonstrable acinus-S was also detected in the cytoplasmic fraction, which was escalated by staurosporine. By contrast, acinus-L resided exclusively in the nucleus. Compared to control and NGF, staurosporine triggered extensive acinus degradation in both fractions (Figure 2c, upper middle panel). PKC-δ occurred in both fractions and staurosporine enhanced its nuclear distribution. Nevertheless, Mst1 was allocated mostly in the cytoplasmic fraction. Faint Mst1 and PKC-δ degradation was observed in control cells and was elevated upon staurosporine treatment (Figure 2c, right panels). The identities and purity of each fraction were verified by the specific markers: PARP and α-tubulin. PARP distributed solely in the nuclear fraction and was cleaved upon staurosporine treatment (Figure 2c, middle and right bottom panels). Taken together, these data demonstrate that acinus forms a complex with PKC-δ in the nucleus and also strongly associates with Mst1 in the cytoplasm. It is noteworthy that the active acinus p17 potently binds to both Mst1 and PKC-δ in the perinuclear region.

The active acinus p17 enhances both Mst1 and PKC-δ kinase activity. To explore whether Mst1 post-translational modification mediates its association with acinus, we cotransfected a variety of GST-acinus fragments into HEK293 cells with myc-Mst1 and pulled down acinus proteins. The beads-associated proteins were monitored with anti-myc and Mst1 phospho-specific antibodies, respectively. Full-length acinus and C-terminus of acinus (228–583) displayed comparable affinity to Mst1 upon staurosporine stimulation, but the strongest interaction

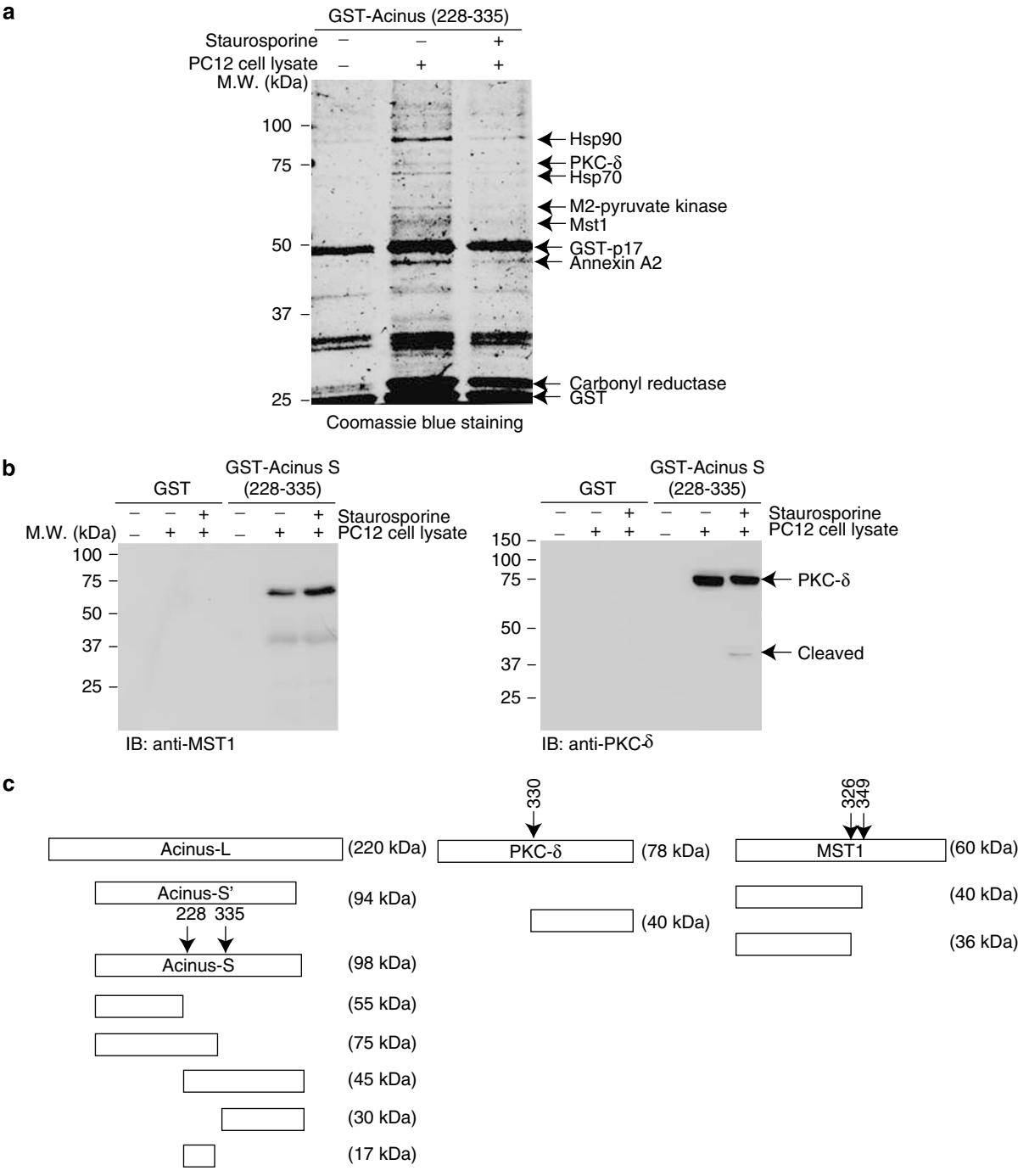


Figure 1 Acinus associates with both Mst1 and PKC-δ. (**a**) The active acinus p17 form interacts with Mst1 and PKC-δ. The GST-recombinant p17 protein was incubated with cell lysate from PC12 cells, pretreated with 250 nM staurosporine or control. After extensive wash, the beads-associated proteins were resolved on SDS-PAGE and stained with Coomassie blue. The proteins selectively bound to p17 were cut from the gel and analyzed by proteomic methods. Their identities are: Hsp90, Hsp70, PKC-δ, Mst1, Annexin A2, M2-pyruvate kinase, and carbonyl reductase. (**b**) Both Mst1 and PKC-δ strongly interact with GST-p17. (**c**) A diagram of acinus isoforms: Acinus-L, -S, and S', PKC-δ, and Mst1 apoptotic cleavage fragments. The numbers on the top of diagrams are apoptotic cleavage sites for acinus-S, PKC-δ, and Mst1

occurred to the active p17 fragment, which associated with Mst1 regardless of its phosphorylation status. The caspase-3 cleavage-resistant Mst1 (D326N) also interacted strongly with p17. Notably, p17 interacted robustly with the N-terminus of Mst1 (a.a.1–326) (Figure 3a, top left panel). Immunoblotting analysis reveals that full-length Mst1 was phosphorylated when cotransfected with p17, which was not detected when full-length or C-terminal acinus was employed. Both the N-terminus and caspase-uncleavable D326N mutant of Mst1 were also potently phosphorylated in p17 samples (Figure 3a, second left panel). In-gel kinase assay demonstrated that p17 upregulated Mst1 kinase activity on histone 2B *in vitro*, correlating with its phosphorylation status. The phosphorylation mimicking mutant T183E also strongly phosphorylated

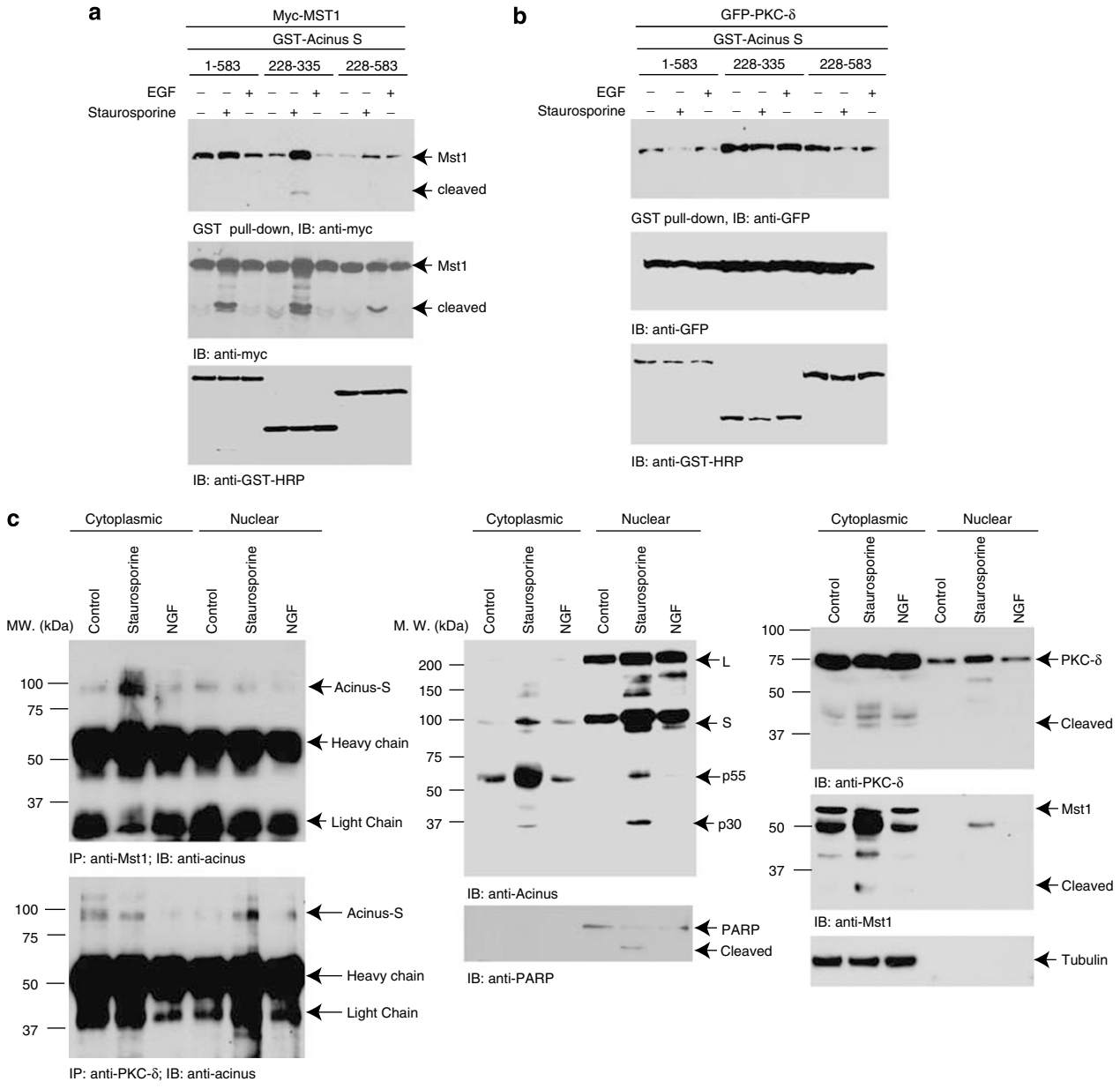


Figure 2 Apoptotic stress stimulates acinus to interact with both Mst1 and PKC-δ. (**a**) Staurosporine and EGF reveal opposite effects on Mst1 binding to acinus. Myc-Mst1 was cotransfected into HEK293 cells with various GST-Acinus fragments, followed by EGF or staurosporine stimulation. Compared to control, staurosporine strongly elevated the association between Mst1 and various acinus recombinant proteins, which was substantially decreased after EGF stimulation. Remarkably, p17 robustly bound to Mst1 in response to staurosporine treatment (upper panel). The pulled down Mst1 and acinus recombinant proteins were verified (middle and bottom panels). (**b**) PKC-δ binds to acinus p17. GFP-PKC-δ was cotransfected into HEK293 cells with various GST-acinus fragments, followed by EGF or staurosporine stimulation. Compared to control, neither EGF treatment nor staurosporine significantly affected the association between PKC-δ and various acinus fragments. Remarkably, p17 robustly bound to PKC-δ regardless of any treatment (top panel). The expression of both PKC-δ and acinus recombinant proteins was verified (middle and bottom panels). (**c**) Acinus binds Mst1 and PKC-δ in the cytoplasm and the nucleus of PC12 cells. PC12 cells were treated with NGF for 30 min or 250 nM staurosporine for 24 h, and the cytosolic and nuclear fractions were prepared. Mst1 and PKC-δ were immunoprecipitated. The co-precipitated proteins were analyzed by acinus antibody. Compared with control and NGF, staurosporine strongly promoted Mst1 binding to acinus in the cytoplasm. In the nuclear fraction, the binding of Mst1 to acinus was negligible regardless of any treatment (upper left panel). By contrast, staurosporine robustly elicited acinus to bind PKC-δ in the nuclear fraction. In the cytoplasm, acinus interacted with PKC-δ under both control and staurosporine conditions, and the complex was disrupted upon NGF stimulation (lower left panel). Acinus-S predominantly distributed in the nucleus, but it was also demonstrable in the cytoplasm, and staurosporine triggered its apoptotic cleavage (upper middle panel). PKC-δ resided in both the cytoplasm and the nucleus, and staurosporine treatment also enhanced its nuclear translocation (top right panel). Mst1 localized mainly in the cytoplasm, and staurosporine triggered both Mst1 and PKC-δ apoptotic cleavage. The identity and purity of the cytosolic and nuclear fractions were verified by the specific markers tubulin and PARP, which were selectively cleaved upon staurosporine treatment (bottom middle and right panels)

H2B *in vitro* (Figure 3a, bottom left panel). Therefore, p17 stimulates Mst1 kinase activity by binding to its N-terminal kinase domain.

To investigate the effect of p17 on PKC-δ kinase activity, we cotransfected a variety of GST-acinus fragments into HEK293 cells with GFP-PKC-δ construct. *In vitro* kinase

assay with immunoprecipitated PKC-δ demonstrated that p17 exhibited stronger effect on PKC-δ kinase activity on H2B than did full-length acinus (Figure 3b, bottom panel). The stimulatory effect by acinus correlated with its affinity to PKC-δ. GST pull-down assay revealed that PKC-δ strongly bound to p17, whereas acinus full-length and acinus fragment (a.a. 228–583) also interacted with PKC-δ; by contrast, C-terminal segment (a.a. 340–583) lacking p17 domain failed, underscoring that p17 is essential for its association with PKC-δ. As a control, PKC-ε did not bind to either full-length acinus or p17 (Figure 3b, top panel).

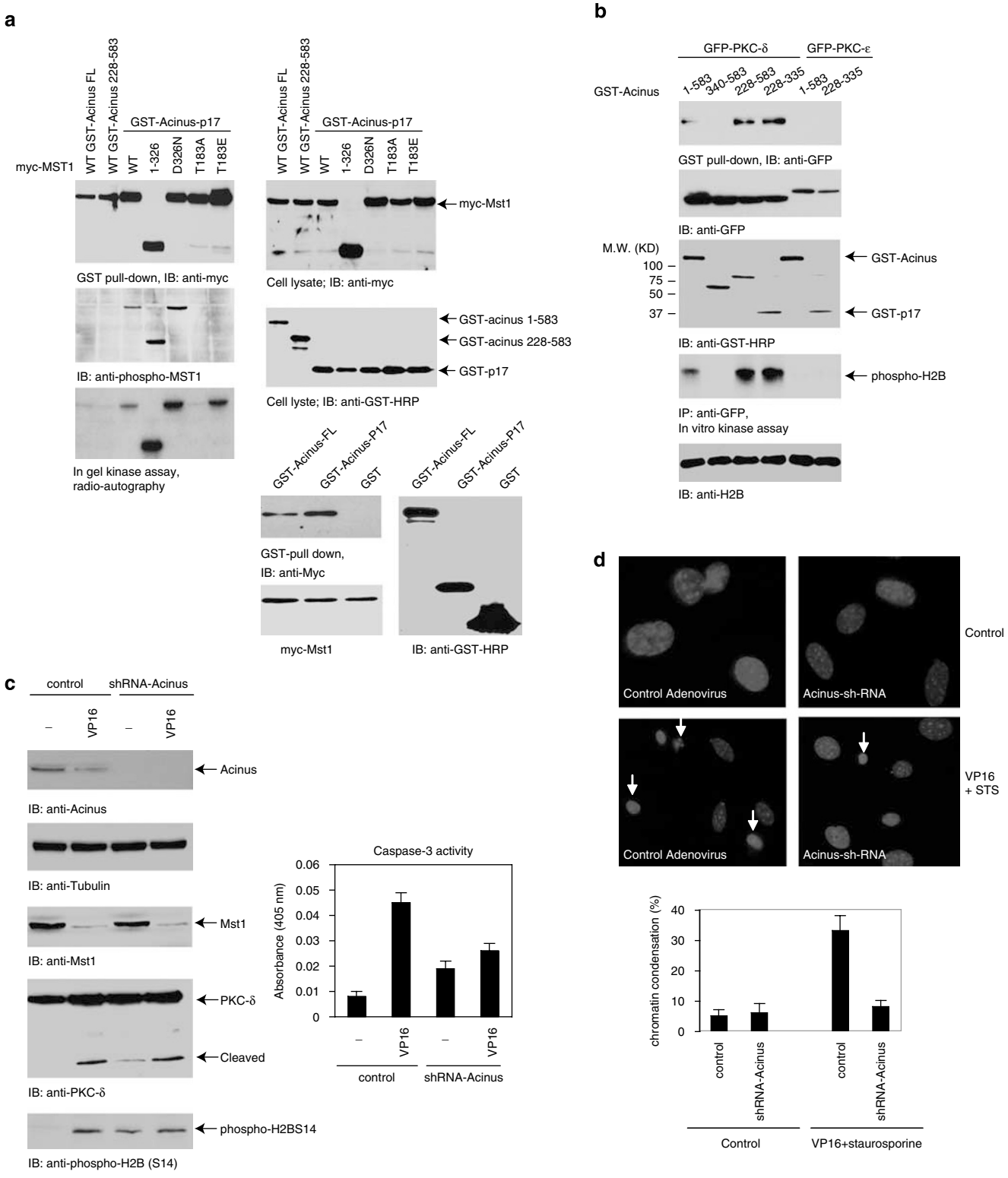
To further explore the effect of acinus on Mst1 and PKC-δ's kinase activity, we depleted acinus in PC12 cells with adenovirus expressing sh-RNA of acinus and treated the cells with VP16 (etoposide). Compared with control, acinus was obviously diminished by its shRNA, and VP16 treatment triggered its degradation (Figure 3c, top left panel). Noticeably, Mst1 was slightly decreased when acinus was depleted, and it was completely decayed upon VP16 treatment. Surprisingly, when acinus was knocked down, PKC-δ was selectively cleaved even in the absence of VP16 treatment, suggesting that depletion of acinus also incurred caspase-3 activation. VP16 elicited profound PKC-δ cleavage in both control and sh-RNA acinus adenovirus-infected cells (Figure 3c, third and fourth left panels). Remarkably, when acinus was knocked down, H2BS14 phosphorylation was detectable even in the absence of VP16 treatment, and VP16 provoked more potent H2B phosphorylation in control cells than in acinus-depleted cells (bottom left panel). Caspase-3 activity assay confirmed that depletion of acinus was sufficient to activate caspase-3; VP16 provoked more potent caspase-3 activity in control cells than acinus-knocked down cells (Figure 3c, right panel), underscoring that acinus also somehow regulates caspase-3 activity. These results demonstrate that acinus protein level modulates both Mst1 and PKC-δ kinase activities, indicating that acinus plays a critical role in activating Mst1 and PKC-δ during the apoptosis. Chromatin condensation assay demonstrates that when acinus was knocked down, chromatin condensation was concurrently reduced (Figure 3d), coupling to H2B phosphorylation activity. Therefore, these findings demonstrate that acinus selectively upregulates both Mst1 and PKC-δ kinase activities.

Dominant-negative PKC-δ but not Mst1 inhibits acinus-induced H2B phosphorylation. To establish the physiological role of Mst1 and PKC-δ in mediating H2BS14 phosphorylation, we employed wild-type and acinus S422A mutant stably transfected PC12 cells. Acinus S422A, a mutant unable to be phosphorylated by Akt, exhibits extensive degradation with demonstrable p17 after etoposide/staurosporine (Eto/STS) (100 μM etoposide, followed by 1 μM staurosporine for another 9 h) stimulation.[5] We transfected the acinus stable cells with myc-tagged, kinase-dead, dominant-negative PKC-δ (K376R), Mst1 (K59R), and catalytic domain of Mst1 (1–326, K59R), respectively, followed by Eto/STS stimulation. The transfected cells were labeled with anti-Myc and phospho-H2BS14 antibodies, and the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Quantitative analysis showed that more than 50% of acinus S422A cells were H2B positive compared to approximately 30% in wild-type cells. Transfection of kinase-deficient PKC-δ (K376R) substantially diminished H2B phosphorylation in both cells. By contrast, dominant-negative Mst1 (K59R) or its catalytic N-terminus (1–326, K59R) demonstrated modest inhibitory effect on both cells, suggesting that PKC-δ plays an essential role in mediating acinus-provoked H2BS14 phosphorylation (Figure 4a). Chromatin condensation assay revealed similar results (data not shown). To further confirm that PKC-δ is critical for this effect, we transfected wild-type and PKC-δ-null mouse embryonic fibroblast (MEF) cells with the same constructs. Compared with wild-type MEF cells, immunoblotting analysis revealed that PKC-δ was completely eliminated in $-/-$ cell. However, acinus and tubulin remained at similar levels in both cells. Interestingly, the expression levels of Mst1 appeared less in wild-type cells than in PKC-δ-null cells (Figure 4b, left panels). In wild-type cells, kinase-dead PKC-δ strongly suppressed Eto/STS-provoked H2BS14 phosphorylation, whereas kinase-deficient Mst1 and its catalytic domain failed. In PKC-δ $-/-$ cells, less than 10% cells were H2BS14 positive in PKC-δ $-/-$ cells, regardless of any plasmid transfection (Figure 4b, right panel). Taken together, these observations support that PKC-δ but not Mst1 accounts for acinus-initiated apoptotic effects.

Depletion of Mst1 provokes H2B phosphorylation and chromatin condensation through activating PKC-δ. Early work suggests that Mst1 phosphorylates H2B on serine 14 and triggers chromatin condensation.[2] To determine the role of Mst1 in mediating acinus-provoked chromatin condensation, we infected PC12 cells with control adenovirus or adenovirus expressing sh-RNA of Mst1, and treated the cells with or without VP16. Compared with control adenovirus, Mst1 was substantially eradicated by its sh-RNA (Figure 5a, top panel). As expected, both acinus and PKC-δ remained intact in the control cells, and VP16 provoked their proteolytic degradation and H2BS14 phosphorylation (lanes 1 and 3, third, fourth and fifth panels). Surprisingly, when Mst1 was depleted, acinus and PKC-δ were prominently cleaved, and H2BS14 was robustly phosphorylated even in the absence of VP16 stimulation. VP16 treatment further enhanced H2BS14 phosphorylation (lanes 2 and 4, third, fourth, and fifth panels). These results demonstrate that Mst1 is not required for H2BS14 phosphorylation in intact cells. Strikingly, when Mst1 was eliminated, caspase-3 was activated in the absence of VP16 treatment, and it was escalated upon VP16 stimulation. Caspase-3 activation pattern tightly correlated with acinus and PKC-δ apoptotic cleavage (Figure 5a, bottom panel). As both proteins are caspase-3 substrates, Mst1 depletion might initiate caspase-3 activation, triggering acinus and PKC-δ apoptotic degradation.

PKC-δ has previously been shown to phosphorylate H2B on serine 14.[24] To examine whether S14 phosphorylation in Mst1-depleted samples attributes to PKC-δ activation, we knocked down Mst1 in PC12 cells, followed by treatment with a variety of PKC-δ kinase inhibitors. Compared to control, Mst1 was substantially depleted by its sh-RNA (Figure 5b, top

panel). H2BS14 was strongly phosphorylated when Mst1 was depleted, which was robustly inhibited by PKC inhibitor but not by MEK1 inhibitor PD98059, suggesting that PKC-δ is responsible for H2B phosphorylation (Figure 5b, second panel). Moreover, PKC-δ and acinus were extensively cleaved and activated when Mst1 was eliminated, and the apoptotic degradation was not affected by the pharmacological agents (Figure 5b, third and fourth panels). To further examine whether PKC-δ is the physiological kinase contributing to H2B phosphorylation, we transfected PC12 cells with



PKC-δ and ε siRNA, and treated cells with VP16. Compared with control and PKC-ε RNAi, knocking down of PKC-δ evidently decreased its active fragment generation. Concomitantly, H2B phosphorylation is apparently diminished. Both acinus and Mst1 were degraded in response to VP16 stimulation in all samples (Figure 5c). Therefore, these findings support that PKC-δ but not Mst1 is indispensable for H2BS14 phosphorylation in apoptotic cells.

PKC-δ is required for acinus-provoked H2BS14 phosphorylation and chromatin condensation. PKC-δ nuclear translocation and H2BS14 phosphorylation were suggested to be implicated in spontaneous death of resting B cells.[24] To investigate whether p17-elicited chromatin condensation involves PKC-δ, we transfected both wild-type and knockout MEF cells with GST-p17, and stained them with phospho-H2BS14-specific antibody. Chromatin condensation was monitored with DAPI staining. P17 elicited profound H2B phosphorylation in wild-type cells but not in PKC-δ-deficient cells. The H2BS14-positive cells revealed condensed and aggregated chromatin, a hallmark of apoptosis. P17 resided in the nucleus of PKC-δ $+/+$ cells, whereas it distributed in the cytoplasm of PKC-δ $-/-$ cells (Figure 6a). Presumably, PKC-δ is implicated in tethering p17 in the perinuclear region or the nucleus. The N-terminus of Mst1 1–326 fragment possesses active kinase activity on H2B *in vitro*, and provokes chromatin condensation in some of the transfected cells.[12] However, overexpression of myc-Mst1 (1–326) failed to initiate H2B phosphorylation or chromatin condensation in PKC-δ $-/-$ cells, although modest H2B phosphorylation was observed in some of the transfected PKC-δ $+/+$ cells (Figure 6b). By contrast,

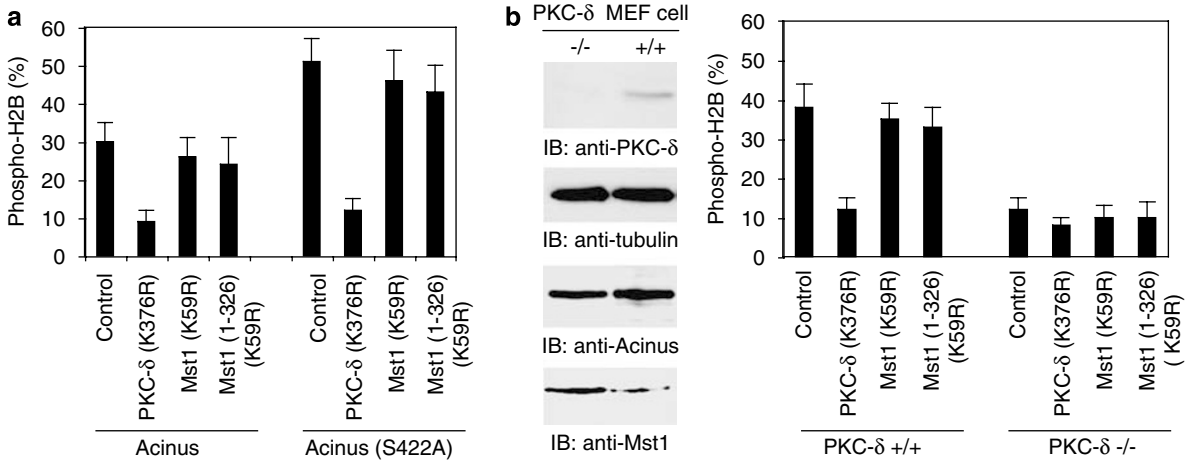


Figure 4 Dominant-negative PKC-δ but not Mst1 inhibits acinus-induced H2B phosphorylation and chromatin condensation. (**a**) PKC-δ but not Mst1 is essential for acinus-induced H2B phosphorylation. PC12 cells, stably transfected with wild-type or S422A mutant, were transfected with myc-tagged, kinase-dead PKC-δ (K376R), Mst1 (K59R), or its N-terminus (1–326, K59R). The transfected cells were treated with 100 μM etoposide, followed by 1 μM staurosporine for another 9 h (Eto/STS). The cells were fluorescently labeled with anti-myc and anti-phospho-H2BS14 antibodies. In addition, the cells were also stained with DAPI. The transfected cells were also analyzed for chromatin condensation. (**b**) PKC-δ plays a critical role for H2BS14 phosphorylation. Wild-type and PKC-δ-null MEF cells were transfected with the above constructs, followed by the same treatment. In wild-type MEF cells, kinase-dead PKC-δ substantially abolished H2BS14 phosphorylation, whereas kinase-deficient Mst1 failed; by contrast, less than 10% cells were H2BS14 positive. More than 200 transfected cells in different fields were analyzed (right panel). Numbers of cells with positive anti-phospho-H2BS14 staining were calculated as means ($\pm$S.D.) of three experiments. PKC-δ was depleted in PKC-δ knockout cells, whereas similar expression levels of acinus and tubulin occurred in both $+/+$ and $-/-$ cells. Mst1 expression was slightly decreased in $+/+$ cells than $-/-$ cells (left panels)

Figure 3 The active acinus p17 form enhances both Mst1 and PKC-δ kinase activity. (**a**) P17 of acinus binds Mst1 and stimulates its kinase activity. Various GST-acinus fragments and Myc-Mst1 were cotransfected into HEK293 cells and the GST-fusion proteins were pulled down, and its associated proteins were analyzed with anti-myc antibody. Acinus fragments containing p17 interacted with Mst1. P17 itself also interacted directly with various Mst1 proteins regardless of its phosphorylation status (top left panel). In the presence of p17, Mst1 is highly activated and phosphorylated on T183. In-gel kinase assay with H2B as a substrate confirmed these observations (middle and bottom left panels). The expression of transfected constructs was verified (top and second right panels). As a control, GST alone did not bind to Mst1 (lower right panels). (**b**) P17 of acinus binds PKC-δ and stimulates its kinase activity. Various GST-acinus fragments and GFP-PKC-δ were cotransfected into HEK293 cells, and the GST-fusion proteins were pulled down, and its associated proteins were analyzed with anti-GFP antibody. GST-acinus fusion proteins strongly associated with GFP-PKC-δ as a control but they did not bind to PKC-ε (top panel). The expression of transfected constructs was verified (second and third panels). Compared with full-length acinus, p17 and 228–583 fragment strongly promoted PKC-δ to phosphorylate H2B *in vitro* (fourth panel). The exogenously added H2B was verified by immunoblotting assay (bottom panel). (**c**) Depletion of acinus diminishes H2B phosphorylation upon VP16 treatment. PC12 cells were infected with control or acinus adenovirus expressing its shRNA for 36 h, and then treated with DMSO or 100 μM VP16 (etoposide) for 24 h. Compared with the control, acinus was selectively knocked down by its RNAi. VP16 elicited acinus degradation (top left panel). Equal amount of proteins was loaded (second left panel). Mst1 was slightly decayed in acinus-knocked down cells than control cells, and VP16 treatment completely eliminated it (third left panel). PKC-δ was prominently cleaved upon acinus depletion in the absence of VP16 (lane 3), and VP16 provoked its apoptotic cleavage in both control and acinus-depleted cells (fourth left panel). H2BS14 was phosphorylated when acinus was depleted, and VP16 triggered stronger H2B phosphorylation in control cells than in acinus-depleted cells (bottom left panel). Caspase-3 activity correlated with H2BS14 phosphorylation pattern (right panel). Absorption was calculated as means ($\pm$S.D.) of three triplicate experiments. (**d**) VP16 and staurosporine-triggered chromatin condensation is diminished when acinus is knocked down. PC12 was subjected to the same treatment, and its chromatin condensation was analyzed under fluorescent microscope after DAPI staining. The condensed or fragmented nuclei were labeled with white arrows. Numbers of cells with positive condensed nuclei were calculated as means ($\pm$S.D.) of three experiments

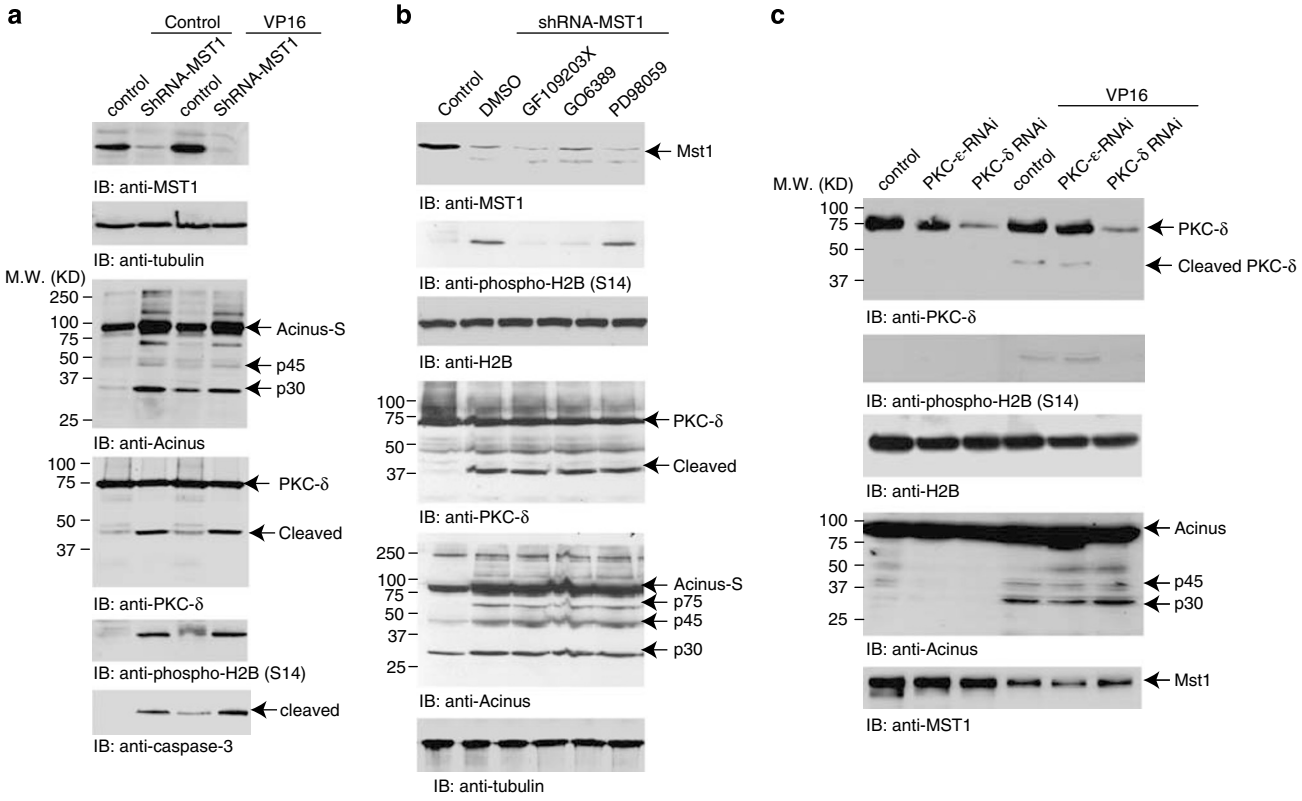


Figure 5 Depletion of Mst1 provokes H2BS14 phosphorylation through activating PKC-δ. (**a**) Depletion of Mst1 triggers acinus and PKC-δ degradation. PC12 cells were infected with control or sh-RNA MST1 adenovirus, followed by VP16 treatment. Mst1 RNAi potently knocked down its protein expression (lane 2, top panel). Equal amount of protein was loaded (second panel). Compared with control adenovirus, depletion of Mst1 alone robustly provoked acinus and PKC-δ proteolytic degradation, and VP16 treatment further enhanced their cleavage (third and fourth panels) slightly. Interestingly, in the absence of VP16, H2BS14 was evidently phosphorylated when Mst1was depleted (second lane in fifth panel). As expected, VP16 treatment elicited H2B phosphorylation in the control sample, and further enhanced it in Mst1-depleted cells (fifth panel). Knocking down of Mst1 also triggered caspase-3 activation even in the absence of VP16 (bottom panel). (**b**) Mst1 depletion-triggered H2BS14 phosphorylation can be antagonized by PKC inhibitors. PC12 cells were infected with control or shMst1 adenovirus, then treated with 10 μM GF109203X, 5 μM Go6389, 10 μM MEK1 inhibitor PD98059 for 30 min. Compared with control, Mst1 was markedly decreased by its shRNA (top panel). Depletion of Mst1-incurred H2BS14 phosphorylation was markedly blocked by PKC inhibitors but not by MEK1 inhibitor (second panel). Extensive PKC-δ and acinus degradation was observed when Mst1 was depleted (third and fourth panels). Equal amount of protein was employed (bottom panel). (**c**) Knockdown of PKC-δ decreases H2BS14 phosphorylation. PC12 cells were transfected with control or siRNA of PKC-ε and -δ, respectively, followed by VP16 treatment for 24 h. A portion of PKC-δ was selectively depleted, and VP16 treatment elicited PKC-δ degradation. The amount of cleaved PKC-δ was decreased when PKC-δ was knocked down (top and second panels). H2BS14 phosphorylation was also reduced when PKC-δ was depleted (third panel). VP16 treatment also provoked acinus and Mst1 degradation (fourth and fifth panels). As a control, equal amount of tubulin was present in all samples (bottom panel)

transfection of catalytically active fragment of PKC-δ (a.a. 331–676) induced evident H2B phosphorylation in both PKC-δ +/+ and −/− cells (Figure 6c, left panels). However, kinase-dead counterpart (K376R) failed to initiate H2B phosphorylation in either cell (Figure 6c, right panels). Quantitative analysis revealed that more than 70% of GST-p17-transfected cells were H2BS14 positive in wild-type cells, which was decreased to 15% in PKC-δ −/− cells. Nevertheless, approximately 15 and 10% were H2BS14 positive for Mst1 (1–326)-transfected PKC-δ +/+ and −/− cells, respectively. Strikingly, constitutively active PKC-δ-CF incurred H2B phosphorylation in more than 80–90% of transfected cells in both cell lines. By contrast, kinase-dead PKC-δ-CF failed to provoke any significant H2B phosphorylation in either cell line (Figure 6d). Collectively, these findings indicate that Mst1 is neither required nor sufficient to stimulate H2BS14 phosphorylation or chromatin condensation, for which PKC-δ is indispensable.

Discussion

In this study, we report that PKC-δ but not Mst1 is responsible for triggering H2BS14 phosphorylation and chromatin condensation in response to active acinus p17 fragment. P17 binds to both Mst1 and PKC-δ, and selectively upregulates both kinase activities. However, Mst1 is not required for provoking H2BS14 phosphorylation or chromatin condensation. Instead, PKC-δ plays an essential role in these processes. Unexpectedly, knocking down of Mst1 elicits acinus and PKC-δ proteolytic cleavage, promoting H2BS14 phosphorylation. This process might be regulated by caspase-3 activation upon Mst1 depletion. It has been shown before that PKC-δ facilitates apoptosis by phosphorylating multiple cellular proteins implicated in apoptosis. Recently, it has been shown that PKC-δ phosphorylates H2B in B lymphocyte and mediates its cell death.[24,27] Here we show that PKC-δ acts as a downstream effector of acinus, which

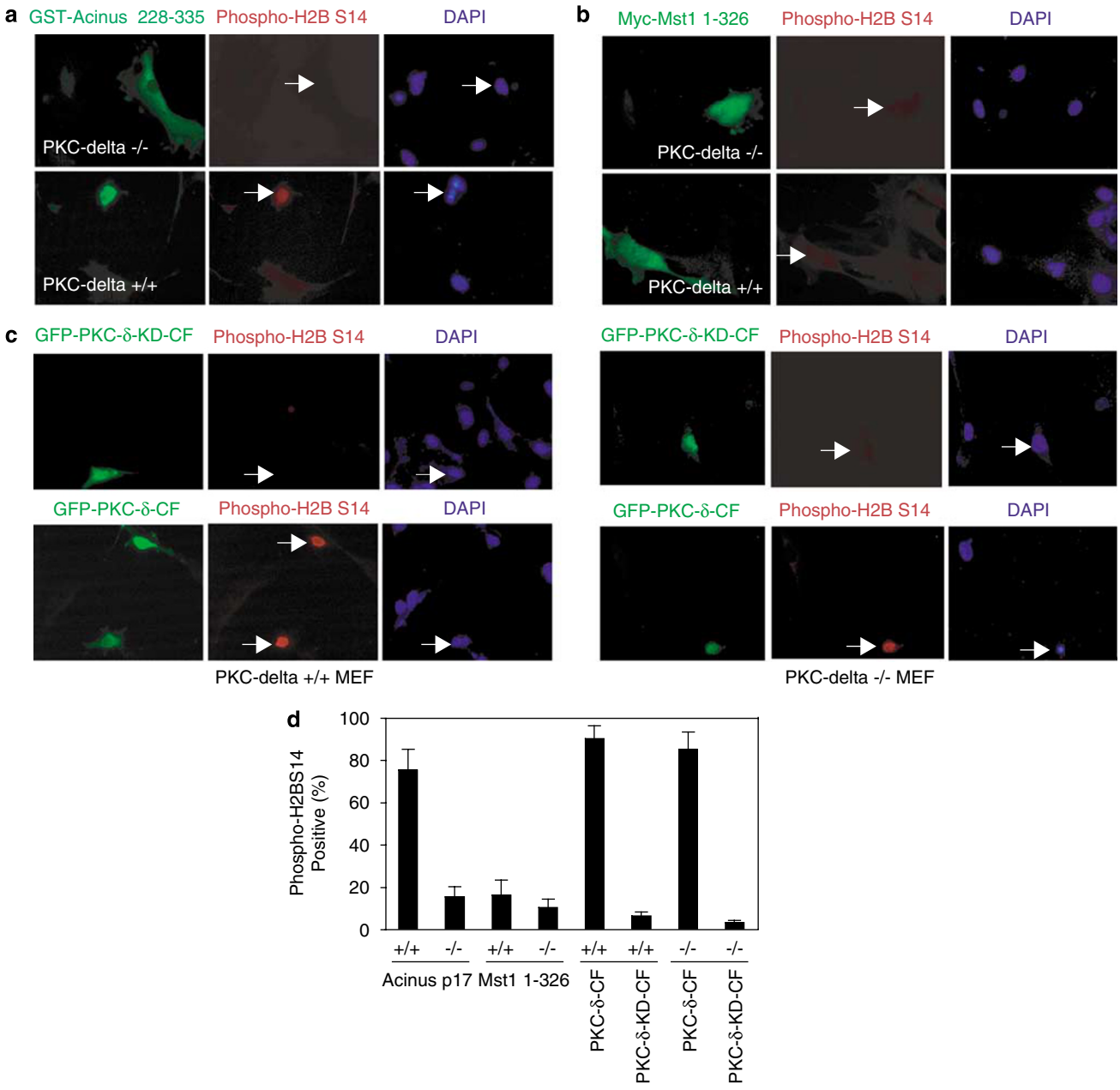


Figure 6 PKC-δ is required for H2B phosphorylation and chromatin condensation. (**a–c**) Immunofluorescent staining of MEF cells transfected with various acinus p17, Mst1 (1–326), and PKC-δ (CF) constructs. PKC-δ wild-type and knockout MEF cells were transfected with GST-acinus p17, Myc-Mst1 (a.a.1–326) and GFP-PKC-δ (CF (catalytic fragment) and CF-KD (kinase-dead catalytic fragment), respectively, and stained with the corresponding antibody to visualize the transfected cells. H2BS14 phosphorylation was monitored with its specific antibody. The nuclei were stained with DAPI. Acinus p17 provoked pronounced chromatin condensation in transfected $+/+$ cells, which was substantially decreased in $-/-$ cells (**a**). By contrast, active Mst1 (1–326) elicited modest chromatin condensation in the transfected wild-type cells, which was negligible in $-/-$ cells (**b**). On the contrary, constitutively active PKC-δ (CF) incurred chromatin condenstion in about 90% of transfected cells, regardless of wild-type or knockout cells. By contrast, no significant H2B phosphorylation was detected in kinase-dead PKC-δ (CF-KD)-transfected cells (**c**). (**d**) Quantitative analysis of chromatin condensation provoked by acinus p17, Mst1 (1–326), and PKC-δ (CF). Approximately 200 transfected cells in different fields were calculated as means ($\pm$S.D.) of three experiments

directly interacts with PKC-δ in the nucleus and promotes its kinase activity in apoptotic cells. Our discovery provides further evidence showing that PKC-δ phosphorylates H2BS14 in intact cells and mediates the apoptotic actions of acinus, inducing chromatin condensation.

Mst1 is homologous to a member of the yeast MAPK cascade, but it is not involved in the regulation of Erk1 or 2. Interestingly, PP2A treatment upregulates Mst1's kinase activity, whereas EGF treatment decreases its kinase activity, suggesting that phosphorylation on the resting Mst1 negatively modulates its kinase activity. Mitogenic signal might trigger its further phosphorylation, resulting in reduction of its kinase activity.[6] Here we show that acinus directly interacts with Mst1 and that its apoptotic active form p17 strongly binds

the N-terminus of Mst1 and escalates its kinase activity. However, little is known about the immediate downstream targets of Mst1 in the cytoplasm. Staurosporine enhances the association between acinus and Mst1 in apoptotic cells, and this interaction is suppressed upon EGF or NGF stimulation (Figure 2). These observations demonstrate that survival signal-mediated phosphorylation on Mst1 sequesters it from the apoptotic effector, acinus.

Mst1 is a physiological substrate of caspase-3. Surprisingly, we found that depletion of Mst1 triggers caspase-3 activation. Concomitantly, both acinus and PKC-δ are potently cleaved, and H2BS14 is strongly phosphorylated (Figure 5). Interestingly, knocking down of acinus elicits similar effect on Mst1 and PKC-δ degradation, probably through activating caspase-3 (Figure 3). Recently, it has been reported that knockdown of acinus in HeLa cells leads to a reduction in cell growth and inhibition of DNA fragmentation, but chromatin condensation was not significantly attenuated.[28] This finding underscores that acinus expression level is essential for cell growth and survival, consistent with our observations (Figure 3). Nonetheless, acinus and Mst1 remain intact in PKC-δ knockout cells (Figure 5). It remains unknown how Mst1 or acinus elimination alone elicits the onset of caspase-3 activation. As overexpression of Mst1 induces cell death in numerous cell types,[10,12,15,17,29] here we show that depletion of Mst1 also ignites apoptosis. Thus, the expression level of Mst1 has to be tightly monitored in the living cells, probably through RASSF1 and NORE1.[30,31]

During apoptosis, numerous factors couple to chromatin condensation, for example, perturbations of $Na+$ and $K+$ homeostasis,[32] release of nuclear actin,[33] AIF,[34] and acinus, etc. The exact contribution of each factor remains controversial. Presumably, different apoptotic stages or stimuli and subcellular contexts may determine which factors trigger chromatin condensation. Moreover, it has been well established that histone modification also plays a critical role in the broad context of chromatin condensation. Phosphorylation of the histone tails is intimately associated with regulation of chromatin structure. Histone H1 and H3 phosphorylation associated with chromatin condensation during mitosis has been studied extensively. Phosphorylation of the flexible N-termini of nucleosome core histones appears to play a more significant role in chromatin condensation than that of the linker histone H1.[35] Among modifications of the core histones, phosphorylation of Ser-10 of histone H3 is a hallmark of chromatin condensation during mitosis. H2A.X phosphorylation at serine 139 correlates with double-stranded DNA breaks induced by numerous stimuli, and H2AX phosphorylation has also recently been shown to associate with chromatin condensation.[36] Moreover, induction of phosphorylation of histone H3 on Ser-10 and H2AX on serine 139 has been observed during premature chromatin condensation (PCC).[35,37]

Allis and co-workers recently reported that caspase-cleaved Mst1 acts as a H2BS14 kinase *in vitro* and *in vivo*, and that the onset of H2BS14 phosphorylation is dependent upon Mst1 cleavage by caspase-3.[2,38] However, we show that knocking down of Mst1 does not abolish H2BS14 phosphorylation. On the contrary, it provokes marked H2BS14 phosphorylation in the intact cells in the absence of apoptotic stimulation. This finding indicates that Mst1 is not necessary for H2BS14 phosphorylation during apoptosis. Depletion of Mst1 provokes both acinus and PKC-δ degradation and yields a catalytic active form of PKC-δ, leading to H2BS14 phosphorylation. This activity can be blocked by PKC inhibitors but not MEK1 inhibitor (Figure 5), underscoring that PKC-δ activation accounts for this effect. Most recently, they showed that Mst1 is responsible for yeast H2BS10 phosphorylation, an event triggering cell death in yeast.[38] However, yeast H2B N-terminus has no homology to the mammalian H2B counterpart. S14 is well conserved among mammalian species, but does not exist in H2B from yeast or fly. Mst1 might be responsible for yeast H2BS10 phosphorylation, but our evidence and others' results clearly prove that Mst1 is dispensable for H2BS14 phosphorylation in mammalian cells. However, it is also possible that other Mst kinase family members might have redundant functions. The other member might compensate the depletion of Mst1 by phosphorylating H2BS14 during apoptosis. Moreover, we manifest that acinus p17 can provoke H2B phosphorylation and chromatin condensation in PKC-δ $+/+$ but not in $-/-$ cells. The previously claimed *in vivo* H2B kinase Mst1 (1–326) fails to elicit H2BS14 phosphorylation or chromatin condensation in either cell. In contrast, PKC-δ CF (catalytic fragment) but not CF-KD (kinase-dead catalytic fragment) provokes H2BS14 phosphorylation in both cells (Figure 6). These findings demonstrate that PKC-δ is the major kinase responsible for phosphorylating H2BS14 in cells. This observation is supported by a recent report that H2BS14 phosphorylation is almost negligible in PKC-δ-deficient B cells compared to wild-type cells.[24] Although Mst1 expression is relatively higher in PKC-δ-null cells than in wild-type cells, no H2BS14 phosphorylation was detected in PKC-δ $-/-$ cells, even though Mst1 was robustly phosphorylated and activated upon VP16 treatment (data not shown), further supporting that Mst1 is not a main kinase phosphorylating H2B in intact cells.

In summary, we propose the following model: in response to apoptotic stimuli, acinus is cleaved by active caspase-3, yielding the active p17. The active p17 binds and activates Mst1 and PKC-δ, stimulating their apoptotic cleavage by caspase-3, leading to PKC-δ activation. The catalytic fragment of PKC-δ phosphorylates H2B on serine 14, triggering chromatin condensation. Although numerous Mst1-binding proteins have been identified, little is known about the immediate downstream targets in either the cytoplasmic or the nuclear compartment. The identification of nuclear substrates for the translocated active Mst1 will further our understanding about the physiological role of this interesting kinase in apoptosis.

Materials and Methods

Cells and reagents. PC12 cells were maintained in medium A (DMEM with 10% fetal bovine serum (FBS), 5% horse serum, and 100 U penicillin–streptomycin) at 37°C with 5% CO_2 atmosphere in a humidified incubator. All PC12 cells are in naive but not differentiated form. The Myc-Acinus and Myc-NLS-Akt stably transfected PC12 cells (Tet-off cell line) were cultured in medium B (DMEM, 10% horse serum, 5% FBS, 100 μg/ml G418, 100 μg/ml hygromycin B, 2 μg/ml tetracycline, and 100 U penicillin–streptomycin). The transfected genes were induced by culturing in medium B without tetracycline for 24 h. Both wild-type and PKC-δ knockout MEF cells were cultured in 10% FBS in DMEM, supplemented with nonessential amino acids and 50 μM β-mercaptoethanol. NGF was from Roche.

Anti-caspase-3 and α-tubulin antibodies were from Santa Cruz Biotechnology Inc. Anti-Myc, anti-acinus, anti-Phospho-Akt-473, anti-Akt, and anti-PKC-δ antibodies were from Cell Signaling. Active PKC-δ protein, anti-Mst1, and anti-phospho-Mst1 antibodies were from Upstate Biotechnology, Inc. All the chemicals not included above were from Sigma. The siRNA sequences were: PKC-α, TCCTTGTCCAA GGAGGCTG; PKC-δ, CCATGAGTTTATCGCCACC; the shRNA for rat Mst1 was 5′-GATCCCGGCTGACATCCATCCAATGAGGGCAATATTGAAGCTTGAATATTG CCCTCATTGGATGGATGTCAGCTTTTTT-3′. The silence resistant mutant Mst1 primer sequence was 5′-GCCGATATACACCCGATGAGGGCAATATT-3′.

***In vitro* binding assays.** GST fusion proteins were prepared and coupled to glutathione-sepharose beads. PC12 cells were treated with or without 250 nM staurosporine for 12 h, and the cells were washed once in PBS, lysed in 1 ml lysis buffer A (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A), and centrifuged for 10 min at 14 000 × *g* at 4°C. The cell lysates were incubated with GST proteins-conjugated glutathione beads at 4°C for 3 h. After incubation, the beads were washed three times with 500 μl lysis buffer each time. The agarose was then resuspended in 30 μl sample buffer separated by SDS-PAGE, followed by immunoblotting analysis.

Cytochemical staining of apoptotic cells. Stably transfected PC12 cells were induced in tetracycline-free medium overnight. In the presence or absence of NGF, cells were treated with 10 μM etoposide for 24 h, followed by 1 μM staurosporine for another 5 h. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with DAPI as described.[39] Totally, about 500 nuclei were counted under different fields. The picture was taken on an Olympus IX71 invert fluorescent microscope.

In-gel kinase assay. Cells treated/untreated with various drugs were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5% Triton X-100, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 6 mM dithiothreitol, and 2% aprotinin). To assay the total cell lysate, equal amounts of protein (50–100 μg) were electrophoresed in 10% SDS-polyacrylamide gels containing 0.5 mg/ml histone H2B as a substrate. Following electrophoresis, SDS was removed from the gel, the protein was renatured, and a kinase assay was carried out by incubating the gel in buffer (40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM EGTA) containing 20 μM γ-[^{32}P]ATP. Gels were washed and dried, and the incorporated radioactivity was analyzed by autoradiography.

***In vitro* kinase assay.** Endogenous MST or various forms of ectopic expressed MST were immunoprecipitated with 2 μg of antibody. After extensive washing, the immunocomplex was employed in a kinase assay. The immunoprecipitates were incubated with 2 μg of histone H2B in 20 μl of kinase reaction buffer (20 mM Tris, pH 7.5 with 10 mM $MgCl_2$) containing 25 μM ATP and 2.5 μCi of γ-[^{32}P]ATP for 20 min at 30°C. Reactions were terminated by adding 7 μl of Laemmli's sample buffer and boiling for 5 min. For PKC-δ kinase assay, the beads were washed three times in a PKC buffer (10 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM DTT). The immunocomplex was resuspended in PKC kinase buffer supplemented with 8 mM MOPS, pH 7.2, 10 mM β-glycerol phosphate, 1 mM EGTA, 0.4 mM Na_3VO_4, 0.5 mM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 0.01 mg/ml diacylglycerol, and 2.5 μCi of γ-[^{32}P]ATP. A portion of the sample (15 μl) was separated on a 15% SDS-polyacrylamide gel and autoradiographed or analyzed by PhosphorImage analyzer.

Caspase-3 activity assay. Caspase-3 activity was measured by means of the CaspACE Assay System Fluorometric Kit (Promega Corporation, Madison, WI, USA). Cells were initially seeded at a density of 1×10^6 in 10-cm dishes. After infection with control or sh-RNA acinus adenovirus, followed by 35 μM VP16 treatment for 24 h, caspase-3 activity was measured by the cleavage of the fluorometric substrate Ac-DEVD-AMC according to the manufacturer's instructions.

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