

DAP kinase activates a p19^{ARF}/p53-mediated apoptotic checkpoint to suppress oncogenic transformation

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DAP kinase is a pro-apoptotic calcium-regulated serine/threonine kinase, whose expression is frequently lost in human tumours. Here we show that DAP kinase counteracts oncogene-induced transformation by activating a p19^{ARF}/p53-dependent apoptotic checkpoint. Ectopic expression of DAP kinase suppressed oncogenic transformation of primary embryonic fibroblasts by activating p53 in a p19^{ARF}-dependent manner. Consequently, the fibroblasts underwent apoptosis, characterized by caspase activation and DNA fragmentation. In response to c-Myc or E2F-1, the endogenous DAP kinase protein was upregulated. Furthermore, functional or genetic inactivation of the endogenous DAP kinase reduced the extent of induction of p19^{ARF}/p53 and weakened the subsequent apoptotic responses to c-Myc or E2F-1. These results establish a role for DAP kinase in an early apoptotic checkpoint designed to eliminate pre-malignant cells during cancer development.

A functional approach to gene cloning has identified DAP kinase as a positive mediator of apoptosis^{1,2}. This protein kinase was shown to modulate cell death induced by interferon- γ , Fas, tumour necrosis factor- α , and detachment from extracellular matrix^{3–5}, indicating its wide involvement in apoptosis. DAP kinase is a Ca²⁺/calmodulin (CaM)-regulated serine/threonine kinase that carries a conserved death domain⁶ and associates with actin microfilaments³. The enzyme's active site is regulated by an adjacent CaM-binding domain, the deletion of which generates a constitutively active kinase (DAPK- Δ CaM) with enhanced ability to induce apoptosis³. A second mode of autoinhibition engages the serine-rich carboxy-terminal tail, spanning the last 17 amino acids of the protein⁷. The downstream effectors and the biochemical pathways through which DAP kinase executes its pro-apoptotic actions are not known.

A link between DAP kinase and cancer was recently established. It was found that expression of DAP kinase mRNA and protein were frequently lost in human cancer cell lines, often as a result of silencing by DNA methylation⁸. Further analysis of DNA extracted from human tumour samples showed high incidence of hypermethylation of the DAP kinase 5'-untranslated region (UTR) in B-cell malignancies⁹, non-small-cell lung carcinoma¹⁰ and head and neck cancer¹¹. The anti-tumorigenic effect of DAP kinase was demonstrated in mouse model systems where the reintroduction of DAP kinase into highly metastatic mouse lung carcinoma cells strongly reduced their metastatic capacity⁵. Thus, it appears that loss of DAP kinase confers a selective advantage to cancer cells and may have a causative role in tumour progression. We investigated whether DAP kinase could also affect the initial steps of oncogenesis. We found that it suppresses oncogene-induced transformation of primary embryonic fibroblasts by activating p53 in a p19^{ARF}-dependent manner. Moreover, we showed that this effect of DAP kinase reflects its involvement in an intrinsic p53-dependent apoptotic checkpoint that is turned on by oncogenes at the initial stages of transformation.

Results

Suppression of oncogenic transformation by DAP kinase requires p53. Quantitative primary cell transformation assays using various combinations of oncogenes were used to assess the anti-oncogenic action of DAP kinase and to functionally position it along known growth control pathways. As shown in Fig. 1a, addition of activated DAP kinase (DAPK- Δ CaM) to Myc and Ras or E1a and Ras co-transformation assays in rat embryo fibroblasts (REFs) caused a significant reduction in the number of foci. In contrast, transformation by a combination of SV40 large T antigen (T-Ag) and Ras was completely refractory to inhibition by activated DAP kinase (Fig. 1a), in spite of its continuous expression in the transfectants (data not shown). These results suggested that T-Ag may inactivate DAP kinase protein itself or a protein critical for its action.

T-Ag binds and affects a large number of cell proteins, including the two key tumour suppressors—retinoblastoma protein (Rb) and p53 (refs 12–14). Interestingly, DAP kinase regained its suppressive effects on transformation when a deletion mutant of T-Ag (dl1001)¹⁵, which lacks the C-terminal p53-binding region, was used instead of the full-length protein (80% reduction in the number of foci, $P < 0.05$; Fig. 1a). To test more directly whether functional p53 is necessary for the anti-oncogenic activity of DAP kinase, we used two different dominant-negative mutants of p53—Phe132 (ref. 16) and the DD miniprotein¹⁷. Addition of either of these neutralized the suppressive effects of DAP kinase in the focus assays (Fig. 1a). Moreover, in p53-null mouse embryo fibroblasts (MEFs), the introduction of active DAP kinase completely failed to inhibit focus formation induced by a combination of T-Ag dl1001 and Ras (Fig. 1b) or Myc and Ras (data not shown). This stood in contrast to wild-type MEF cultures, where transfection with activated DAP kinase caused a striking reduction (about 90%, $P < 0.05$) in the number of foci (Fig. 1b). These results unequivocally establish an essential role for p53 in DAP kinase-induced suppression of oncogenic transformation.

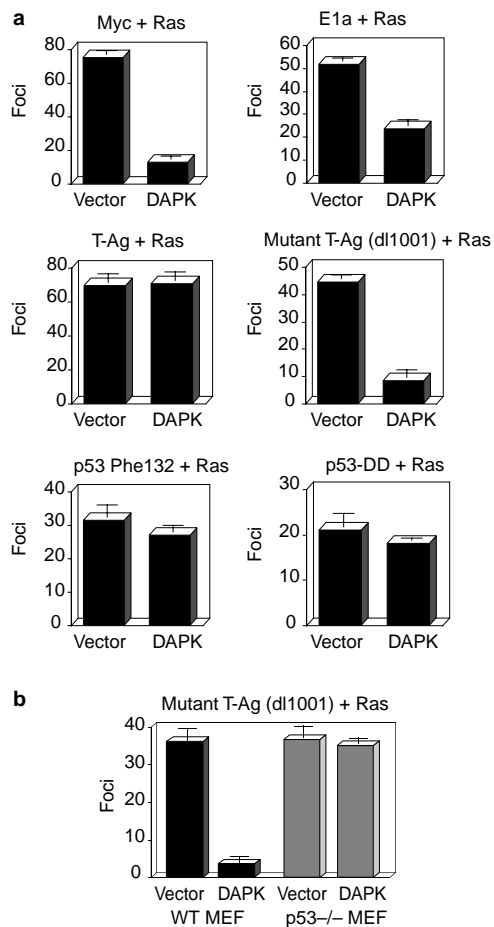


Figure 1 Suppression of transformation by DAP kinase in primary rodent fibroblasts. **a**, Primary rat embryo fibroblasts (REFs) were transformed with the indicated combination of oncogenes, with the addition of either an empty vector (pBabe-puro), or the same vector carrying human activated DAP kinase (Δ CaM). Foci were counted 14–16 days later. Each assay was done in triplicate and the bars represent mean (\pm s.d.) numbers of foci per 9-cm plate from a representative experiment. **b**, Mouse embryo fibroblasts (MEFs) derived from either wild-type (WT) or p53^{-/-} embryos (from a similar genetic background) were transformed with a combination of mutant SV40 large T antigen (T-Ag (dl1001)) and Ras, and the effect of DAP kinase on numbers of foci was assessed as in **a**.

DAP kinase activates p53 via p19^{ARF}. The requirement for wild-type p53 suggested that DAP kinase could activate p53 to achieve its tumour suppressive effects. To test this hypothesis we measured p53-dependent transcriptional activation in a reporter assay^{18,19}. To this end, 10(1) cells²⁰ that lack endogenous p53 were transiently co-transfected with the activated DAP kinase, p53 and a reporter plasmid driving luciferase gene transcription from the p53-responsive *mdm2* promoter. As shown in Fig. 2a, DAP kinase stimulated transcription from the *mdm2* promoter in a dose-dependent and p53-dependent manner, whereas no significant effects on transcription from an irrelevant promoter (rous sarcoma virus (RSV)) were detected.

We next introduced the activated DAP kinase into MEFs by high-efficiency retroviral infection, and examined its effect on the level of endogenous p53 and p53-induced proteins. The activated DAP kinase caused an increase in the amount of p53, as well as its targets, Mdm2 and p21, exclusively in wild-type and not in p53-null MEFs (Figs 2b, c). Among the various mechanisms that activate p53, a role for p19^{ARF} has been established in which it stabilizes

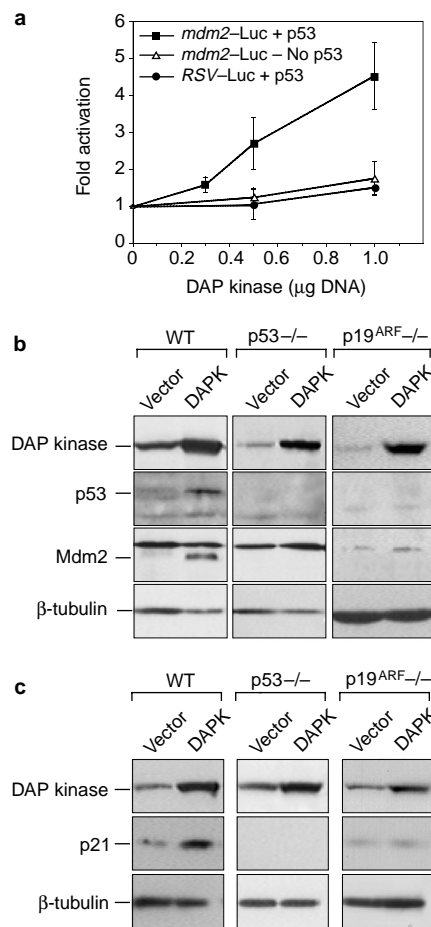


Figure 2 Activation of p53 by DAP kinase. **a**, 10(1) cells (10^5 per 35-mm dish) were transfected with 300 ng of a reporter plasmid carrying luciferase cDNA driven by the p53-responsive promoter of human Mdm2 (*mdm2*-Luc), or by RSV promoter (RSV-Luc), together with the indicated amounts of pBabe-puro carrying human activated DAP kinase cDNA. Where indicated, 10 ng of plasmid expressing human wild-type p53 was included. Luciferase activity in cell lysates was determined 16 h post-transfection. The graph represents mean (\pm s.d.) values, from three experiments, of the fold increase in luciferase activity upon addition of activated DAP kinase. The actual basal values of *mdm2*-Luc activity were enhanced by two orders of magnitude upon addition of wild-type p53 alone. **b**, Early-passage wild-type (WT), p53-null or p19^{ARF}-null MEFs were infected with retroviruses carrying either an empty vector (pBabe-puro) or a vector coding for activated DAP kinase. Forty-eight hours later puromycin was added, and cells remaining on the plate after 5 days of selection were collected. Equal amounts of protein extracts were analysed by western blotting for expression levels of DAP kinase, p53 and Mdm2 proteins. β -tubulin was used as a loading control. **c**, MEFs (wild-type, p53-null or p19^{ARF}-null) were infected as in **b**, and harvested 24 h later. The levels of DAP kinase and p21 proteins were analysed by western blotting.

p53 by binding to Mdm2 and preventing p53 degradation^{21,22}. To test whether activation of p53 by DAP kinase is dependent on p19^{ARF}, similar infections with retroviral vectors carrying activated DAP kinase were carried out in p19^{ARF}-/- primary MEFs. Neither p53 itself (Fig. 2b) nor its target the p21 protein (Fig. 2c), were induced by activated DAP kinase in these cells, suggesting that the effects of DAP kinase on p53 are mediated by p19^{ARF}. Together, these results reinforce a functional interaction, placing DAP kinase upstream of p53, and also indicate that DAP kinase executes its anti-oncogenic action through activation of p53 in a mechanism that requires p19^{ARF}.

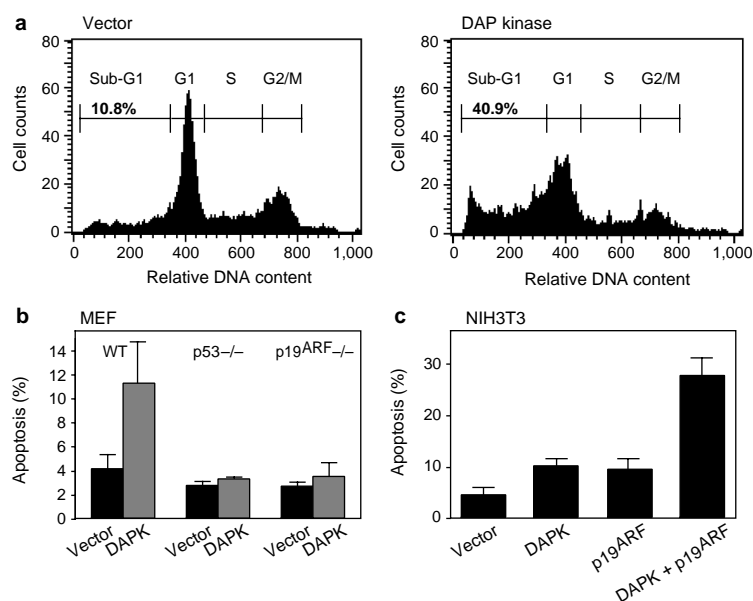


Figure 3 DAP kinase induces p19^{ARF}/p53-dependent apoptosis. **a**, REFs were transfected with either an empty vector (pcDNA3), or the same vector carrying activated DAP kinase together with a plasmid coding for membrane-bound GFP. Forty-eight hours later, the DNA content of GFP-positive cells was analysed by FACS. Cells with sub-diploid DNA content (Sub-G1) were defined apoptotic. **b**, Wild-type, p53-null and p19-null MEFs were transfected with the indicated plasmids and

apoptosis was assessed by FACS as in **a**. The histogram represents mean (\pm s.d) values from at least three separate experiments. **c**, NIH3T3 cells were transfected with GFP and either an empty vector (pcDNA3), or the same vector carrying p19^{ARF} or activated DAP kinase cDNA, and apoptotic cells were scored microscopically 24 h later according to morphological hallmarks, including cell shrinkage and membrane blebbing.

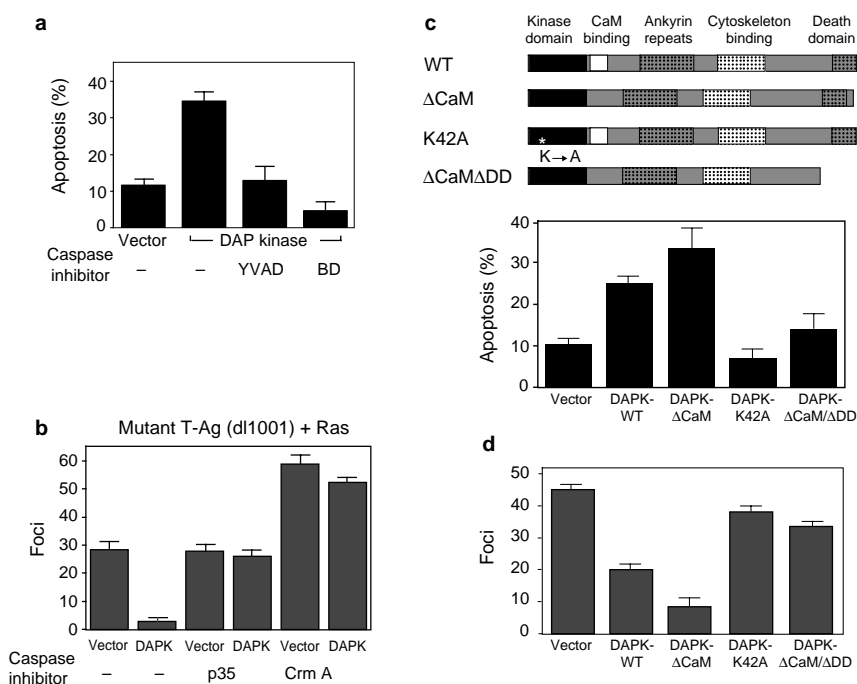


Figure 4 DAP-kinase-induced apoptosis requires the catalytic activity of the kinase, and involves caspase activation. **a**, REFs were transfected with the indicated plasmids and GFP, and the fraction of GFP-positive apoptotic cells carrying sub-G1 DNA content was analysed by FACS. Where indicated, the peptide caspase inhibitors YVAD-fmk and BD-fmk were added to the culture medium. **b**, REFs were transfected with the combination of mutant SV40 large T antigen (T-Ag) (dl1001) and Ras, with the addition of either an empty vector (pBabe-puro) or a vector carrying the activated DAP kinase. Where indicated, expression plasmids coding for the protein caspase inhibitors

p35 or Crm A were added as well. Each bar represents mean (\pm s.d) numbers of foci per 9-cm plate. **c**, REFs were transfected with 5 μ g of empty vector (pcDNA3), or with pcDNA3 carrying cDNAs of the different DAP kinase forms. GFP was used as a marker, and apoptosis was analysed by FACS as in **a**. The histogram represents data averaged from three separate experiments. **d**, REFs were transfected with the oncogene combinations T-Ag(dl1001) + Ras with the addition of empty vector, or a vector coding for one of the indicated DAP kinase forms. The values represent averaged counts of foci from a representative experiment done in triplicate.

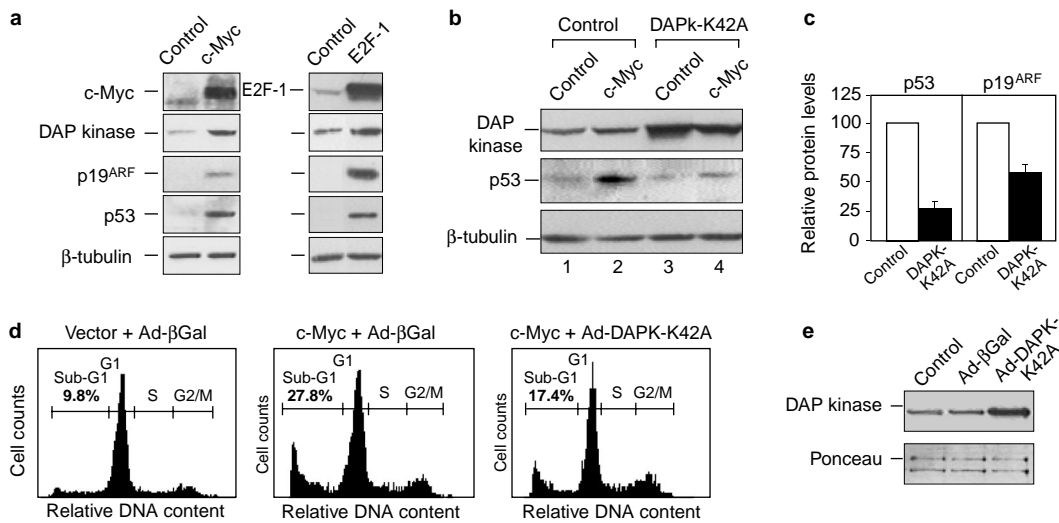


Figure 5 Inactivation of DAP kinase by a dominant-negative mutant reduces induction of p53 and apoptotic responses to c-Myc. **a**, Early-passage wild-type MEFs were infected with retroviruses carrying either an empty vector (pBabe-puro) or the same vector coding for c-Myc or E2F-1. Forty-eight hours later, infected cells were selected with puromycin for the following 3 days. Cells were then harvested and lysed, and equal amounts of protein extracts were analysed by western blotting for expression levels of different proteins, as indicated. β -tubulin was used as a loading control. **b**, Early-passage wild-type MEFs were infected with retroviruses carrying either a control vector (pBabe-puro) or the same vector coding for DAP kinase dominant-negative mutant (DAPK-K42A). Twenty-four hours later the cells were infected with either an empty vector (pBabe-Bleo), or the same vector expressing c-Myc. The next day, cells were selected with puromycin to ensure mutant DAP kinase expression, and 3 days later the cells were harvested. Equal amounts of protein extracts were analysed by western blotting for expression levels of the indicated proteins. Anti-DAP kinase monoclonal antibodies recognize both the endogenous

protein and the exogenous DAPK-K42A mutant. β -tubulin was used as a loading control. **c**, Relative protein levels were determined by densitometry of the signals. Results averaged from two separate experiments are presented, and Myc-induced p53 and p19^{ARF} levels measured in the control cell population are referred to as 100%. **d**, REFs were infected with adenovirus carrying either a β -galactosidase cDNA (Ad- β Gal) or a dominant-negative mutant of DAP kinase (Ad-DAPK-K42A). After one day the cells were transfected with either an empty vector (pcDNA3), or a vector coding for c-Myc together with a plasmid carrying a membrane-targeted GFP marker. Twenty-four hours later, the cells were shifted to medium containing 0.1% serum. Apoptosis was measured after additional 24 h by FACS, as the fraction of GFP-positive cells with sub-G1 DNA content. This experiment was repeated three times and the average extent of protection was $41.6 \pm 3.4\%$. **e**, Western blot analysis of DAP kinase in cells infected with Ad- β Gal or Ad-DAPK-K42A. Ponceau staining is presented as a loading control.

DAP kinase induces p19^{ARF}/p53-dependent apoptosis. DAP kinase could exert its p53-mediated suppressive effects on oncogenic transformation by imposing growth arrest, apoptosis or both^{23,24}. To distinguish between these possibilities we introduced activated DAP kinase into REFs, and examined their fate by FACS analysis of their DNA content. As shown in Fig. 3a, at 48 hour post-transfection, almost 40% of the transfected REFs had a sub-G1 DNA content indicative of DNA fragmentation, showing that DAP kinase evoked an apoptotic programme in those cells. Furthermore, no alterations in cell-cycle distribution of the viable transfectants were apparent, indicating that, in this setting, DAP kinase operates exclusively through induction of apoptosis. Similar apoptotic responses were observed when DAP kinase was co-transfected into REFs with Myc and Ras (data not shown). In contrast to the effect in wild-type cells, transfection of activated DAP kinase into p53-null, or p19^{ARF}-null MEFs did not significantly increase the fraction of cells with sub-G1 DNA content (Fig. 3b), suggesting that each of these two genes were essential for the apoptotic effects of DAP kinase. Notably, in these p53- and p19^{ARF}-deficient primary fibroblasts, DAP kinase caused the appearance of multi-nucleated cells, probably as a result of inhibition of cytokinesis (data not shown), reflecting activation of additional p53-independent pathway(s).

We next tested whether the reintroduction of p19^{ARF} into cells deficient for this protein could restore the apoptotic responses to DAP kinase. Transfection of activated DAP kinase alone into NIH3T3 cells, which are deficient for the *INK4a* locus and lack p19^{ARF} expression²⁵, exerted weak apoptotic effects in these cells, as determined by morphological alterations (Fig. 3c). Ectopic morphological alterations expression of p19^{ARF} in these cells induced

primarily G1 arrest (data not shown) and very little apoptosis (Fig. 3c), consistent with previous reports²⁵, and indicating that the mere activation of p53 by p19^{ARF} is not sufficient to drive fibroblasts towards apoptosis. Yet, the co-transfection of p19^{ARF} and DAP kinase greatly enhanced the extent of apoptosis (Fig. 3c). These results reinforce a functional link between DAP kinase and p19^{ARF}, and indicate that other DAP-kinase-activated events might cooperate with the p19^{ARF}/p53 pathway by creating the appropriate cellular context that channels activated p53 towards apoptosis.

Induction of apoptosis by DAP kinase in wild-type REFs was mediated by caspase activation, as demonstrated by the elimination of the sub-G1 cell population after addition of the caspase peptide inhibitors YVAD-fmk or BD-fmk to the culture medium (Fig. 4a). Furthermore, caspase inhibition by the viral proteins crmA or p35 completely abrogated the negative effects of DAP kinase on focus formation, thus tightly linking suppression of transformation to apoptosis (Fig. 4b). We therefore conclude that DAP kinase suppresses oncogenic transformation by inducing a classical type of apoptosis that depends on p19^{ARF} and p53, and involves caspase activity and DNA fragmentation, with no detectable effects on cell-cycle progression. Because activation of p53 by DAP kinase, as assessed by the reporter assays, was not prevented by caspase inhibitors (data not shown), it is assumed that the caspases operate mainly downstream of activated p53.

Functional examination of several DAP kinase mutants revealed that the ability of DAP kinase to induce apoptosis and suppress focus formation depended on the potency of its catalytic activity and on the presence of the death domain. In both assays, the DAPK- Δ CaM mutant, whose kinase domain is constitutively

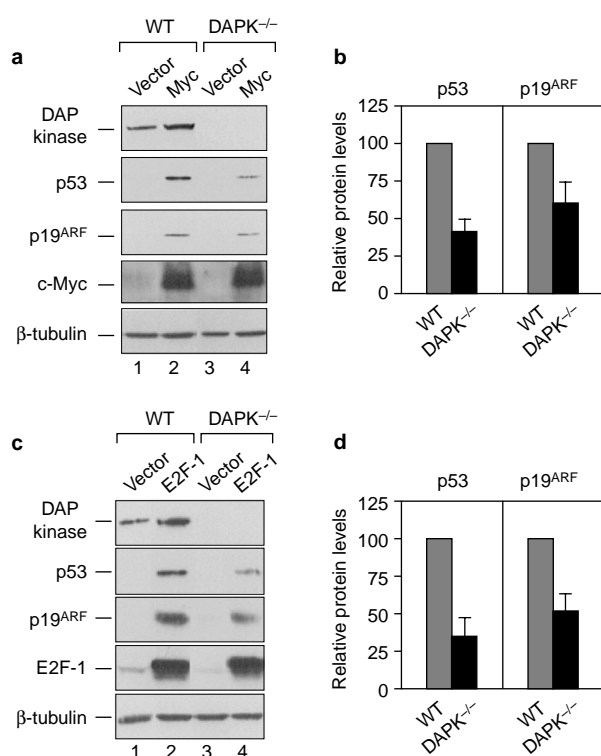


Figure 6 DAP-kinase-deficient MEFs display reduced extent of p53 induction by c-Myc or E2F-1. **a, c,** Early-passage wild-type (WT) or DAPK^{-/-} MEFs were infected with retroviruses carrying either an empty vector (pBabe-puro) or the same vector coding for c-Myc (a) or E2F-1 (c). Twenty-four hours later, puromycin was added to select for infected cells. After 3 days in selection, cells were harvested and lysed. Equal amounts of protein extracts were analysed by western blotting for expression levels of DAP kinase p53, p19^{ARF} and c-Myc (a) or E2F-1 (c) proteins. β -tubulin served as a loading control. **b, d,** Densitometric analysis of p53 and p19^{ARF} protein levels induced by c-Myc (b) or E2F-1 (d). The levels of these proteins in c-Myc or E2F-1-infected wild-type cells are referred to as 100%.

active, was more effective than wild-type DAP kinase, whereas the ‘dead’ kinase mutant, K42A, was ineffective (Fig. 4c, d). Deletion of the death domain from DAPK- Δ CaM, reduced both the induction of apoptosis and the inhibition of focus formation (Fig. 4c, d). These results stress the functional importance of these two domains in DAP kinase, and provide independent support for the notion that induction of apoptosis underlies the anti-oncogenic activity of DAP kinase.

Involvement of DAP kinase in the Myc-induced rise in p53 and apoptosis. The effects of DAP kinase shown here could reflect its possible function in an intrinsic apoptotic checkpoint, which is turned on by oncogenes early during cellular transformation to protect cells against uncontrolled hyperproliferative signals^{26,27}. We therefore tested the regulation and functional involvement of DAP kinase during oncogene-induced apoptosis. First, we found that when either c-Myc or E2F-1 were introduced into MEFs by retroviral infection, endogenous DAP kinase protein levels were elevated, along with the expected increase in p19^{ARF} and p53 proteins (Figs 5a, 6a and c; the increase in DAP kinase protein ranged between two- to fourfold in the different experiments). Second, the contribution of the endogenous DAP kinase to Myc-induced elevation of p53 protein levels was examined by infection of MEFs with retroviral vectors carrying the catalytically inactive mutant, K42A, previously shown to function in a dominant-negative manner³. As shown in Fig. 5b, the levels of the DAPK-K42A mutant protein significantly

exceeded those of the endogenous wild-type protein, suggesting that the mutant can compete with the endogenous DAP kinase (compare lanes 3 and 4 to 1 and 2 in Fig. 5b). Examination of p53 levels in the different infected cell populations showed that in cells expressing DAPK-K42A, inhibition of DAP kinase attenuated the c-Myc-induced increase in p53 protein levels (Fig. 5b, compare lanes 2 and 4). Quantitative assessments indicated that in the presence of the DAPK-K42A mutant, the extent of p53 and p19^{ARF} induction by c-Myc was lowered by 70% and 40%, respectively (Fig. 5c).

To test whether the functional inactivation of DAP kinase eventually interferes with the cellular responses to c-Myc, we used REFs, which are more amenable than MEFs for the direct assessment of apoptosis. REFs were infected with a recombinant adenovirus carrying the mutant kinase (Ad-DAPK-K42A) or with a control virus. The DAPK-K42A mutant was expressed at greater levels than the endogenous protein (Fig. 5e). Subsequently, the infected cells were co-transfected with the genes for c-Myc and green fluorescent protein (GFP), and 24 hours later shifted to low-serum conditions to trigger apoptosis. Inactivation of DAP kinase by the dominant-negative mutant partially, yet significantly, protected the cells from these apoptotic effects (average protection 41.6 \pm 3.4%; $P < 0.05$; Fig. 5d), demonstrating a role for the endogenous DAP kinase in mediating apoptosis induced by c-Myc.

DAP kinase deficiency weakens the p53 and apoptotic responses to hyperproliferative signals. To examine directly the role of DAP kinase in the molecular and cellular responses to oncogene activation, we have used MEFs derived from DAP-kinase-null (DAPK^{-/-}) mice that were recently generated in our laboratory. First, c-Myc or E2F-1 were ectopically expressed by retroviral infection in early-passage (p4) wild-type (control) and DAPK^{-/-} MEFs, and the consequent rise in levels of p53 and p19^{ARF} in the two cell types was monitored by immunoblotting. Both c-Myc and E2F-1 reached equal levels in wild-type and DAPK^{-/-} cell cultures (Figs. 6a, c). The lack of DAP kinase in the null fibroblasts caused a significant decrease in the rise in p53 and p19^{ARF} compared with wild-type control MEFs (Fig. 6a, c, compare lanes 2 and 4). Densitometric analysis of proteins from several experiments showed that c-Myc-mediated induction of p53 and p19^{ARF} was reduced by 60% and 40%, respectively (Fig. 6b), and that E2F-1-mediated induction of p53 and p19^{ARF} was reduced by 65% and 50%, respectively (Fig. 6d). Thus, the knock-out experiments are consistent with data derived from the use of the dominant-negative mutant, supporting the view that functional DAP kinase is necessary to achieve full activation of p19^{ARF}/p53 by aberrant proliferative signals.

We next tested whether this partial reduction in p53 responses would result in reduced sensitivity of DAP-kinase-deficient cells to apoptosis. To this end, we infected cells with retrovirus carrying E2F-1, which in wild-type MEFs can trigger massive apoptosis without additional stimuli. Wild-type and DAPK^{-/-} MEFs were infected with retroviruses carrying either an empty vector or E2F-1. Sixteen hours after infection, the cells were plated in 96-well dishes (5,000 cells per well), and cell viability was assessed 6 days later by a neutral red uptake assay. The extensive cell killing by E2F-1 in wild-type fibroblasts was significantly weakened in the knock-out MEFs, resulting in an approximately threefold increase in cell viability at this time point (Fig. 7a). The reduced apoptotic responses to E2F-1 were also apparent macroscopically upon crystal violet staining of the plates (Fig. 7b). The analysis of cell lysates, which were prepared from these infected cultures at an earlier time (48 h), showed that E2F-1 protein levels were similar in both wild-type and knock-out cell cultures (Fig. 7c). Also, the infection efficiency, as estimated by selection with puromycin, using the control retroviral vector for infection, was similar in the two cell types (data not shown). Microscopic examination of the puromycin-resistant E2F-1-infected cells at 48 h after plating revealed that in the DAPK^{-/-} cultures, the fraction of cells with rounded apoptotic morphology was smaller than that of the corresponding wild-type cells (Fig. 7d: average values from four separate fields are 40.5 \pm 2% and

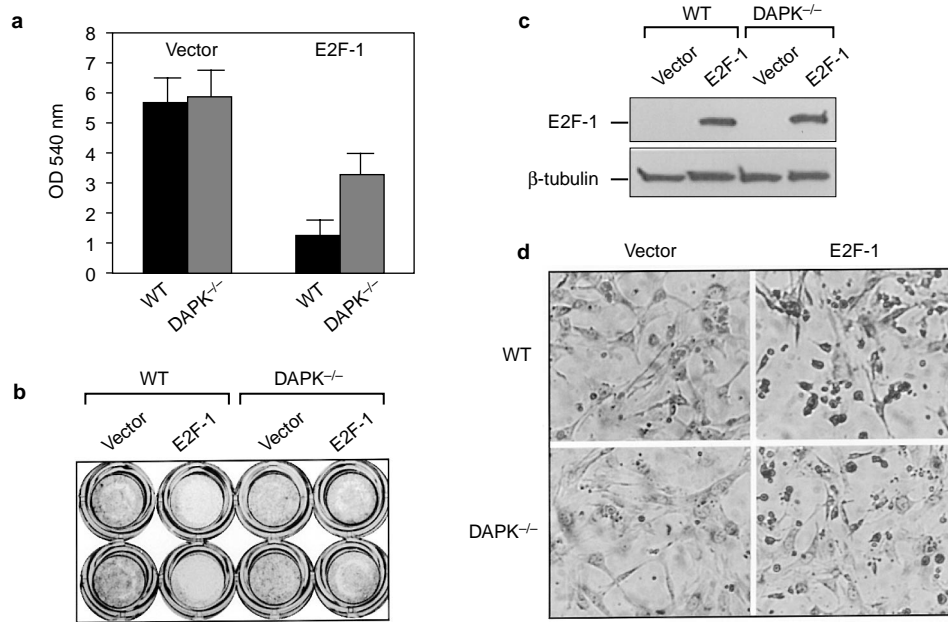


Figure 7 E2F-1-induced apoptosis is attenuated in the absence of DAP kinase. Early passage wild-type (WT) or DAPK^{-/-} MEFs were infected with retroviruses carrying either an empty vector (pBabe-puro) or the same vector coding for E2F-1. **a**, Sixteen hours after infection the cells were plated in a 96-well dish (5,000 cells per well) without puromycin selection. Viability was measured 6 days later by a neutral red uptake assay. **b**, A photograph of the crystal violet stained plate from

the experiment described in **a**. **c**, Analysis of proteins extracted from MEF cultures that were infected in parallel with the same virus suspensions used in **a**. Protein extracts were prepared 48 h after infection, with no drug selection. Equal amounts (40 μg) of protein were loaded, β-tubulin served as a loading control. **d**, The different infected MEFs were photographed under a light microscope 48 h after plating (5,000 cells per well in a 96-well dish) with puromycin selection.

61 ± 3%, respectively). Altogether, it is concluded that the lack of DAP kinase reduces the sensitivity of cells to E2F-1-induced apoptosis.

Discussion

In the course of cancer development, aberrant cell proliferation induced by deregulated activation of oncogenes such as c-Myc, E2F-1 and E1a, or by loss of Rb function, elicits a cellular response in which p53 is activated through the induction of p19^{ARF}, and consequently apoptosis is triggered to eliminate those pre-malignant cells^{28–31}. The experiments described here identify a novel participant—DAP kinase—in this oncogene-induced apoptotic checkpoint. DAP kinase is upregulated by hyperproliferative signals, and operates upstream of p19^{ARF} and p53 to induce apoptosis. Whereas the inactivation or loss of DAP kinase significantly reduces the p53 responses to c-Myc or E2F-1, it does not completely eliminate them, indicating that DAP kinase is not an exclusive player upstream of p19^{ARF}/p53. Of note, similar partial inhibition of c-Myc-induced p53 elevation and apoptosis was also documented after elimination of p19^{ARF} (ref. 29). p53 is therefore wired simultaneously to a few independent upstream branches, one of which includes DAP kinase. This Ca²⁺/CaM-dependent kinase channels the cells towards apoptosis rather than cell-cycle arrest through the induction of additional p19^{ARF}/p53 –independent events. Thus, when induced by oncogenes, DAP kinase contributes both to activation of p53 and to creating a cellular environment that dictates the preferential induction of p53-dependent apoptosis.

The mechanism by which DAP kinase activates p19^{ARF} remains to be elucidated. The anti-oncogenic/apoptotic effects of DAP kinase depend on the status of the catalytic activity, which means that the direct substrate(s) in the system should be identified. *In vitro* kinase assays showed that p19^{ARF} (but not p53 or Mdm2) can be phosphorylated by DAP kinase (data not shown). The physiological relevance of this phosphorylation requires further investigation

as well as the possibility that the activation of p19^{ARF} by DAP kinase may be indirect, through yet-unidentified substrates.

Apoptosis induced by c-Myc hinders the unchecked proliferation of pre-malignant cells. Thus, to achieve full oncogenic transformation, cells must override this tumour suppression mechanism, and may do so by inactivation of its components. Indeed, the p53 and INK4a/ARF genes are frequently mutated in cancer^{22–24}, and directed genetic inactivation of either gene was shown to subvert the apoptotic response, and hence contribute to tumorigenesis^{32–34}. In the light of our present study, loss of DAP kinase in human tumours may provide another means of negating the p19^{ARF}/p53 pathway by interfering with its activation, and hence would be advantageous for cells at early stages of tumour formation.

Note added in proof: The high incidence of hypermethylation of the DAP kinase 5'-UTR in non-small-cell lung carcinoma is reported by Tang, X. *et al.* (Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J. Natl Cancer Inst.* 92, 1511–1516; 200) □

Methods

Cell culture.

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS; Biolab), 2 mM glutamine and 100 U ml⁻¹ penicillin and streptomycin (Gibco BRL). MEF medium also included 0.1 mM non-essential amino acids (Gibco BRL) and 55 μM β-mercaptoethanol (Sigma). Primary REFs or MEFs were prepared from embryonic day (E) 14.5 embryos as described³⁵. The peptide caspase inhibitors YVAD-fmk and BD-fmk (Enzyme Systems Products) were dissolved in DMSO and added to the culture medium at a concentration of 100 μM, whereas DMSO alone (0.2%) was added to control cultures.

DAPK^{-/-} kinase-null mice were generated by targeting the first coding exon of the gene, thereby completely eliminating protein expression from the mutant allele. DAP kinase knock-out mice are viable and fertile (the details on the establishment of the colony and characterization of the deficient mice will be published elsewhere). Homozygous mutant animals were mated to obtain embryos in parallel to their wild-type littermates. MEFs were prepared from E-14.5 DAPK^{-/-} or +/+ embryos (10–15 embryos of each genotype were pooled).

Transformation assays.

Early-passage primary embryonic fibroblasts were plated (3–5 × 10⁵ cells per 9-cm plate), and 24 h later transfected with the indicated plasmids (3 µg each) by a standard CaPO₄ precipitation procedure. Medium was replaced every 3 days and foci were scored after 14–16 days. Vectors containing oncogenic Ras (V12), c-Myc, adenovirus E1a and SV40 large T antigen were described²⁸. Plasmids carrying a deletion mutant of T antigen (dl1001) as well as dominant-negative mutants of p53, Phe132 and DD, were kindly provided by Moshe Oren. The generation of various DAP kinase mutants has been described³⁴. The plasmids coding for p35 and CrmA have been described⁴.

Apoptosis analysis of transfected cells.

REFs or MEFs (10⁶ cells) were transfected with 5 µg of the indicated plasmids, together with 0.5 µg cDNA coding for membrane-targeted GFP³⁶. Transfection was performed using Fugene reagent (Boehringer Mannheim). Forty-eight hours after transfection cells were harvested for analysis. Cells were trypsinized, washed once in cold PBS, fixed in 5 ml cold methanol (–20 °C) for 30 min, centrifuged and resuspended in 0.5 ml PBS containing RNase A (50 µg ml^{–1}) and propidium iodide (PI; 100 µg ml^{–1}). The cells were then subjected to flow cytometric analysis, where GFP-positive cells were gated by their high fluorescence intensity (FL1). For each sample, 5,000 GFP-positive cells were collected and cell-cycle distribution was analysed according to relative DNA content (PI staining).

NIH 3T3 cells (10⁶ cells) were transfected using Fugene reagent, with 1 µg EGFP (Clontech) together with either 5 µg of control vector (pcDNA3) or 2.5 µg of pcDNA3-p19ARF or pcDNA3-DAPK-ΔCaM. Total DNA was brought to 6 µg with pcDNA3.

Viral infections.

Retroviruses were produced in 293 cells by transient transfection³⁷. Culture supernatants were collected 24–72 h post-transfection at 12-h intervals and pooled. MEFs at passage 4–5 (4 × 10⁵ per 9-cm plate) were infected with filtered supernatants in the presence of 8 µg ml^{–1} polybrene (Sigma), and fresh viral suspensions (3 ml per plate) were added twice at 3-h intervals. The medium was then changed and the cells were incubated for the indicated times before harvesting. For selection of infected cells, 1 µg ml^{–1} puromycin (Sigma) was added 48 h post-infection. Cell viability was measured by a neutral red uptake assay as previously described⁴.

Replication-defective adenovirus that carries DAPK-K42A was generated on the basis of the commercial pΔE1sp1A shuttle vector (Microbix Biosystems), which was modified by replacement of its multiple cloning sites with an expression cassette taken from the mammalian expression vector pcDNA3 (Invitrogen). Infectious viruses were generated in 293 cells and purified as described³⁸. For infection, viruses were suspended in serum-free medium and added to REFs at a multiplicity of infection (MOI) of 1,000. After 30 min incubation, fresh medium was added to the cells. In every experiment, adenovirus containing a gene for β-galactosidase (β-Gal) was used for control, and infection efficiency was verified by standard X-Gal assay.

Reporter assays.

For reduced stress and uniform high transfection efficiency, the Fugene reagent (Boehringer) was used. Empty pcDNA3 vector was added to the indicated plasmids to adjust total DNA amount to 2 µg per plate. At 16–18 h after transfection, cells were lysed in 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diamino cyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol and 1% Triton X-100. Lysate volumes corresponding to an equal number of cells from each sample were analysed using luciferase assay reagent (Promega). Reporter plasmids (*mdm2*-Luc and *RSV*-Luc) and the plasmid carrying human wild-type p53 under a cytomegalovirus (CMV) promoter were a gift from Moshe Oren.

Protein analysis.

Cell pellets were lysed in PLB buffer, separated by SDS-PAGE and blotted onto nitrocellulose membranes as described³. Filters were blocked in PBS plus 0.1% Tween-20 and 10% w/v skim dry milk, and then incubated with the following antibodies: anti-DAP kinase monoclonal antibodies (Sigma) diluted 1:1,000, anti-p21/WAF1 affinity-purified rabbit polyclonal antibodies (a gift from Claudio Schneider) at 1:500 dilution, anti-β-tubulin monoclonal antibodies (Sigma, 1:5,000), anti-p53, a mixture of two monoclonal antibodies (PAb421 and PAb248, a gift from Moshe Oren) in hybridoma supernatants (1:20), anti-Mdm2 monoclonal antibodies (2A10, a gift from Arnold Levine, provided by Moshe Oren) in hybridoma supernatant (1:20), anti-p19^{ARF} polyclonal antibodies (1:500), anti-c-Myc monoclonal antibodies (3C7) in hybridoma supernatant (1:5) and anti-E2F-1 monoclonal antibodies (SC-251). All filters were washed in PBS-Tween and then incubated with horseradish peroxidase-conjugated protein A (Amersham), or with goat anti-mouse second antibody (Jackson Immunoresearch). Antibodies were visualized by enhanced chemiluminescence (Supersignal; Pierce) according to the manufacturer's instructions.

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Correction: On page 4, col. 1, line 8, “Ectopic morphological alterations expression of p19^{ARF} should read “Ectopic expression of p19^{ARF}”
See the print issue for the correct version.