Direct coupling of the cell cycle and cell death machinery by E2F

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Unrestrained E2F activity forces S phase entry and promotes apoptosis through p53-dependent and -independent mechanisms. Here, we show that deregulation of E2F by adenovirus E1A, loss of Rb or enforced E2F-1 expression results in the accumulation of caspase proenzymes through a direct transcriptional mechanism. Increased caspase levels seem to potentiate cell death in the presence of p53-generated signals that trigger caspase activation. Our results demonstrate that mitogenic oncogenes engage a tumour suppressor network that functions at multiple levels to efficiently induce cell death. The data also underscore how cell cycle progression can be coupled to the apoptotic machinery.

Excessive mitogenic signalling is coupled to apoptosis as part of a fail-safe mechanism that suppresses oncogenic transformation[1]. For example, both the E1A and Myc oncoproteins induce ARF (the alternative reading frame product of the *INK4a/ARF* tumour suppressor locus), which in turn activates p53 to trigger cell death[2]. As a consequence, disruption of the ARF–p53 pathway allows inappropriate proliferation and survival, thereby stimulating tumorigenesis[2,3]. However, oncogenes can also signal to p53 through ARF-independent mechanisms[4–6] and promote apoptosis in ways that are entirely independent of p53 (ref. 1).

The Rb tumour suppressor is another factor that regulates proliferation and survival[3,7]. Rb restrains proliferation, in part, by modulating the activity of E2F transcription factors. In quiescent cells, Rb associates with several E2Fs, resulting in the repression of proliferation-associated genes. As cells progress into the cell cycle, cyclin-dependent kinases phosphorylate Rb, freeing E2F and allowing it to directly transactivate genes required for S phase entry. As a consequence, loss of Rb promotes aberrant proliferation. Disruption of Rb also promotes apoptosis and enforced expression of E2F can induce apoptosis through both p53-dependent and -independent mechanisms[1,3]. In contrast to how E2F promotes S phase entry, however, most studies suggest that E2F controls apoptosis though an indirect mechanism. For example, E2F can transcriptionally activate ARF or p73 (a member of the p53 family), which in turn produces secondary changes in gene expression that eventually trigger apoptosis[8–11].

Caspases are essential components of the apoptotic machinery[12]. These proteases are synthesized as inactive proenzymes and processed to an active state during apoptotic cell death. Initiator caspases (for example, Casp-2, -8 and -9) trigger a cascade that results in activation of the effector caspases (for example, Casp-3 and -7), which in turn produce the characteristic morphological changes associated with apoptosis[12]. The 'extrinsic' apoptotic pathway is triggered by interactions between cytokines and death receptors, resulting in activation of Casp-8. The 'intrinsic' pathway can be initiated by changes to the mitochondria that facilitate the release of cytochrome *c*, which associates with Apaf-1 and Casp-9 to promote caspase activation. How oncogenic signals interact with the apoptotic machinery is poorly understood, but it may involve changes to both pathways[1,13,14].

Results

E1A induces caspases in an ARF- and p53-independent manner. To determine whether oncogenes could influence caspase expression, we examined the effect of the *E1A* oncogene on caspase expression. E1A was introduced into early passage mouse embryo fibroblasts (MEFs) and normal diploid human fibroblasts (IMR90) by retroviral-mediated gene transfer. Infected cells were harvested for immunoblotting with antibodies specific for initiator (Casp-8 and Casp-9) or effector (Casp-3 and Casp-7) caspases. In both human and murine cells, E1A expression induced a 5–15-fold increase in the expression of all caspases examined (Fig. 1a, compare lanes 1 and 2 with lane 3 and 4). Similar results were also observed for Casp-2 (data not shown). Therefore, E1A coordinates the upregulation of multiple caspases, perhaps through a common mechanism.

E1A can promote apoptosis through the ARF–p53 tumour suppressor pathway, and p53 directly regulates expression of Apaf-1 (refs 15–17), a cofactor of Casp-9. To determine whether ARF and/or p53 also regulate caspase expression in response to oncogenes, we investigated whether these proteins are required for E1A-induced caspase expression. E1A induced expression of all four caspases in cells deficient in either p53 or ARF (Fig. 1b, compare lane 2 with lanes 4 and 6). Therefore, E1A regulates caspase expression through a p53-independent mechanism.

E1A induces caspases by inactivating Rb and deregulating E2Fs. Rb is an essential target of E1A in apoptosis; furthermore, E1A mutants that are unable to inactivate Rb are defective at inducing ARF and p53, and promoting cell death[15,18]. These E1A mutants also failed to increase caspase levels (data not shown). To determine whether inactivation of Rb is sufficient to increase caspase expression, we examined caspase levels in MEFs derived from *Rb*-deficient mice ($Rb^{-/-}$). Indeed, $Rb^{-/-}$ MEFs expressed much higher levels of Casp-3, -7, -8 and -9 when compared with wild-type cells (Fig. 2a). Conversely, reintroduction of Rb into Saos-2 cells (an *Rb*-null, *p53*-null human osteosarcoma line) repressed caspase expression to almost undetectable levels (Fig. 2b). This repression correlates with the ability of Rb to bind to E2F, as a tumour-derived Rb mutant defective in this activity was unable to suppress caspase levels (RbΔ22; data not shown). Interestingly, the impact of Rb on

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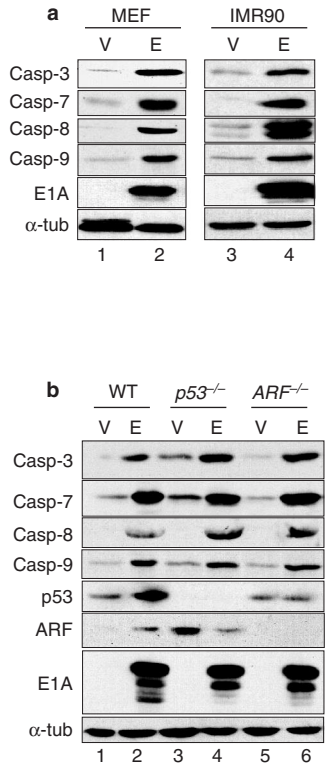

Figure 1 **E1A induces caspases independently of ARF and p53.** The levels of caspase proenzymes were assessed in cells transduced with a control retrovirus (V) or a retrovirus expressing *E1A* (E) by immunoblotting using antibodies directed against the indicated proteins. α-tubulin (α-tub) was used as a loading control. **a**, Casp-3, -7, -8 and -9 levels in MEFs and IMR90 cells. **b**, Casp-3, -7, -8 and -9 levels in early-passage wild-type, *p53*$^{-/-}$ and *ARF*$^{-/-}$ MEFs.

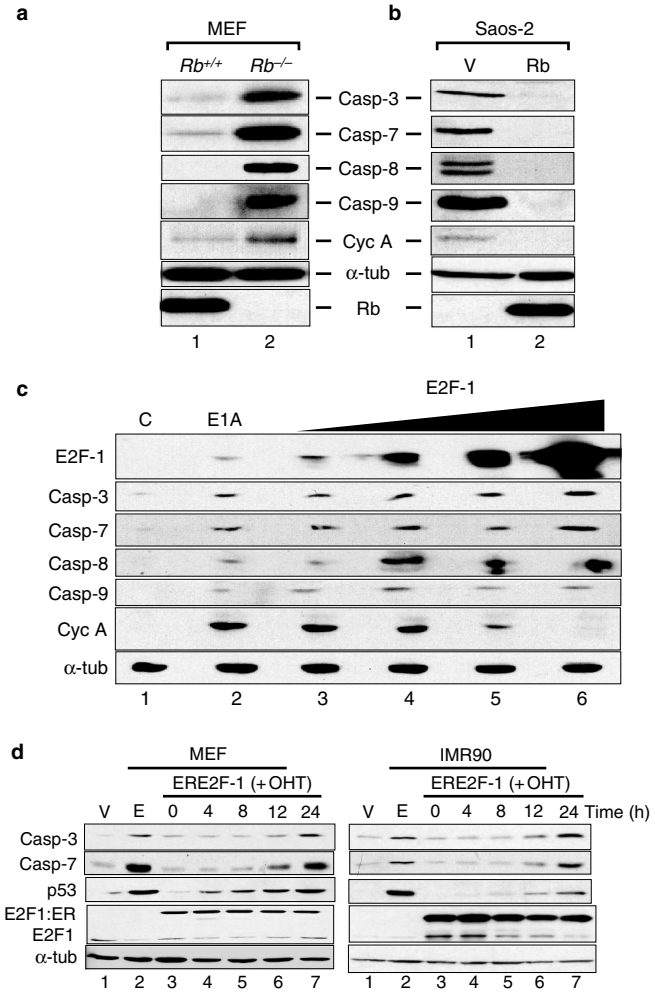

Figure 2 **Rb controls caspase expression.** Casp-3, -7, -8, -9 and cyclin A (Cyc A) expression levels were compared in wild-type (*Rb*$^{+/+}$) and *Rb*$^{-/-}$ MEFs (**a**) or in *Rb*-deficient Saos-2 cells after transfection with vector (V) or Rb constructs (**b**). *Rb* deficiency and equal loading were confirmed by examining expression of Rb and α-tubulin (α-tub), respectively. **c**, IMR90 fibroblasts were infected with adenoviruses expressing GFP, E1A and E2F-1 (triangle indicates increasing moi). Casp-3, -7, -8, -9, E2F-1, Cyc A and p53 levels were determined as above. The decline in *Cyc A* expression at high E2F levels presumably reflects the 'squelching' phenomenon often observed after using supra-physiological levels of transcription factors[36]. **d**, MEFs and IMR90 cells were infected with control (V), *E1A*-expressing (E) or *E2F-1*-expressing (ERE2F-1) retroviruses. At time-zero, medium containing 500 nM of 4-hydroxytamoxifen (OHT) was added and Casp-3, Casp-7 and p53 levels were determined at the indicated times. A mutant ERE2F-1 lacking the transactivation domain failed to induce caspases (data not shown).

expression of caspases paralleled the effects on expression of cyclin A (Fig. 2), a component of the cell-cycle machinery.

As loss of Rb can promote apoptosis through unrestrained E2F activity[2,7], we asked whether enforced expression of E2F could also induce caspase expression. E2F-1, the E2F family member most closely associated with apoptosis, was introduced into IMR90 cells using increasing amounts of a recombinant adenovirus. Caspase expression was determined 36 h later. As controls, parallel cultures were infected with adenoviruses expressing either green fluorescent protein (GFP; control) or E1A. Expression of E2F-1 was sufficient to induce caspase expression, even when produced at levels comparable to endogenous E2F in cells expressing E1A (Fig. 2c, compare lanes 2 and 3). With the exception of Casp-8, higher E2F-1 levels did not induce further increases in caspase expression (Fig. 2c, lanes 3–6). This effect was not specific to E2F-1 or to adenovirus-mediated gene delivery, as preliminary studies suggest that E2F-2 and -3 can also increase caspase expression (Z.N. & S.W.L., unpublished observations). Furthermore, both IMR90 cells and MEFs stably expressing a well-characterized E2F-1/oestrogen-receptor fusion construct[19] (ERE2F-1) accumulated caspase proteins in response to treatment with tamoxifen, an oestrogen-receptor ligand (Fig. 2d). Interestingly, the impact of E2F-1 on caspase expression correlates well with its ability to induce cyclin A, a *bona fide* E2F target-gene[7] (Fig. 2b, c).

Caspases can be direct transcriptional targets of E2F. E2F-1 directly controls the expression of genes involved in cell-cycle progression and S phase entry[7]. To determine if E2F regulates caspase transcription, we examined *caspase* mRNA expression in IMR90 cells expressing E1A or ERE2F-1 (in the presence and absence of tamoxifen) by northern blotting. Both E1A and E2F-1 increased expression of *caspase* mRNA 5–15-fold when compared with controls (Fig. 3a, compare lanes 1 and 2 for E1A and lanes 3 and 4 for E2F-1), which is consistent with cDNA microarray studies using cells expressing E1A (Z.N. & S.W.L., unpublished observations) or E2F-1 (ref. 20). The fact that E1A deregulates endogenous E2F activity and induces *caspase* mRNA expression implies that caspases are physiological targets of E2F-1. In further support of this view, the level of caspase expression was low in quiescent cells and higher as cells entered S phase, precisely paralleling the expression profile of cyclin A (Fig. 3b). However, the upregulation was less pronounced for Casp-8 and -9 when compared with Casp-3 and -7, suggesting that sig-

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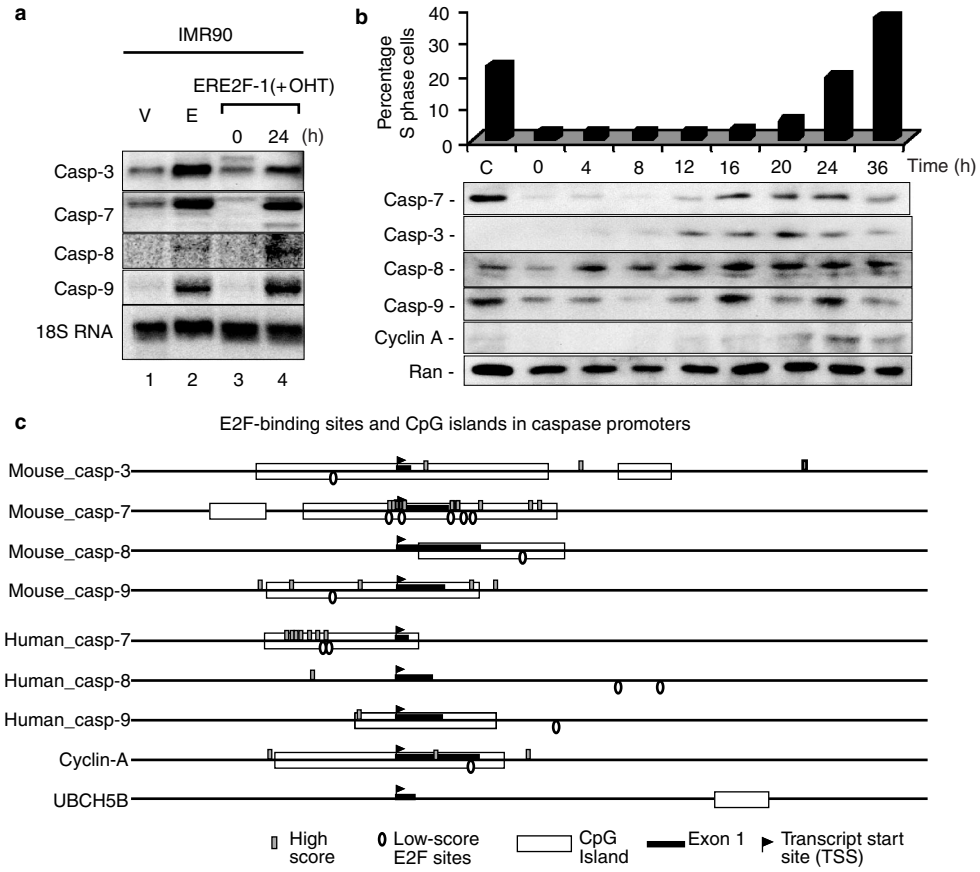

Figure 3 **Caspases display characteristics of E2F target-genes. a**, Northern blotting of total RNA isolated from IMR90 cells infected with the empty vector (V), *E1A*- (E) or *ERE2F-1*-expression constructs using probes specific for the indicated caspase. 18S RNA was used as a loading control. E2F1 was induced by incubating cells with 500 nM OHT for 24 h. **b**, Expression of caspases was determined by immunoblotting in control (C) and serum-starved cells ($t = 0$), and at various times after serum addition. The percentage of cells in S phase at various times is shown. *Ran* expression is shown as a loading control. *Caspase 3* and *caspase 7* mRNAs were also elevated in growing cells when compared with arrested cells (data not shown). **c**, The genomic regions spanning 1000 bp upstream of the predicted caspase transcriptional start site and 500 bp into the first intron of mouse *casp-3, -7, -8, -9*, human *casp-7, -8, -9*, *Cyclin A* and *UBCH5B* were retrieved from genome databases and analysed for potential E2F-binding sites using SiteScan. Predicted E2F-binding sites for caspase genes are presented in this schematic diagram. Squares and dots represent high-score and low-score sites, respectively (see Supplementary Information Table).

nalling caspases require more sustained E2F activity to respond.

To further evaluate whether caspases are E2F target-genes, we investigated whether caspase promoters contain predicted E2F-binding sites using the SiteScan program, which identifies E2F-binding sites on the basis of consensus sequence and additional contextual motifs[21] (see Supplementary Information Table). Caspase genomic regions were assembled from sequence information in public and private databases before analysis using SiteScan. Each of the caspase promoters had features that have been observed in previously characterized E2F-responsive promoters, including at least one predicted E2F-1-binding site (Fig. 3b; Supplementary Information Table). As expected, SiteScan also identified the well-characterized E2F sites in the cyclin A promoter and did not identify sites in housekeeping genes, such as the ubiquitin-ligase enzyme, UBCH5B, which is not regulated by E2F.

If caspase genes are direct transcriptional targets of E2F, then E2F proteins should physically associate with these promoters when caspase expression is high and caspase promoter sequences should confer E2F responsiveness to heterologous genes. To verify binding of E2F-1 to caspase promoters *in vivo*, we used chromatin immunoprecipitation (ChIP)[22], which allows the detection of proteins bound to specific regions of DNA. We focused on the binding of E2F-1 to the *casp-7* promoter, which has an aggregate of putative E2F-1 sites (Fig. 3c). Vector- and E1A-expressing IMR90 cells were fixed in formaldehyde to crosslink proteins to the DNA. After sonication to generate ~500-base pair fragments of genomic DNA, E2F-1:DNA complexes were immunoprecipitated using an antibody against E2F-1. The DNA was released from immunoprecipitates and analysed by quantitative PCR using primers that flanked the putative E2F-binding elements.

casp-7 promoter sequences were readily detected in immunoprecipitates from *E1A*-expressing cells (Fig. 4b, top, lane 2). As controls, the same procedure amplified sequences corresponding to the *bona fide* E2F site in the cyclin A promoter, but not from the 3′ end of the *cyclin A* gene, which lacks E2F sites (Fig. 4a, and Fig. 4b, lanes 1 and 2). Although *casp-7* sequences were not amplified from normal cells (Fig. 4b, lane 1), it is possible that E2F-1 is bound to the promoter at low levels or in a manner that is not recognized by the antibody. Nevertheless, E2F-1 is bound to the *casp-7* promoter in cells prone to apoptosis.

To determine whether the *casp-7* promoter was responsive to E2F, a genomic fragment containing the predicted E2F sites (sequences −546 to +86 upstream of the transcriptional start site) was isolated and subcloned into a luciferase reporter plasmid (Casp-7-luc). The construct was then cotransfected into *p53*-deficient Saos-2 cells with either an empty vector (LPC), or increasing

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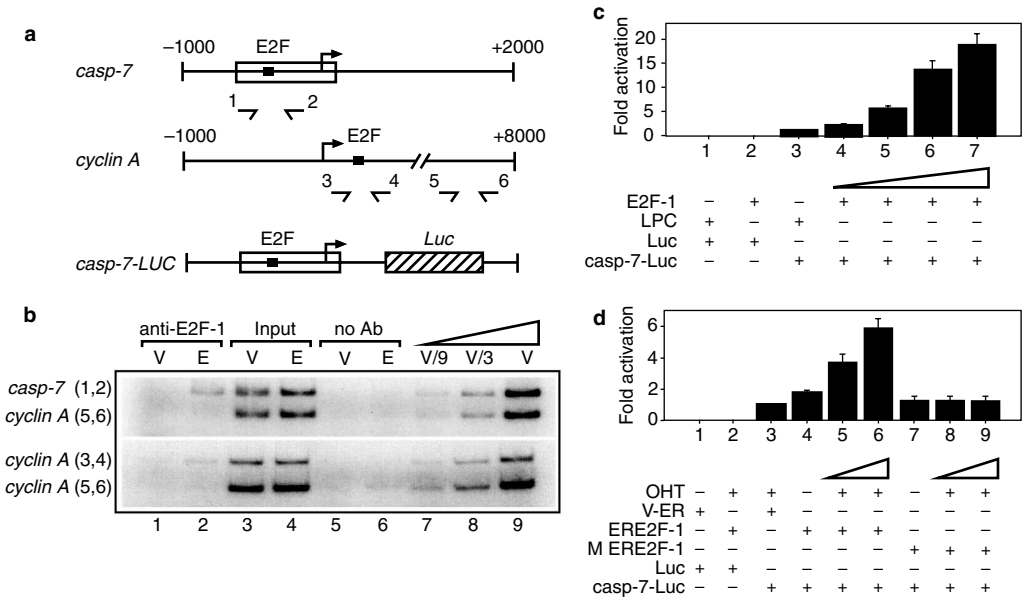

Figure 4 **Caspase 7 is a direct E2F-1 target. a**, Schematic diagrams depicting genomic regions of human *casp-7* and *cyclin A* genes, as well as the *casp-7*-luciferase construct. Open box represents the putative *casp-7* promoter region cloned into the luciferase reporter plasmid. Numbers correspond to primers with homology to the indicated sites (see Supplementary Information Table). **b**, *In vivo* detection of promoter occupancy by E2F using ChIP. Chromatin was prepared from IMR90 cells infected with control vector (V) or *E1A*-expressing (E) retroviruses and immunoprecipitated with antibodies specific to E2F-1. Duplex PCR amplification was performed on corresponding templates using the indicated primer sets. Input corresponds to PCR reactions containing 0.5% of total chromatin used in immunoprecipitation reactions. Parallel immunoprecipitation without antibody failed to yield detectable signals after equivalent autoradiographic exposure. Equal loading of templates in E1A and vector samples is shown in the input lanes, and a titration of DNA template was performed to demonstrate the linearity of PCR amplification. **c**, Luciferase reporter assays using Saos-2 cells cotransfected with empty vector (LPC) or E2F-1 plasmid in addition to a luciferase reporter plasmid containing the *casp-7* promoter (casp-7-Luc) or empty luciferase vector (Luc). The presence (+) or absence (–) of particular plasmids in the transfection is indicated and the triangle represents increasing amounts of *E2F-1* plasmid (20 ng, 100 ng, 500 ng and 1 μg, respectively). **d**, Luciferase experiments were performed exactly as in **c**, except that the reporter plasmids were cotransfected with empty vector (V-ER), ERE2F-1 or mutant ERE2F-1 (M ERE2F-1). Here, the triangles represent increasing concentrations of the ER fusion construct (500 ng and 1 μg, respectively). 500 nM OHT was added for 12 h to activate E2F-1.

concentrations of E2F-1 expression plasmid (Fig. 4c) and a normalization control. Luciferase activity was plotted as the fold induction relative to basal luciferase activity (that is, luciferase activity in cells receiving the reporter plasmid but no exogenous E2F-1). The results indicate that E2F-1 can induce an almost 18-fold increase in reporter activity over basal levels. We also cotransfected Saos-2 cells with the reporter construct and a plasmid encoding the aforementioned ERE2F-1 or the ERE2F-1 mutant. As expected, the *casp-7* promoter was responsive to E2F-1 after addition of tamoxifen, resulting in a sixfold increase in signal over basal levels (Fig. 4d). In contrast, the reporter was unresponsive to the ERE2F-1 mutant, even in the presence of tamoxifen (Fig. 4d, compare lane 7 with lanes 8 and 9). Taken together, our data demonstrate that E2F-1 transactivates the *casp-7* promoter. Given the coordinate regulation of Casp-2, -3, -7, -8 and -9 by E2F and the existence of E2F sites in the all caspase promoters examined, we suspect the other caspases are also direct targets of E2F.

Cells with high caspase levels are sensitized to apoptosis after release of cytochrome *c*. The fact that E1A utilizes the Rb–E2F pathway to induce apoptosis and caspase expression implies that high caspase levels facilitate oncogene-induced cell death. Accordingly, wild-type MEFs expressing *E1A* are sensitized to diverse apoptotic stimuli, including serum withdrawal and adriamycin treatment (activators of the Casp-9 pathway[23]) and the death ligand tumour necrosis factor α (TNF-α, an activator of the Casp-8 pathway[24]; Fig. 5a). However, accumulation of caspases is not sufficient to induce apoptosis, as *p53*-null cells expressing *E1A* are resistant to serum withdrawal and adriamycin treatment[25] but express elevated caspase levels, although they still respond to TNF-α. Therefore, caspase accumulation may potentiate cell death in the presence of an apoptotic stimulus.

Together with previous reports, our results indicate that E2F targets the apoptotic programme at multiple levels: for example, E2F can facilitate caspase activation through p53-dependent signals, resulting in mitochondrial release of cytochrome *c*, while simultaneously increasing caspase expression through a direct mechanism that is independent of p53. This model predicts that enforced cytoplasmic delivery of cytochrome *c* should bypass the requirement for p53 during oncogene-induced apoptosis, allowing the sensitization effect of elevated caspase expression to be uncoupled from the upstream processes required for caspase activation (see Fig. 5d). To test this hypothesis, we conducted two complementary experiments. First, we examined the ability of Bax — a pro-apoptotic member of the Bcl-2 family that functions downstream of p53 to facilitate cytochrome *c* release[25,26] — to potentiate apoptosis in $p53^{-/-}bax^{-/-}$ cells expressing *E2F*. Second, we microinjected cytochrome *c* directly into early passage $p53^{-/-}$ and $p53^{-/-}Rb^{-/-}$ MEFs. In both settings, the absence of p53 eliminated any p53-dependent component from our analysis, whereas enforced E2F expression or the absence of Rb provided a stimulus to increase caspase expression.

As predicted, cells that co-express E2F-1 and Bax underwent substantially more apoptosis when compared with cells expressing either E2F-1 or Bax alone (Fig. 5b). Moreover, at all cytochrome *c* concentrations tested, $p53^{-/-}Rb^{-/-}$ cells were significantly more sensitive to apoptosis than $p53^{-/-}$ cells (Fig. 5c). Of note, E2F-expressing cells were also more sensitive to cytochrome *c*-induced apoptosis when compared with controls (M.J. and D.B., unpublished observa-

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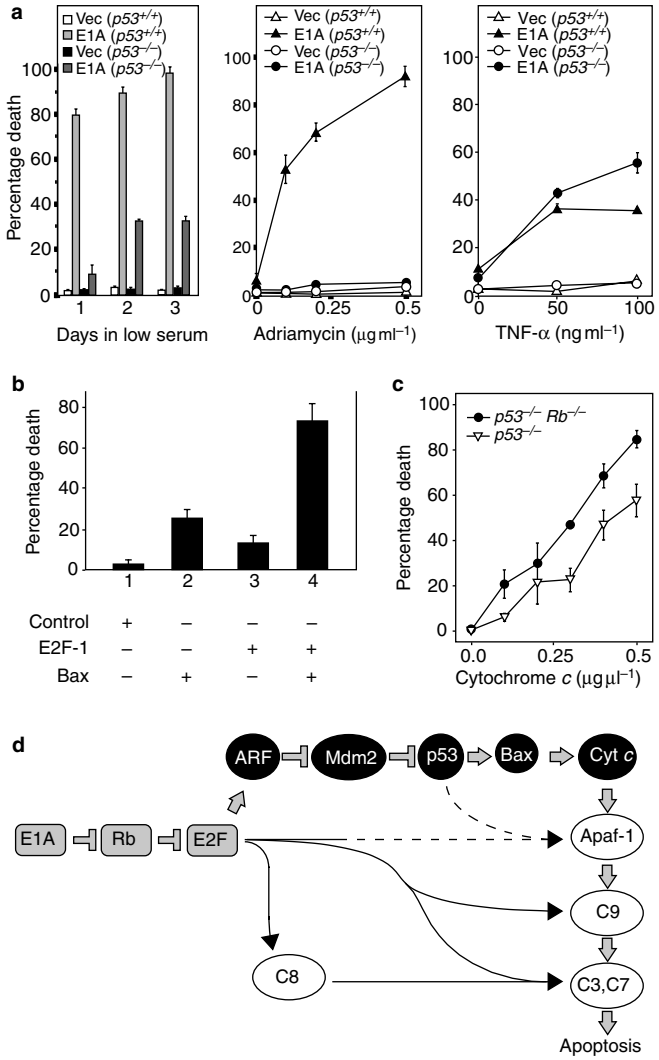

Figure 5 **Loss of Rb sensitizes cells to apoptosis after cytochrome *c* release. a**, *p53*$^{+/+}$ and *p53*$^{-/-}$ MEFs expressing a control vector (Vec) or E1A were incubated in the presence of 0.1% serum, adriamycin or TNF-α and the percentage of dead cells was determined at 24 h or at the indicated time. **b**, *p53*$^{-/-}$*bax*$^{-/-}$ MEFs expressing control vectors, Bax, E2F-1 or co-expressing Bax and E2F-1 were produced by gene transfer using a *bax*-expressing retrovirus and an *E2F-1*-expressing adenovirus. Cell death was assessed 36 h after adenovirus infection. The presence (+) or absence (–) of the particular genes is indicated. **c**, Cytochrome *c* was microinjected into *p53*$^{-/-}$ or *p53*$^{-/-}$*Rb*$^{-/-}$ MEFs at the indicated concentration. After 1 h, the injected cells were scored for apoptosis using morphological criteria. FITC–dextran was co-injected with cytochrome *c* to facilitate identification of injected cells. Each data point represents a total of 85–225 cells and the data represents the mean and standard deviation of three experiments. **d**, A model for oncogene-induced apoptosis involving both p53-dependent and p53-independent pathways. E2F signalling indirectly to p53 facilitates caspase activation and simultaneously produces increases in caspase expression. These two pathways function in concert to ensure efficient cell death. The dashed line is extrapolated from previous work suggesting that Apaf-1 is a transcriptional target of both E2F-1 and p53 (ref. 16). Both E2F-1 and p53 have additional targets not shown on this scheme, all of which may function in concert to produce efficient cell death.

tions). Given that cytochrome *c* functions late in the apoptotic programme, our results strongly suggest that E2F-mediated increases in caspase expression are responsible for this sensitization effect.

Discussion

In summary, we show that deregulation of the Rb–E2F pathway by E1A, loss of Rb or enforced E2F expression coordinately increases caspase expression, at least in part, through a direct transcriptional mechanism. Although not sufficient for apoptosis, high levels of signalling caspases may increase the probability that a death-inducing signal overcomes endogenous caspase inhibitors (for example, inhibitor of apoptosis (IAP) proteins) to trigger a caspase cascade, whereas high levels of effector caspases may ensure that apoptosis proceeds efficiently once initiated. Hence, p53-generated signals resulting in caspase activation (for example, cytochrome *c* release from mitochondria) cooperate with p53-independent increases in caspase expression to induce apoptosis (Fig. 5d). Alternatively, increases in caspase expression — particularly Casp-8 — may sensitize cells to death-inducing ligands[27,28]. Together, our results may help explain the ability of E2F-1 to cooperate with p53 in apoptosis[29] and the increased sensitivity of oncogene-expressing cells to pro-apoptotic cytokines, such as TNF-α[30]. More importantly, they imply that oncogene-induced apoptosis proceeds through a highly coordinated series of events, ensuring that cell death is efficiently executed.

Oncogene-induced apoptosis limits the transforming potential of excessive mitogenic signalling[1,31], but precisely how cells 'sense' aberrant proliferation has been unclear. Here, we have shown that this can be achieved, in part, by coupling cell-cycle progression to caspase expression through E2F. Hence, E2F transmits mitogenic signals to the cell-cycle machinery by directly regulating the transcription of genes required for S phase entry and simultaneously upregulates the cell death machinery through a similar mechanism. As a consequence, cycling cells are primed for apoptosis should proliferation be perceived as aberrant. Together, our results demonstrate that oncogenes can coordinately upregulate caspase expression through a direct mechanism, providing an example of how apoptosis can be 'hardwired' to cell-cycle progression. □

Methods

Cells and gene transfer

IMR90, U2OS and Saos-2 cells were obtained from the American Type Culture Collection (ATCC). Primary MEFs from wild type, *p53*$^{-/-}$, *ARF*$^{-/-}$, *Rb*$^{-/-}$, *p53*$^{-/-}$ *Rb*$^{-/-}$ and *p53*$^{-/-}$ *bax*$^{-/-}$ mice were isolated and cultured as described[32]. IMR90 cells (expressing the ectopic retrovirus receptor[32]) and MEFs were infected with high-titre recombinant retroviruses expressing *E1A* (LPC-12S), *ERE2F-1* (pBabeHAERE2F-1 (ref. 19)) and a transactivation-defective mutant of E2F-1 (pBabeHAERE2F-1(1–374)[16]) as described[32]. For ER constructs, E2F-1 activity was induced by the addition of 500 nM 4-hydroxytamoxifen (OHT; Sigma, St Louis, MO). *E1A* and *E2F-1* were also introduced into IMR90 cells using recombinant adenoviruses[33] at a multiplicity of infection (moi) of 100 plaque-forming units (pfu) cell^{-1} for Ad-E1A$_{12s}$ and Ad-GFP, and 3, 12.5, 50, and 200 pfu cell^{-1} for Ad-E2F1. For reintroduction of *Rb* into Saos-2 cells, calcium phosphate transfection was performed using Rb (pSVE) and GFP (pBABE) expression plasmids at a ratio of 4:1, respectively. Two days after transfection, cells were sorted by FACS (Elite ESP; Coulter, Miami, FL) and the GFP-positive cells collected and lysed for immunoblotting. For *Bax/E2F-1* co-infection, *p53*$^{-/-}$ *bax*$^{-/-}$ MEFs were infected with high-titre *Bax* (pLPC-HABax) retroviruses. After selection, cells received either Ad-E2F1 or Ad-GFP (moi of 250) and viability was determined 36 h later using a trypan blue exclusion assay.

Protein and gene expression

Protein expression was assessed by separating 30 μg of total cell lysate on 10% polyacrylamide gels before immunoblotting. Blots were probed with antibodies directed against Casp-2 (dilution 1:250; 13951A, PharMingen, San Diego, CA), Casp-3 (1:1000; C76920, Transduction Laboratories, Lexington, KY), Casp-7 (1:1000; clone 1-1-10), Casp-8 (1:500; 97-7-15), Casp-9 (1:500; AAP-119, Stressgen, Victoria, BC), E1A (1:50; M58 (ref. 34)), p53 (1:1000; CM5, CM1, Novocastra, Newcastle, UK), ARF (1:1000; NB 200-106, Novus Biologicals, Littleton, CO), Rb (1:1000; 14001A, PharMingen), E2F-1 (1:100; KH95, Santa Cruz Biotechnology, Santa Cruz, CA), Cyclin A (1:200; BF683, Santa Cruz) or α-tubulin (1:2000; B-5-1-2, Sigma). Anti-mouse or anti-rabbit horseradish peroxidase (1:5,000; Amersham, Piscataway, NJ) were used as secondary antibodies. Proteins were visualized using the ECL detection system (Amersham). mRNA levels were assessed by northern blotting using 10 μg of total RNA (Ultraspec RNA system, Biotecx, Houston, TX).

Caspase promoter analysis

mRNA sequences of human and mouse caspases (NM_001227, NM_001228, NM_001229, NM_009810, NM_007611, BC006737 and NM_015733) were retrieved from NCBI nucleotide database and aligned with genomic sequences by local alignment program BLAST. First exons and their flanking regions, 1000 bp upstream promoter region and 500 bp downstream intron region, were then retrieved from the GenBank and Celera databases and analysed for potential E2F-binding sites with the SiteScan program (http://compel.bionet.nsc.ru/FunSite/SiteScan.html). SiteScan generated

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composite transcriptional modules consisting of a binding site for E2F transcription factors and additional contextual motifs[21] (see Supplementary Information Table).

Functional analysis of caspase promoters

ChIP was performed as previously described[22] using an E2F-1-specific antibody (E2F-1 sc-193, Santa Cruz). DNA released from precipitated complexes was amplified using sequence-specific primers to detect *casp-7* promoter elements (Fig. 4, oligonucleotides 1 and 2). As controls, oligonucleotides corresponding to the indicated positions (Fig. 4, oligonucleotides 3–6) were used to amplify sequences within the *cyclin A* gene. Genomic fragments corresponding to the *casp-7* promoter were amplified by PCR from HeLa cells and cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). U2OS or Saos-2 cells were transfected using FuGene 6 (Roche, Basel, Switzerland) with 2 μg of the reporter construct, 20–1000 ng of the E2F1 expression plasmid and 1.2 μg of the pRL-β-globin control plasmid (Promega). Cells were harvested 36 h after transfection. Luciferase activity was assayed using a luminometer and normalized to the transfection control. The sequences of oligonucleotides used in the ChIP assay and for amplifying the *casp-7* promoter are available from the authors on request.

Cell-cycle analysis

IMR90 fibroblasts were incubated in DMEM (Invitrogen, Carlsbad, CA) medium supplemented with a suboptimal concentration of foetal bovine serum (0.1%) for 4 days. Normal serum level (10%) was restored and cells harvested at 0, 4, 8, 12, 16, 20, 24 and 36 h after release from low serum. Nuclei from each time-point were stained with DAPI and analysed for DNA content by flow cytometry.

Microinjection

Cytochrome *c* (C-3131; Sigma), diluted in 0.5× PBS containing 2 mg ml^{-1} fluorescein isothiocyanate (FITC)–dextran (FD-20; Sigma) was injected into MEFs using an Eppendorf microinjector (5242) at the indicated concentrations, as described previously[35]. The cells were incubated for 1 h at 37 °C before scoring. Apoptosis was determined using blebbing and cell rounding as morphological markers, whereas FITC–dextran staining permitted identification of successfully injected cells.

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COMPETING FINANCIAL INTERESTS

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