TGF-β induces apoptosis through Smad-mediated expression of DAP-kinase

Chuan-Wei Jang, Chun-Hau Chen, Chun-Chieh Chen, Jia-yun Chen, Yi-Hsien Su and Ruey-Hwa Chen*

Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei 100, Taiwan
**e-mail: rhchen@ha.mc.ntu.edu.tw*

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Transforming growth factor-β (TGF-β) and TGF-β-related factors induce apoptosis in a variety of tissues; however, the mechanism underlying this induction is largely unknown. Here, we demonstrate that TGF-β induces the expression of the death-associated protein kinase (DAP-kinase) as an immediate early response in cells that undergo apoptosis in response to TGF-β. DAP-kinase is a positive mediator of apoptosis induced by certain cytokines and oncogenes. We show that the DAP-kinase promoter is activated by TGF-β through the action of Smad2, Smad3 and Smad4. Overexpression of DAP-kinase triggers apoptosis in the absence of TGF-β, whereas inhibition of DAP-kinase activity protects cells from TGF-β-induced apoptosis, blocks TGF-β-induced release of cytochrome *c* from mitochondria and prevents TGF-β-induced dissipation of the mitochondrial membrane potential. Our findings indicate that DAP-kinase mediates TGF-β-dependent apoptosis by linking Smads to mitochondrial-based pro-apoptotic events.

The TGF-β superfamily consists of a large number of cytokines that regulate essential cellular functions, such as proliferation, differentiation and apoptosis[1,2]. TGF-β-dependent apoptosis is important in the elimination of damaged or abnormal cells from normal tissues *in vivo*. For example, TGF-β is a potent inducer of apoptosis in hepatoma cell lines and in regressing and cirrhotic liver[3–5]. The apoptotic effect of TGF-β is implicated in the elimination of immature lymphocytes[6], and prostatic epithelial cells after castration[7]. TGF-β-related factors also induce apoptosis during development, and are critical in the regulation of embryonic programmed cell death and in the maintenance of normal development. Examples include limb development[8,9], interdigital space formation[10,11], myeloid cell development[12], and hair follicle cycling[13]. However, the signalling pathways that are activated by these ligands, and lead to apoptosis, have not been well characterized.

TGF-β exerts its biological effects by binding to a cell surface receptor complex of type I and type II receptors[1]. Upon ligand binding, the type II receptor phosphorylates the type I receptor, which subsequently phosphorylates Smad2 and Smad3. Receptor-activated Smad2 and Smad3 undergo a conformational change that allows heteromerization with a common partner, Smad4 (refs 15–18). This complex is subsequently translocated to the nucleus[15–18]. In the nucleus, Smad complexes act as TGF-β-sensitive transcriptional co-activators or corepressors through their interaction with a variety of transcription factors[19–22]. TGF-β responses can also be modulated by Smad6 and Smad7, which bind to the activated receptors or pathway-restricted Smads, thereby preventing further propagation of TGF-β signalling[23,24].

Although the mechanism of TGF-β signalling that leads to the transcriptional activation of immediate early genes, such as plasminogen activator inhibitor-1 and $p15^{InK4B}$, is well documented, little is known about the signalling pathway that allows TGF-β and TGF-β-related factors to induce apoptosis. Several pro-apoptotic events, such as induction of oxidative stress[25], downregulation of Bcl-2 or Bcl-X_L[26–28], and activation of caspase 3 (refs 3,27), have been implicated in TGF-β-dependent apoptosis. Recently, a mitochondrial septin, ARTS, was shown to be involved in apoptosis induced by TGF-β[28,29]. Furthermore, TGF-β triggers the translocation of ARTS from the mitochondria into the nucleus[28,29]. How TGF-β signalling leads to the activation of these pro-apoptotic events is currently unknown. In addition, although Smads may be involved in TGF-β-dependent apoptosis[30,31], the Smad targets that mediate and dictate the apoptotic pathway of TGF-β have not yet been identified.

TGF-β elicits multiple cellular effects, depending on the type and state of the cell to which it binds. Accordingly, its apoptotic effect is largely dependent on the cellular context. Such cell-type-specific responses to TGF-β are caused largely by the cross-talk of TGF-β signalling with a variety of other signalling pathways, at the level of Smads[32]. In the nucleus, Smads cooperate with numerous transcription factors, where they function as transcriptional comodulators to regulate pre-existing gene expression patterns[20–23]. Thus it is important to identify context-dependent Smad targets to understand the ability of TGF-β to induce apoptosis.

Here, we identify DAP-kinase as an effector of TGF-β-dependent apoptosis. DAP-kinase is a target of transcriptional activation by Smads. Previous studies have identified DAP-kinase as a calcium/calmodulin-regulated serine/threonine kinase that is localized to the cytoskeleton, and that participates in several apoptotic processes[33–36]. We found that ectopic expression of DAP-kinase in TGF-β-sensitive hepatoma cells is sufficient to trigger apoptotic cell death, whereas expression of DAP-kinase dominant negative mutants or antisense inhibition of DAP-kinase expression blocks TGF-β-dependent apoptosis. Finally, we provide evidence that DAP-kinase acts upstream of mitochondrial-based pro-apoptotic events, along the apoptotic pathway of TGF-β. Thus, DAP-kinase participates in TGF-β-induced apoptosis by acting immediately downstream from Smads and upstream of mitochondrial pro-apoptotic events.

Results

Induction of DAP-kinase expression during TGF-β-dependent apoptosis. Hep3B hepatoma cells undergo apoptosis in response to TGF-β, resulting in cell death 18 h after TGF-β treatment (Fig. 1a, b)[3]. A complementary DNA microarray approach identified genes with mRNA levels altered during TGF-β-induced apoptosis. In cells

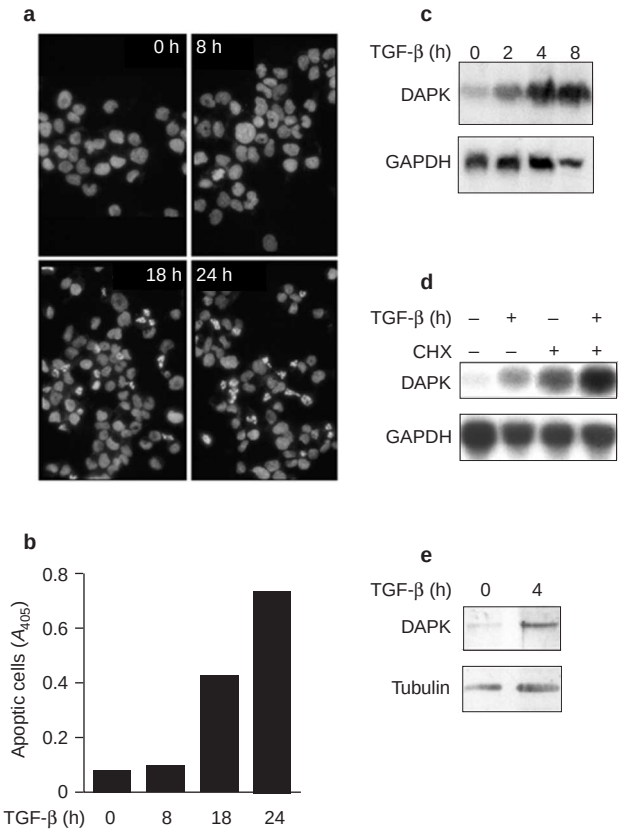


Figure 1 **Upregulation of DAP-kinase during TGF-β-induced apoptosis. a**, **b**, Induction of apoptotic death in Hep3B cells by TGF-β. Cells were treated with TGF-β (5 ng ml^{-1}) for various times and apoptotic nuclei and DNA fragmentation were detected by Hoechst 33258 staining (**a**) and ELISA assays[49] (**b**), respectively. **c**, Northern blot analysis of mRNAs prepared from Hep3B cells, treated with or without TGF-β for various times, with probes specific to DAP-kinase or GAPDH, as indicated. **d**, Induction of DAP-kinase is an immediate early response to TGF-β. Hep3B cells were treated with or without TGF-β and/or 10 μg ml^{-1} cycloheximide (CHX), as indicated. Northern blot analysis was performed to detect expression of DAP-kinase or GAPDH. **e**, Western blot analysis of Hep3B cells, treated with or without TGF-β for 4 h, with an antibody specific to DAP-kinase or tubulin, as indicated.

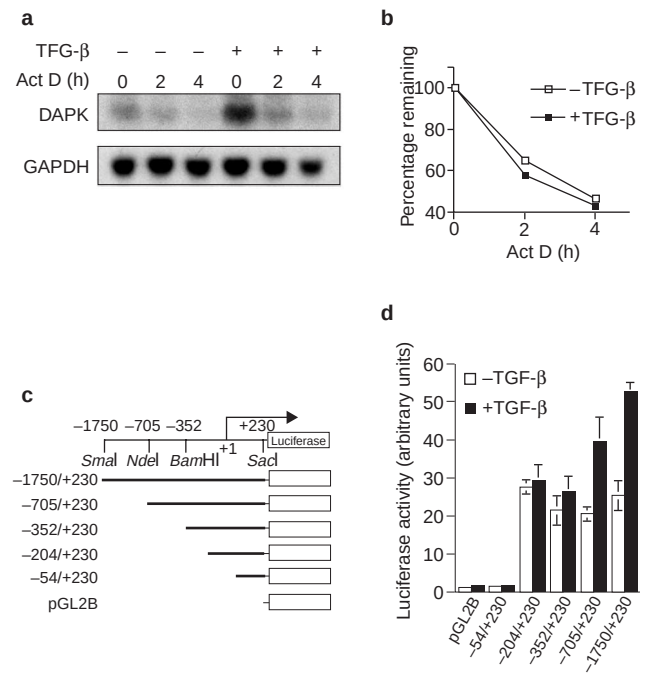


Figure 2 **TGF-β regulates DAP-kinase promoter activity but not mRNA stability. a**, Hep3B cells were treated with or without TGF-β for 4 h and then exposed to ActD (5 μg ml^{-1}) alone, or ActD in combination with TGF-β for various times, as indicated. Total RNA was isolated and subjected to northern blot analysis with a probe specific to DAP-kinase or GAPDH. **b**, The levels of DAP-kinase mRNA shown in **a** were quantified, normalized to the amount of GAPDH mRNA, and converted to the percentage of DAP-kinase mRNA at time zero to determine the stability of DAP-kinase mRNA. **c**, Illustration of luciferase reporters driven by various regions of the DAP-kinase promoter. **d**, Regulation of the DAP-kinase promoter by TGF-β. Hep3B cells were transiently transfected with various promoter constructs and then treated with or without TGF-β for 18 h. Luciferase activities were normalized for transfection efficiency and cell survival against β-galactosidase activities derived from the cotransfected pRK–βGal plasmid. Values are mean ± s.d. of triplicate assays.

treated with TGF-β for 2 h, a number of genes were upregulated, including c-*fos*, *jun*B, fibronectin and tissue inhibitor of metalloproteinase I (data not shown). The levels of most genes that have already been implicated in apoptosis were not significantly altered during TGF-β-induced apoptosis, and this was confirmed by northern and/or western blot analyses on a subset of these genes (see Supplementary Information, Table S1). However, one gene that was induced by TGF-β treatment is DAP-kinase, a death-domain-containing kinase that functions in apoptosis induced by several cytokines and oncogenes[33–36]. Northern blot analysis of RNAs isolated from Hep3B cells treated with TGF-β for various times demonstrated that DAP-kinase mRNA was present in these cells, and rapidly induced after treatment with TGF-β (Fig. 1c). An approximately eight-fold induction of DAP-kinase mRNA was detected 8 h after TGF-β treatment, previously established as a time point that precedes the onset of apoptosis (Fig. 1a, b). To investigate whether the induction of DAP-kinase expression by TGF-β requires *de novo* protein synthesis, we assessed the effect of the protein synthesis inhibitor cycloheximide. Although cycloheximide itself caused an increase in DAP-kinase mRNA levels, a significant superinduction was seen in cells treated with cycloheximide and TGF-β (Fig. 1d), suggesting that DAP-kinase is a primary target of TGF-β signalling. The increase of DAP-kinase mRNA levels in the presence of cycloheximide was probably caused by mRNA stabilization, as has been observed for mRNAs with an instability sequence in their 3′ untranslated region[37]. Accordingly, the 3′ untranslated region of DAP-kinase mRNA contains two copies of the mRNA instability signal[33].

To determine whether the rapid induction of DAP-kinase mRNA could lead to an increase in its protein level, a polyclonal antibody was raised against the carboxy-terminal portion of DAP-kinase. Immunoblot analysis with the anti-DAP-kinase antibody specifically identified a band of the same size as the DAP-kinase protein (Fig. 1e)[33]. Furthermore, a band with the same mobility, but higher intensity, was detected in cells overexpressing DAP-kinase (see below). With this antibody, we examined the levels of DAP-kinase in Hep3B cells treated with or without TGF-β. TGF-β caused an increase in levels of DAP-kinase protein (Fig. 1e). Thus, we conclude that both the mRNA and protein levels of DAP-kinase are upregulated during TGF-β-induced apoptosis.

Induction of the DAP-kinase promoter by TGF-β signalling. To investigate whether the upregulation of DAP-kinase mRNA by TGF-β was caused by mRNA stabilization, we examined the half-life of DAP-kinase mRNA in the presence or absence of TGF-β. Hep3B cells were treated with or without TGF-β for 4 h and chased in the presence of actinomycin D (ActD), an inhibitor of transcription, for various times. Northern blot analysis revealed that the

degradation rate of DAP-kinase mRNA in cells treated with TGF-β resembled that in untreated cells (Fig. 2a, b). These results indicate that the effect of TGF-β on DAP-kinase mRNA accumulation does not occur at the level of mRNA degradation, and imply that TGF-β induces transcription of the DAP-kinase gene.

To determine whether TGF-β-induced expression of DAP-kinase involves direct transcriptional activation of the DAP-kinase promoter, the 5′ flanking region of the DAP-kinase gene was isolated and characterized. Polymerase chain reaction (PCR)-based primer extension identified the transcriptional initiation site as 352 base pairs upstream from the start codon (data not shown, Fig. 2c). A DNA fragment corresponding to the −1,750 to +230 region of the DAP-kinase promoter region was inserted upstream of a luciferase reporter gene, to determine the induction of transcription from the DAP-kinase promoter by TGF-β. When transfected into Hep3B cells, this reporter conferred promoter activity and inducibility by TGF-β (Fig. 2d). To identify the minimum promoter region required for induction by TGF-β, a series of 5′ promoter deletion constructs were generated (Fig. 2c). Deletions up to position −705 still conferred responsiveness to TGF-β. However, deletion of the −705 to −352 sequence abolished TGF-β induction, without affecting the constitutive transcriptional activity. These findings suggest the existence of a TGF-β-responsive sequence in the −705 to −352 promoter region.

TGF-β-induced DAP-kinase transcription requires Smad2–4. Next, we determined whether the effect of TGF-β on induction of the DAP-kinase promoter is mediated by Smad proteins. Cotransfection of Hep3B cells with Smad3, Smad4 and the −705 to +230 or the −1750 to +230 reporter regions resulted in a 4–5-fold increase in the luciferase activity (data not shown). However, the −352 to +230 promoter region was not transactivated by Smad3 or Smad4 (data not shown), consistent with the finding that this promoter is not regulated by TGF-β (Fig. 2d). .TGF-β signalling. Expression of C-terminally truncated forms of Smad3 or Smad4, which have been shown to act as specific dominant negative inhibitors[17], caused a reduction in TGF-β-induced DAP-kinase promoter activity, whereas the combination of C-terminally truncated Smad2 and Smad4, or Smad3 and Smad4, completely abolished inducibility by TGF-β (Fig. 3b). Furthermore, expression of Smad7 reduced promoter activity to a level that was considerably lower than that in cells which were not treated with TGF-β (Fig. 3b). This strong inhibition is likely to reflect the ability of Smad7 to counteract the autocrine activity of endogenous TGF-β produced by Hep3B cells (data not shown). The autocrine response to endogenous TGF-β may also contribute to the constitutive level of transcription from the DAP-kinase promoter in the reporter assays (Figs 3 and 4). Altogether, our results indicate that DAP-kinase is a transcriptional target of Smad2–4.

The function of Smads in TGF-β-induced activation of the DAP-kinase promoter was examined further in cells lacking Smad3 or Smad4. Smad3-deficient mouse embryonic fibroblasts did not respond to TGF-β-dependent activation of the DAP-kinase promoter; however, reintroduction of Smad3 into these cells restored inducibility by TGF-β (Fig. 3c). Similar findings were observed with SW480.7 colon carcinoma cells, which lack endogenous Smad4. Again, TGF-β failed to induce DAP-kinase promoter activity; however, ectopic expression of Smad4 rescued induction (Fig. 3c). These results, in conjunction with the data obtained from Hep3B cells, highlight the importance of Smads in mediating TGF-β-dependent DAP-kinase induction.

To verify that Smads function in the transcriptional induction of endogenous DAP-kinase, Hep3B cells were infected with recombinant adenoviruses expressing Flag-tagged Smad2 and Smad4, Smad3 and Smad4 or a control virus containing only the vector. The expression of Flag-tagged Smads in infected cells was confirmed by western blot analysis (Fig. 3d). Examination of endogenous DAP-kinase mRNA levels in infected cells revealed that cells infected with Smad2 and Smad4, or Smad3 and Smad4, expressed a higher level of DAP-kinase than those infected with the control virus (Fig. 3e). This result is in agreement with the reporter assay data and supports the function of Smads in the induction of DAP-kinase.

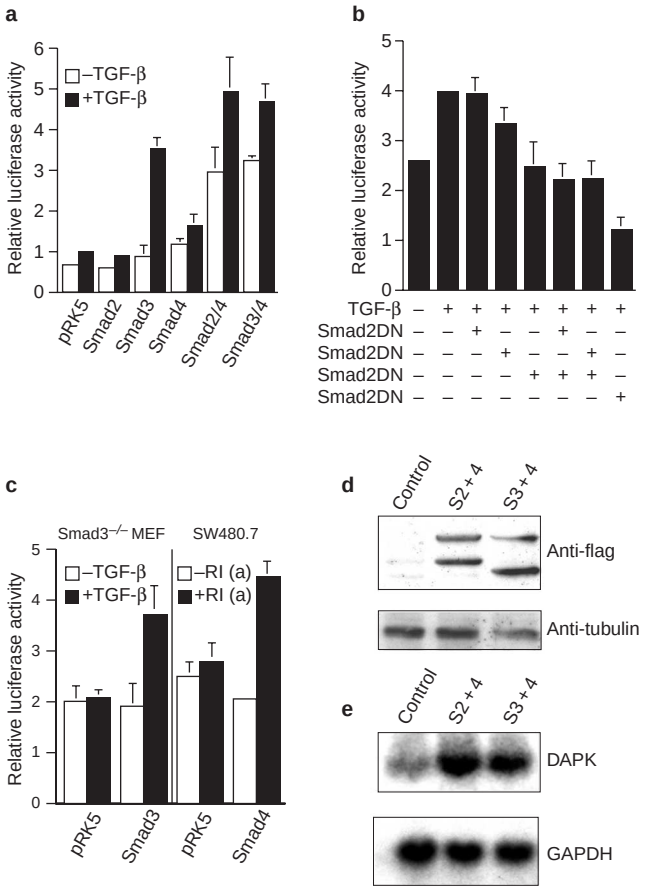


Figure 3 **TGF-β-induced DAP-kinase transcription requires Smad2, Smad3 and Smad4. a**, Transcriptional activation of the DAP-kinase promoter by Smads. Hep3B cells were cotransfected with the −705 to +230 DAP-kinase reporter plasmid and various Smad expression plasmids, as indicated. Luciferase activities were measured after treatment of transfected cells with or without of TGF-β (5 ng ml^{-1}). **b**, Inhibition of TGF-β-induced transcription from the DAP-kinase promoter by dominant negative Smads and Smad7. Hep3B cells were cotransfected with the −705 to +230 reporter and expression vectors for various C-terminally truncated Smads or Smad7, as indicated. Cells were treated with TGF-β and assayed for luciferase activity, as described in **a**. **c**, Smad3 and Smad4 are both required for TGF-β-induced transcription of DAP-kinase. *Smad3*$^{-/-}$ mouse embryonic fibroblasts and SW480.7 Smad4-defective cells were transfected with the −705 to +230 reporter in the presence or absence of expression vector for the active type I TGF-β receptor (RI(a))[50], Smad3 or Smad4, as indicated. **d**, Expression of various Flag-tagged Smads in Hep3B cells infected with Smad adenoviruses. Hep3B cells were infected with recombinant adenoviruses expressing various Smads or control adenovirus at a total MOI of 100, as indicated. Two days after infection, cells were harvested for western blot analysis. **e**, Overexpression of Smad2 and Smad4, or Smad3 and Smad4, induces the expression of DAP-kinase. Hep3B cells were infected with various adenoviruses as described in **d**. One day after infection, cells were cultured in serum-free medium for 24 h and then harvested for northern blot analysis.

Smad-binding elements (SBEs) are required for DAP-kinase induction by TGF-β. Examination of the TGF-β-responsive region of the DAP-kinase promoter revealed the existence of four copies of the consensus SBE (Fig. 4a). Mutation of the four SBEs in the −705 to +230 promoter region completely abrogated TGF-β responsiveness, without affecting the basal transcription level (Fig. 4b).

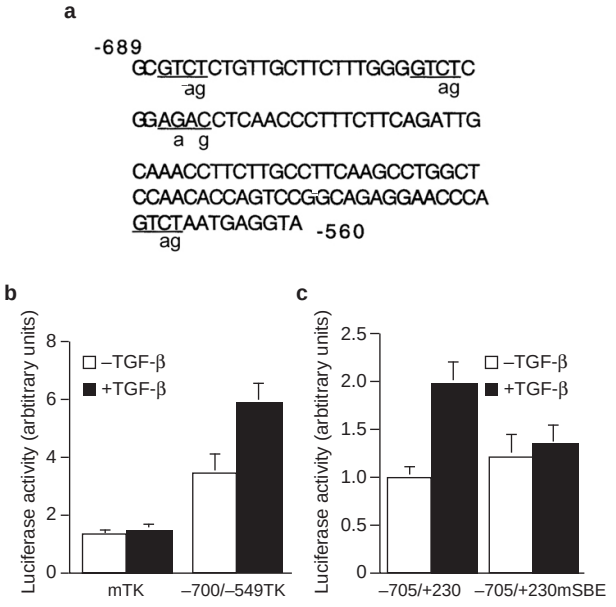


Figure 4 **Smad-binding elements are required for DAP-kinase induction by TGF-β. a**, Sequence of the human DAP-kinase promoter region from –689 to –560. The consensus SBE sites are underlined and their mutations are shown in lower-case characters. **b**, Mutational analysis of the DAP-kinase promoter. The four copies of SBEs in the –705 to +230 promoter were all mutated as shown in **a** to generate the –705 to +230 mSBE promoter. TGF-β responsiveness of the wild-type or mutant reporter in Hep3B cells was measured by luciferase activity. Transfections, TGF-β treatment and luciferase assays were performed as in Fig. 3. **c**, The SBE-containing region confers TGF-β responsiveness. Hep3B cells were transfected with the –700 to –549 mTK promoter or the control mTK reporter, treated with or without TGF-β, and subjected to luciferase assays.

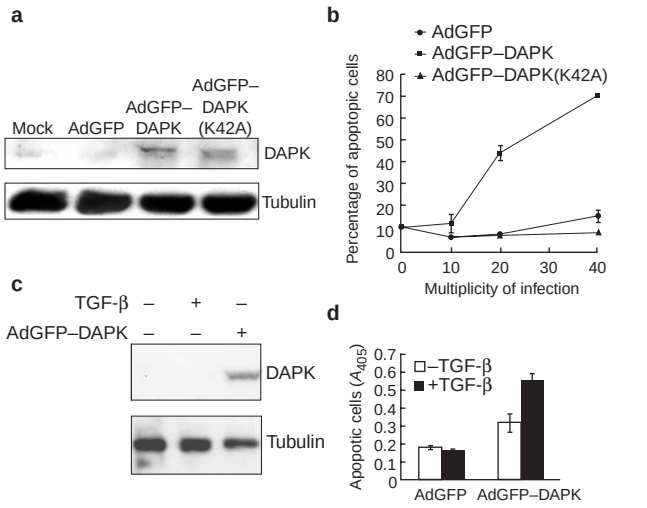


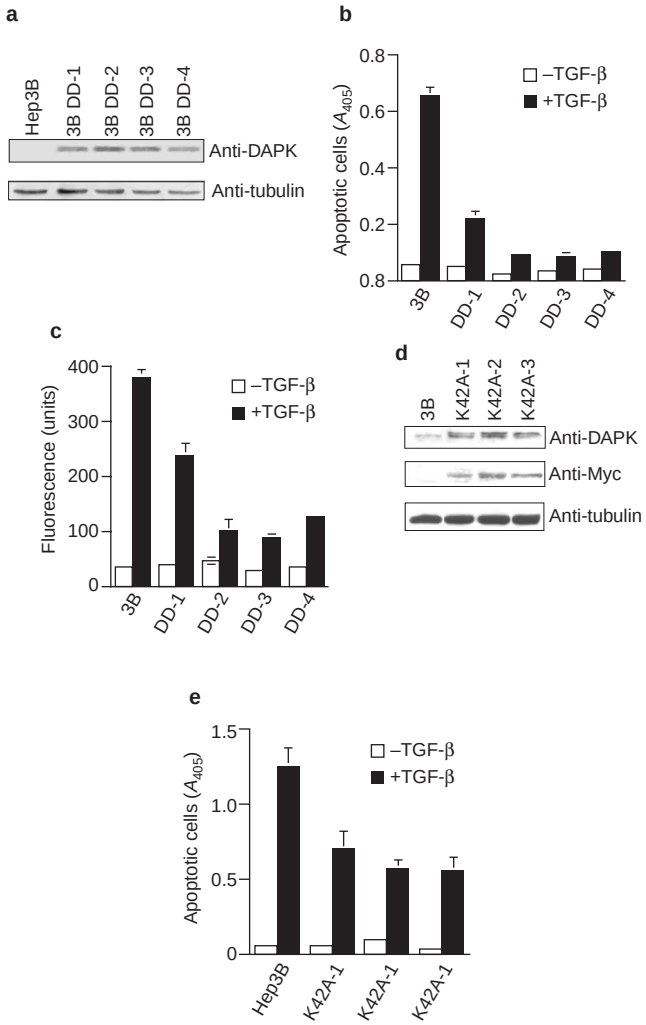
Figure 5 **DAP-kinase expression induces apoptosis and sensitizes cells to TGF-β-dependent apoptosis. a**, Hep3B cells were infected or mock-infected with adenovirus AdGFP, AdGFP–DAPK, or AdGFP–DAPK(K42A) at an MOI of 40. Cells were then cultured in serum-free medium for 48 h before western blot analysis to detect expression of DAP-kinase. **b**, Hep3B cells infected with adenovirus at various MOIs, as indicated, were cultured as in **a** for 96 h and then assayed for apoptotic populations by FACS. Percentage of cells with sub-G1 DNA content are indicated. **c**, Raji cells do not express endogenous DAP-kinase. Lysates of Raji cells treated with TGF-β, or infected with AdGFP–DAPK at an MOI of 100, were subjected to western blot analysis. **d**, Ectopic expression of DAP-kinase restores the apoptotic effect of TGF-β on Raji cells. Raji cells were infected with 100 MOI of recombinant adenovirus, as indicated. Cells were treated with or without TGF-β for 48 h, harvested, and levels of apoptosis measured with the Cell-Death Detection ELISA assay. Error bars represent s.d.

To determine whether the region containing the SBEs is sufficient to direct transcription in response to TGF-β, a DNA fragment corresponding to –700 to –549 of the DAP-kinase promoter was inserted upstream of a TK minimal promoter (mTK) driving a luciferase reporter gene. When transfected into Hep3B cells, this reporter conferred TGF-β responsiveness, while mTK alone did not (Fig. 4c). Thus, the SBE-containing region of the DAP-kinase promoter is both necessary and sufficient for induction of the DAP-kinase promoter by TGF-β.

Ectopic expression of DAP-kinase induces apoptosis. Having demonstrated that DAP-kinase expression is induced by TGF-β signalling through the action of Smads, we next investigated whether ectopic overexpression of DAP-kinase could lead to apoptosis in the absence of TGF-β stimulation. Recombinant adenoviruses expressing green fluorescent protein (AdGFP) or GFP together with the wild-type DAP-kinase (AdGFP–DAPK) or its kinase-dead mutant (AdGFP–DAPK(K42A)) were generated, and Hep3B cells were infected at various multiplicities of infection (MOIs). At an MOI of 40, AdGFP–DAPK infection increased DAP-kinase expression to levels achieved by TGF-β (Figs 5a and 1e). The cells infected with AdGFP–DAPK(K42A) cells overexpressed a similar level of the mutant protein, while cells infected with the control virus did not show DAP-kinase overexpression (Fig. 5a). The fate of infected or mock-infected cells was examined by analysing their DNA content with FACS. Infection of Hep3B cells with AdGFP–DAPK at increasing MOIs resulted in a dose-dependent increase in apoptotic cells with sub-G1 DNA content, whereas control AdGFP or the AdGFP–DAPK(K42A) virus did not lead to apoptosis (Fig. 5b), the latter consistent with previous studies[34,36]. Thus, the increase of DAP-kinase concentration to a level similar to that observed in TGF-β-stimulated cells is sufficient to trigger apoptosis in Hep3B cells. This suggests that induction of DAP-kinase by TGF-β is a critical event in the initiation of TGF-β-dependent apoptosis.

To further confirm that the induction of DAP-kinase is a determining factor in the sensitivity of cells to the apoptotic effect of TGF-β, we studied TGF-β-dependent apoptosis in cells that do not express DAP-kinase. The Raji Burkitt's lymphoma cell line has no endogenous DAP-kinase expression, owing to hypermethylation of the DAP-kinase promoter region[38]. Treatment of these cells with TGF-β did not cause an increase in DAP-kinase expression or an induction of apoptosis (Fig. 5c, d). However, expression of exogenous DAP-kinase by adenoviral-mediated gene transfer led to an increase of basal apoptosis level, and a restoration of sensitivity to TGF-β-dependent apoptosis (Fig. 5d). Altogether, our results indicate that the induction of DAP-kinase expression by TGF-β is an essential step in the apoptotic pathway of TGF-β.

Involvement of DAP-kinase in TGF-β-dependent apoptosis. The death-domain of DAP-kinase (DAPK-DD) is required for the apoptosis-promoting activity of this kinase, and, when expressed alone, it interferes with the full-length protein in a dominant negative manner[35]. To further evaluate the function of DAP-kinase in TGF-β-induced apoptosis, we generated Hep3B cells that stably express DAPK-DD (Fig. 6a). By comparison with parental Hep3B cells, cells stably expressing DAPK-DD were significantly less sensitive to the apoptotic effect of TGF-β, as determined by enzyme-linked immunosorbent assay (ELISA) measuring the intranucleosomal fragmentation of DNA (Fig. 6b). Furthermore, TGF-β-induced caspase 3 activity was reduced in these transfectants (Fig. 6c). In both DNA fragmentation and caspase 3 activity assays, the reduced sensitivities to TGF-β roughly correlated



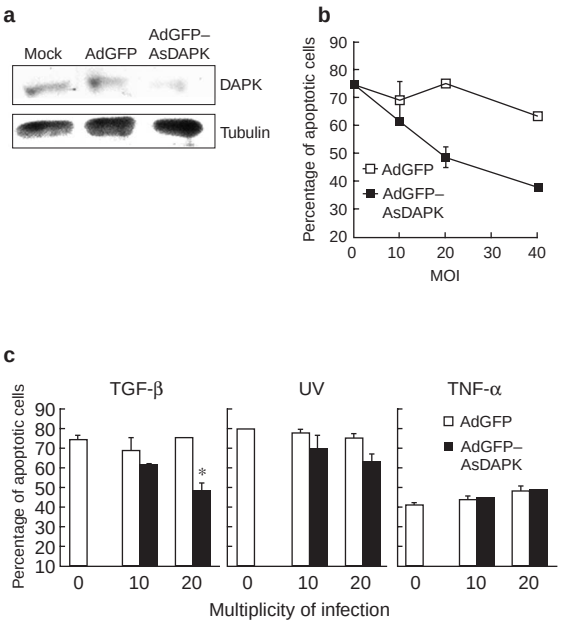


Figure 7 **Antisense inhibition of DAP-kinase expression specifically prevents TGF-β-induced apoptosis. a**, Hep3B cells were infected or mock-infected with recombinant adenovirus expressing antisense DAP-kinase (AdGFP–AsDAPK) or the control virus (AdGFP) at an MOI of 40. One day after infection, cells were cultured in serum-free medium for 24 h and then harvested for western blot analysis with an anti-DAPK or anti-tubulin antibody. **b**, Antisense inhibition of DAP-kinase preferentially prevents apoptosis induced by TGF-β. Hep3B cells infected with adenovirus at various MOIs were cultured as in **a** for 21 h and then treated with TGF-β (5 ng ml^{-1}) for 24 h, or TNF-α (2 ng ml^{-1}) and cycloheximide (10 μg ml^{-1}) for 72 h, or irradiated by UV at 0.01 J cm^{-2} followed by incubation for 12 h. Apoptotic cells were then assayed by FACS analysis and the percentage of cells with sub-G1 DNA content were plotted. Error bars represent s.d. (*, $P < 0.05$ compared with the AdGFP control).

Figure 6 **Expression of DAP-kinase dominant negative mutants protect Hep3B cells from TGF-β-induced apoptosis. a**, Expression of DAPK-DD in four stable transfectants. Western blot analysis of cell lysates from parental Hep3B or its stable transfectants, with an antibody specific to DAP-kinase or tubulin, as indicated. **b**, DAPK-DD suppresses TGF-β-induced apoptosis. Cells were treated with or without TGF-β for 17 h and apoptotic cells were determined with the Cell-Death Detection ELISA. Data from triplicate experiments are presented as mean ± s.d. **c**, DAPK-DD inhibits TGF-β-induced caspase-3 activity. Caspase-3 activities from cells treated with or without TGF-β for 12 h were measured by the amount of fluorescence generated from the cleavage of Ac–DEVD–AMC. **d**, Overexpression of the Myc-tagged DAPK(K42A) in Hep3B stable transfectants. Western blot analysis with an antibody to DAP-kinase, Myc or tubulin as indicated. **e**, DAPK(K42A) impairs TGF-β-induced apoptosis. Cells were treated with or without TGF-β and assayed for apoptosis, as described in **b**.

with the expression levels of DAPK-DD. To exclude the possibility that the anti-apoptotic effect of DAPK-DD is caused by a nonspecific inhibition of other death-domain-containing proteins, we also generated Hep3B stable transfectants that expressed DAPK(K42A) (Fig. 6d)[34]. Like DAPK-DD, expression of the DAPK(K42A) mutant caused a marked reduction of TGF-β-induced apoptosis (Fig. 6e) and caspase 3 activation (data not shown). Thus, our results further confirm that DAP-kinase is a critical component in the apoptotic pathway of TGF-β.

Next, we investigated whether a similar blockage could be achieved by downregulation of DAP-kinase expression with antisense RNA. A recombinant adenovirus expressing antisense DAP-kinase RNA (AdGFP–AsDAPK) was generated. Infection of Hep3B cells with AdGFP–AsDAPK led to a decrease in levels of DAP-kinase protein (Fig. 7a), and a dose-dependent inhibition of TGF-β-induced apoptosis (Fig. 7b). However, the control virus did not significantly affect apoptosis.

DAP-kinase also functions in apoptosis induced by other stimuli, including interferon-γ, TNF-α and Fas[33–36]. Thus, we investigated the effect of antisense DAP-kinase RNA on apoptosis induced by other stimuli. Infection of Hep3B cells with AdGFP–AsDAPK at an MOI of 20 did not affect TNF-α- or ultraviolet (UV)-induced apoptosis (Fig. 7c), although infection at higher MOIs (>100) led to a partial protection from these types of apoptosis (data not shown). By contrast, TGF-β-dependent apoptosis was already significantly reduced by infection with AdGFP–AsDAPK at an MOI of 20 (Fig. 7c). Thus, antisense DAP-kinase RNA is more efficient in protecting against apoptosis induced by TGF-β than by TNF-α or UV radiation.

DAP-kinase functions upstream of TGF-β-induced mitochondrial damage. Next, we determined the functional position of DAP-kinase in the apoptotic pathway of TGF-β. One major mechanism of processing and activation of procaspase-3 involves the release of cytochrome *c* from mitochondria, which activates Apaf-1 to form oligomers[39]. Thus, we examined whether TGF-β treatment of Hep3B cells could induce the release of cytochrome *c* and whether this release could be affected by overexpressing DAPK-DD. Immunofluorescence staining of Hep3B cells with an anti-cytochrome *c* antibody gave a punctate staining pattern, characteristic

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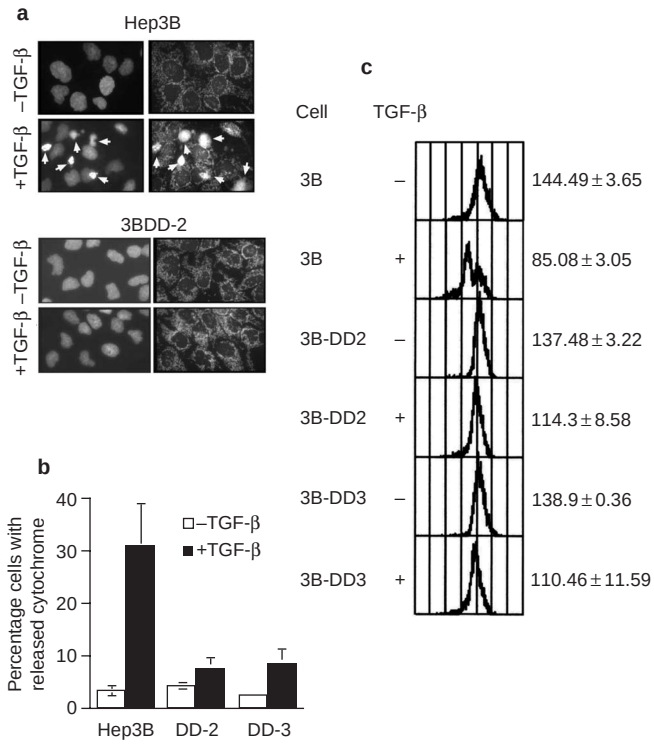

Figure 8 **Dominant negative DAP-kinase protects cells from mitochondrial damage induced by TGF-β. a**, DAPK-DD prevents the release of cytochrome *c* from mitochondria in response to TGF-β. Cytochrome *c* immunostaining (right panel) and Hoechst staining (left panel) of cells were performed 18 h after TGF-β treatment, as indicated. Cells with diffuse cytochrome *c* staining and condensed chromatin are indicated by arrows. **b**, Quantification of cells with diffuse cytochrome *c* staining. Mean ± s.d. of at least 100 cells from three independent experiments is shown. **c**, DAPK-DD prevents alterations in mitochondrial dye uptake induced by TGF-β. Cells treated with or without TGF-β for 24 h were collected and loaded with $DiOC_6$. Fluorescence intensity was determined by FACS analysis and FACS results of a representative experiment are shown on the left. Mean fluorescence intensity (± s.d.) calculated from three independent experiments is shown on the right.

of mitochondrial localization (Fig. 8a). Upon TGF-β treatment, some cells showed nuclear fragmentation and diffuse cytochrome *c* staining (Fig. 8a). Quantification of cells with diffuse cytochrome *c* staining indicated a marked increase in cytochrome *c* release after TGF-β treatment (Fig. 8b). However, in Hep3B cells stably expressing DAPK-DD, TGF-β-induced release of cytochrome *c* was significantly diminished (Fig. 8a, b). A similar finding was observed with cells stably expressing the DAPK(K42A) mutant (data not shown).

We also investigated whether expression of DAPK-DD could prevent other mitochondrial-based pro-apoptotic events occurring during TGF-β-induced apoptosis. Loss of mitochondrial inner membrane potential ($\Delta\Psi_m$) has been implicated in the execution of apoptosis, which is probably because of alterations in permeability transition pores located between the inner and outer membranes[39]. $\Delta\Psi_m$ was assessed with the mitochondrial-specific dye $DiOC_6$. TGF-β treatment of parental Hep3B led to a significant change in $\Delta\Psi_m$ (Fig. 8c). However, TGF-β-induced dissipation of $\Delta\Psi_m$ was almost completely abolished in DAPK-DD stable transfectants. Taken together, our results indicate that dominant negative inhibition of DAP-kinase blocks TGF-β-induced mitochondrial damage, suggesting that DAP-kinase acts upstream of these mitochondrial-based pro-apoptotic events.

Discussion

In this study, we demonstrate that DAP-kinase is a critical component in the apoptotic pathway of TGF-β. Several lines of evidence support this conclusion. First, TGF-β rapidly induces the expression of DAP-kinase. Second, expression of the kinase-dead mutant or the death-domain of DAP-kinase, both of which are capable of inhibiting the function of the full-length protein in a dominant negative manner[35], protects Hep3B cells from TGF-β-induced apoptosis. Third, downregulation of DAP-kinase expression with antisense RNA also significantly inhibits TGF-β-induced apoptosis. Fourth, low-level expression of DAP-kinase in Raji cells, which are normally unable to activate DAP-kinase expression in response to TGF-β, confers an ability to undergo TGF-β-induced apoptosis. Finally, ectopic expression of DAP-kinase is sufficient to trigger apoptotic death of Hep3B cells, in the absence of TGF-β stimulation. Altogether, these data indicate that DAP-kinase functions as an effector of TGF-β-dependent apoptosis. However, this conclusion does not rule out the possibility that other factors may cooperate with DAP-kinase in exerting TGF-β-induced cell death. Given the complexity of the apoptotic process and the cell-context-dependent nature of the apoptotic effect of TGF-β, other TGF-β-inducible factors are likely to be involved. Indeed, our finding that TGF-β could further enhance apoptosis induced by exogenous expression of DAP-kinase in Raji cells implies that such factors exist, as TGF-β cannot induce endogenous DAP-kinase in these cells. Further studies are required to identify these factors and to investigate their interactions.

DAP-kinase is involved in cell death induced by several stimuli[33–36], implying that it lies at a convergence point of multiple apoptotic signals. Although our demonstration that DAP-kinase is involved in TGF-β-induced apoptosis supports this notion, the finding that antisense DAP-kinase RNA inhibits the apoptotic pathway of TGF-β more efficiently than UV- or TNF-α-induced apoptosis suggests that DAP-kinase is more central to TGF-β-dependent apoptosis than other types of apoptosis. This is consistent with the ability of TGF-β to directly activate DAP-kinase expression. The mechanism for this differential contribution is currently unclear. However, the magnitude of DAP-kinase activation or induction in response to an individual apoptotic stimulus, and the combined effects of other pathways induced by the stimulus, are likely to influence the significance of DAP-kinase in each apoptotic pathway.

Although DAP-kinase is likely to be a central to cell death signalling and/or execution, its apoptotic mechanism is poorly understood. A recent study demonstrated that DAP-kinase links certain oncogenes to the $p19^{ARF}$/p53-mediated apoptotic checkpoint[36]; thus activation of the $p19^{ARF}$/p53 pathway represents at least one mechanism by which DAP-kinase exerts its effects. However, the Hep3B cell line in our study contains a homozygous deletion at the p53 locus[40] and yet it is able to respond to the apoptotic effect of DAP-kinase, suggesting that a p53-independent pathway exists.

In addition to demonstrating that DAP-kinase is essential for TGF-β-induced apoptosis, we found that TGF-β-dependent induction of DAP-kinase requires Smads. The rapid induction of DAP-kinase expression by TGF-β and the finding that this induction does not require new protein synthesis indicate that DAP-kinase is a direct target of TGF-β and Smad signalling. Furthermore, the presence of SBEs in the DAP-kinase promoter and their requirement for TGF-β-dependent induction of this promoter supports this model. Therefore, it is likely that Smad-mediated expression of DAP-kinase is the event that initiates TGF-β-induced apoptosis. Accordingly, ectopic expression of DAP-kinase at a level that mimics the induction in response to TGF-β is sufficient to cause apoptosis, whereas downregulation of DAP-kinase expression by antisense RNA prevents cells from undergoing TGF-β-dependent apoptosis. Furthermore, ectopic expression of DAP-kinase in the DAP-kinase-deficient Raji cells restores sensitivity to TGF-β-dependent apoptosis. Taken together, these data indicate that induction of DAP-kinase by TGF-β is essential for TGF-β-induced apoptosis.

The TGF-β-responsive region of the DAP-kinase promoter contains four copies of the SBE[41] and two copies of binding sites for the acute myeloid leukaemia (AML) family of transcription factors. Interestingly, these AML sites are either proximally flanked by, or overlap with, SBEs. Smads activate transcription in response to TGF-β by cooperating with DNA-binding transcription factors[19–22]. Accordingly, Smad3 and Smad4 cooperate with AML transcription factors through an AML–Smad3 interaction, to activate TGF-β-induced transcription from the immunoglobulin-α promoter[42–44]. Thus, it is likely that TGF-β-induced transcription from the DAP-kinase promoter is mediated by the functional synergy of Smad proteins with AML transcription factors. The presence of SBEs in the DAP-kinase promoter suggests that induction of DAP-kinase by TGF-β is not restricted to Hep3B cells. Indeed, TGF-β-dependent activation of the DAP-kinase promoter is also observed in *smad3* or *smad4* null cells, when the corresponding Smad is ectopically reintroduced.

Our demonstration that a dominant negative DAP-kinase mutant blocks TGF-β-induced release of cytochrome *c* and TGF-β-induced dissipation of $\Delta\Psi_m$ suggests that this kinase functions upstream of certain mitochondrial-based pro-apoptotic events. This agrees with a previous finding that Bcl-2 protects cells from apoptotic death induced by an active mutant of DAP-kinase[35]. Interestingly, another mediator of TGF-β-induced apoptosis, ARTS, is a mitochondrial septin that translocates to the nucleus in response to TGF-β[28,29]. Overexpression of ARTS increases apoptotic sensitivity to TGF-β, even in cells that do not normally undergo TGF-β-dependent apoptosis. Whether ARTS and DAP-kinase act along the same pathway, or on parallel pathways, is currently unknown. Despite their distinct subcellular localization, both of their functions are connected with mitochondrial-based events. In addition, both molecules contain P-loop motifs and other motifs found in small GTPases[29,33,45]. The P-loop motif of ARTS is essential for its pro-apoptotic activity[29]. Additional studies will be required to determine the functional relationship between these two proteins. Together, our results indicate that DAP-kinase functions as an apoptotic effector of TGF-β by linking Smad to mitochondrial pro-apoptotic events. □

Methods

Cell culture and transfection. The human hepatoma cell line Hep3B was cultured and treated with TGF-β (R&D Systems, MIN) as described previously[3]. *Smad3*$^{-/-}$ mouse embryonic fibroblasts and SW480.7 human breast carcinoma cells were cultured in DMEM containing 15% and 10% fetal bovine serum, respectively. Transfections were performed with Lipofectamine (Life Technologies, New England) according to the manufacturer's instructions.

cDNA microarray analysis. A high-density cDNA microarray filter membrane[46] was obtained from Konan Peck. cDNA microarray analysis was performed essentially as described[46]. Briefly, mRNA was purified with Oligotex-dT resins (Qiagen, CA) from Hep3B cells treated with TGF-β for 0, 2 or 8 h. The MMLV reverse transcriptase was incubated with 2 μg of each mRNA sample to generate biotin-labelled cDNA probe. The resulting probe was hybridized with arrayed cDNAs on the nylon membrane. The filter was then washed in SSC/SDS solutions. For detection of hybridized probes, the filter was incubated with β-galactosidase-conjugated-Streptavidin (Roche Molecular Biochemicals, Germany) and then stained with X-gal substrate. After developing and scanning the image, the probe was stripped for the next hybridization. Quantitative information on gene expression was generated with image analysis software, as described[46].

Northern blot analysis. Total RNA was isolated from Hep3B cells with Trizol reagents (Life Technologies). RNAs were separated on a formaldehyde agarose gel, transferred to a nylon filter and then hybridized with a probe corresponding to the 3′ untranslated region of the DAP-kinase cDNA or the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The blot was washed with SSC/SDS solutions before autoradiography.

Expression constructs. DAP-kinase cDNA was generated by RT-PCR with RNA purified from Hep3B cells. Four PCR fragments were amplified, corresponding to the DAP-kinase cDNA base pairs 331–1,553, 1554–2,866, 2,867–4,174 and 4,175–4,632. Sequences were verified by sequence analysis. The cDNA fragments were cloned into the pRK5M expression vector[47] to generate C-terminally Myc-tagged, full-length DAP-kinase. DAPK(K42A) was generated by site-directed mutagenesis with the Quick-Change site-directed mutagenesis kit (Strategene, CA). To generate pRK5M–DAPK-DD, the death-domain of DAP-kinase (amino acids 1,271–1,431) was amplified by PCR and cloned into pRK5M.

Preparation of DAP-kinase antigen and antibodies. A His-tagged DAP-kinase C-terminal portion (amino acids 843–1,431) was generated by insertion of an *Xba*I–*Sal*I fragment derived from pRK5M–DAPK into the pET30a vector. This fusion protein was purified from *Escherichia coli* BL21 cells with His–Bind Resin (Novagen, WI) according to the manufacturer's instructions and then used to immunize a rabbit. The resulting polyclonal antiserum was purified further by affinity chromatography.

Generation of reporter constructs and luciferase assay. A bacterial artificial chromosome genomic clone (GenBank accession number 007847) containing the 5′ flanking region of the human DAP-kinase gene was obtained from Genome System. A *Sma*I–*Sac*I fragment corresponding to −1,750 to +230 of the DAP-kinase promoter was cloned to pGL2 vector (Promega, WI). This promoter fragment was digested by *Nde*I and *Bam*HI to generate the −705 to +230 and the −352 to +230 reporter constructs, respectively. The −204 to +230 and the +54 to +230 reporters were made by PCR amplification of the corresponding fragments. Sequences were confirmed by sequence analysis. To analyse the promoter activity of DAP-kinase, these reporter constructs and pRK5–βGal[47] for β-galactosidase expression were transfected into cells in the presence or absence of various Smad expression plasmids. One day after transfection, cells were serum starved for 6 h and then treated with or without TGF-β (5 ng ml^{-1}) for 18 h. Luciferase and β-galactosidase activities were quantified with the Luciferase Assay System (Promega) and the Galacto-Star System (Applied Biosystems, CA), respectively.

Generation of recombinant adenovirus and infection of cells. Recombinant adenoviruses were constructed with the AdEasy system, essentially as described[48]. Briefly, the coding region of DAP-kinase or its kinase-dead mutant, both of which include a C-terminal Myc tag, was excised from pRK5M and inserted into pAdTrack–CMV in a sense or antisense direction. This plasmid, together with the adenoviral vector pAdEasy, were introduced into *E. coli* BJ5138 cells to allow homologous recombination. Early-passage HEK293 cells were infected with the recombinant adenoviral plasmid to produce viral particles. Hep3B cells were infected at various MOIs for 1 h with recombinant adenovirus carrying DAP-kinase (AdGFP–DAPK), the kinase-dead mutant (AdGFP–DAPK(K42A)), or the control virus AdGFP[48]. After washing with PBS, cells were cultured in serum-free medium for 96 h. Cells were then harvested, stained with propidium iodide, and analysed by FACS to detect sub-G1 populations. In other experiments, Hep3B cells were infected with AdGFP–AsDAPK for antisense DAP-kinase expression. Infected cells were cultured in serum-free medium for 21 h and then treated with TNF-α and cycloheximide, TGF-β, or UV and then analysed by FACS.

DNA fragmentation assays and caspase 3 activity assays. DNA fragmentation was quantified with Cell Death Detection ELISA (Roche Molecular Biochemicals) as described[49]. Caspase 3 activities were measured by incubating cell lysates with a fluorogenic peptide substrate, DEVD–AMC, as previously described[49].

Cytochrome *c* immunostaining. Cells were fixed in PBS containing 4% formaldehyde for 15 min and permeabilized in PBS with 0.2% Triton-X100 for 4 min. Cells were then incubated in blocking buffer containing PBS with 10% fetal calf serum for 30 min and washed three times with PBST (PBS containing 0.2% Tween20). Cells were then incubated with a 1:200 dilution of anti-cytochrome *c* (clone 6H2.B4, PharMingen) for 1 h, washed three times with PBST, and then incubated with a 1:500 dilution of fluorescein isothiocyanate-conjugated anti-mouse antibody in the presence of 1 μg ml^{-1} Hoechst 33258 for 1 h. Cells were washed with PBST and the slides were drained and mounted.

Detection of $\Delta\Psi_m$. For detection of $\Delta\Psi_m$, cells were trypsinized, washed with PBS, and incubated in 100 μl of 100 nM $DiOC_6$ (Molecular Probes) at 37°C for 30 min. Cells were then precipitated, resuspended in PBS and analysed by FACS.

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Correspondence and requests for materials should be addressed to R.-H.C.
Supplementary information is available on Nature Cell Biology's website (http://cellbio.nature.com).

Supplementary Information

Table S1: List of genes whose expression was not altered by TGF- treatment of Hep3B cells.

Accession #	Gene Name	Confirmed by
AA100775	STAT1	Northern
AA028975	KHS-1	Northern
L32976	MLK-3	Northern
U17743	JNKK-1	Northern
U09578	mapkap kinase (3pk)	Northern
U45878	IAP-1 (MIHC)	Northern
U45879	IAP-2 (MIHB)	Northern
M25753	cyclin B1	Northern
U13699	caspase 1	Northern
U13737	caspase 3	Northern & Western
U28014	caspase 4	Northern
U56390	caspase 9	Western
L22474	Bax	Western
U66879	BAD	Western
M14745	Bcl-2	Northern & Western *
Z23115	Bcl-XL	Northern
L08246	Mcl-1	Western
M33294	TNF RI	Western
NM003824	FADD	Western

*, no detectable expression

In Wahl & Carr (*Nature Cell Biol.* **3,** E277–E286 (2001)), the empty box next to histone acetyltransferase should contain the words 'p300/CBP' in Fig. 2.

In Deitrick & Rosen (*Nature Cell Biol.* **3,** E31 (2002)), the surname of Mara Kreishman-Deitrick was spelt incorrectly.

In Bourguignon *et al.* (*Nature Cell Biol.* **4,** E22–E23 (2002)), the legends for Figs 1 and 2 were inadvertently reversed.

In Jang *et al.* (*Nature Cell Biol.* **4**, 51–58 (2002)), Fig. 3b should be as below. Fig. 4b and c should also be exchanged, and the *x* axis in Fig. 6e should be Hep3B, K42A-1, K42A-2 and K42A-3.