

Loss of Rb-E2F Repression Results in Caspase-8-mediated Apoptosis through Inactivation of Focal Adhesion Kinase*[§]

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Molecular hardwiring of the cell cycle to the apoptotic machinery is a critical tumor suppressor mechanism for eliminating hyperproliferative cells. Dereglulation of the Rb-E2F repressor complex by genetic deletion or functional inhibition of Rb triggers apoptosis through both the intrinsic (caspase-9 mediated) and extrinsic (caspase-8 mediated) death pathways. Induction of the intrinsic pathway has been studied extensively and involves release of free E2F and direct transcriptional activation of E2F-responsive apoptotic genes such as ARF, APAF1, and CASP9. In contrast, the mechanisms leading to activation of the extrinsic pathway are less well understood. There is growing evidence that Rb-E2F perturbation induces the extrinsic pathway, at least in part, through derepression (as opposed to transactivation) of apoptotic genes. Here, we explore this possibility using cells in which Rb-E2F complexes are displaced from promoters without stimulating E2F transactivation. This derepression of Rb-E2F-regulated genes leads to apoptosis through inactivation of focal adhesion kinase and activation of caspase-8. These findings reveal a new mechanistic link between Rb-E2F and the extrinsic (caspase 8-mediated) apoptotic pathway.

Apoptosis (programmed cell death) plays a critical role in development, tissue homeostasis, and tumor suppression (1). Highly efficient and redundant “hardwiring” between the cell cycle and apoptotic pathways normally ensures the elimination of hyperproliferative cells (2). One of the most important links between cell cycle and apoptosis is the Rb-E2F complex, which triggers cell death by multiple mechanisms when it becomes deregulated by oncogenic mutations (3). The retinoblastoma protein, Rb, interacts with the E2F family of transcription factors (referred to here collectively as E2F) and can inhibit their ability to activate genes by at least two mechanisms (4). Rb directly binds and masks the E2F transactivation (5). This mechanism may be important for regulating genes that require the influence of E2F transactivation for efficient expression. In addition, Rb can actively repress regulatory elements when

recruited to promoters by E2F (6). This mechanism may be important for regulating constitutively repressed apoptotic genes that are expressed only when the Rb-E2F repressor complex is inactivated (7). Both mechanisms appear to be operative in the Rb-E2F-mediated apoptotic response.

Several lines of evidence suggest that loss of Rb leads to an accumulation of free E2F1, transactivation of E2F-responsive apoptotic genes, and induction of the intrinsic apoptotic pathway (8, 9). Free E2F1 transcriptionally activates genes involved in the intrinsic pathway, such as *ARF*,¹ *APAF1*, and *CASP9* (10–12). Rb-null mice die prior to birth from massive apoptosis that is partly dependent on intrinsic pathway proteins such as p53 and APAF1 (13, 14). This apoptosis is partly rescued in mice lacking E2F1, which transactivates genes in the intrinsic pathway (15). Taken together, these findings indicate that some (but not all) apoptosis resulting from Rb loss is because of E2F1 transactivation and intrinsic pathway mechanisms.

Recently, there has been increasing recognition that a portion of the apoptosis resulting from Rb-E2F deregulation does not depend on the transactivation function of E2F and may involve the extrinsic apoptotic pathway. Hsieh and co-workers (16) showed through the use of E2F1 mutants that transactivation was dispensable but DNA binding was required for the apoptotic function of E2F1 (16). Phillips and co-workers (17) showed that an E2F mutant lacking the transactivation domain could induce apoptosis that was independent of the intrinsic pathway protein p53 and involved elements of the extrinsic pathway such as TRAF2 (17). Chromatin immunoprecipitation assays have shown that, contrary to previous concepts, Rb-E2F complexes occupy many promoters throughout the cell cycle (18). Furthermore, Rb-E2F complexes constitutively occupy the promoters of many apoptotic genes, including extrinsic pathway components *FAS* and tumor necrosis factor- α (7). These findings suggest that loss of Rb can cause apoptosis through derepression of basally inhibited extrinsic apoptotic pathway genes. However, no mechanism has provided a molecular explanation for how Rb-E2F is coupled to the extrinsic pathway.

We have used a cell system in which a dominant-negative mutant of E2F1 (dnE2F) that contains the DNA binding domain but lacks the transactivation domain is inducibly activated by a tamoxifen-estrogen receptor system in U2OS-de-

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Fig. S1.

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¹ The abbreviations used are: ARF, alternate reading frame; APAF1, apoptotic protease activating factor-1; CASP9, caspase-9; FAK, focal adhesion kinase; PTP1B, protein-tyrosine phosphatase 1B; PTEN, phosphatase and tensin homolog; PTPN11, protein-tyrosine phosphatase non-receptor type 11; dnE2F, dominant-negative mutant of E2F1; 4-OHT, 4-hydroxytamoxifen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, endoplasmic reticulum; REF, rat embryo fibroblast; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; DAPI, 4',6-diamidino-2-phenylindole.

rived EH1 cells (19). Overexpression of this dnE2F mutant has been shown to displace E2Fs and Rb-E2F complexes from promoters containing E2F sites and to specifically disrupt the functions of Rb that are mediated through its interaction with E2F family members, such as active transcriptional repression and G₁ cell cycle arrest (20). Furthermore, microarray gene expression experiments in this cell system confirmed that dnE2F derepressed many known E2F-responsive genes such as *CCNA2* and *CCNE2* (21). Of particular interest for this study, the dnE2F mutant rapidly induces apoptosis through an Rb-E2F-dependent mechanism (7). A major advantage of this system is that it appears to simultaneously displace all E2F family members and pocket protein-E2F complexes from promoters with similar efficiency (which would be impractical by inhibition or genetic deletion of all individual E2Fs and pocket proteins). Furthermore, the confounding factor of E2F transactivation is eliminated. We show that dnE2F-induced apoptosis is mediated largely through caspase-8, a component of the extrinsic pathway, and involves dephosphorylation and inactivation of focal adhesion kinase (FAK). Our findings provide a novel mechanism to explain the link between Rb-E2F deregulation and extrinsic pathway (caspase 8-mediated) apoptosis.

EXPERIMENTAL PROCEDURES

Cell Cultures—ER-dnE2F cells (gift of D. Dean) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 400 μ g/ml zeocin, 150 μ g/ml hygromycin B, and 300 μ g/ml G418. Treatment with 4-hydroxytamoxifen (4-OHT) (Sigma) resulted in activation of the dnE2F, and treatment with isopropyl 1-thio- β -D-galactopyranoside (1 mM, Sigma) resulted in activation of p16Ink4a, as described previously (21). E2F1-DP1 inducible Rat2 cells (gift of B. Shan and W. H. Lee), originally derived from immortalized rat embryo fibroblast (REF) cells, were maintained in Dulbecco's modified Eagle's medium + 10% fetal bovine serum, 200 μ g of G418, and 1 μ g/ml tetracycline. Withdrawal of tetracycline resulted in overexpression of E2F1 and DP1, as described previously (22). Rat embryo fibroblasts (BioWhittaker) were maintained in Dulbecco's modified Eagle's medium + 10% fetal bovine serum. Cell permeable inhibitors of caspases-8 and -9 were obtained from BD Pharmingen. The tyrosine phosphatase inhibitor bis(*N,N*-dimethylhydroxamido)hydroxooxovanadate (DHMV), which inhibits PTP1B and other members of the protein-tyrosine phosphatase family (23, 24), was obtained from Calbiochem.

Plasmids and Antibodies—Plasmids included: CD2-FAK (FAK fused to CD2 for membrane tethering, gift of G. Whitney) (25), GFP-FAK, GFP-FRNC, GFP-FAT (gifts of D. Illic), GFP-PTP1B (gift of J. Balsamo), and GFP-C1 (Clontech). Transfections were performed using effectene (Qiagen). Antibodies were used that detect caspases-3, -8, and -9, and cleaved caspase-8, AKT-phospho-Ser⁴⁷³, and PTEN (Cell Signaling), FAK (Upstate), and FAK-phospho-Tyr³⁹⁷, -Tyr⁴⁰⁷, -Tyr⁵⁷⁶, and -Tyr⁸⁶¹ (BIOSOURCE). Immunoblots and immunofluorescence assays were performed as described previously (26).

Caspase Activity Assays—Caspase activity was measured by adding specific substrates for caspases-3, -8, and -9 to cell lysates and measuring enzymatic cleavage with a colorimetric assay (Biovision) at 405 nm on a plate reader according to the manufacturer's instructions.

Flow Cytometry—As described previously (26), cells were fixed overnight in cold 70% ethanol, stained with a solution containing 50 μ g/ml propidium iodide and 2 mg/ml RNase A in phosphate-buffered saline, and analyzed for DNA content on a FACS Caliber (BD Biosciences).

Viability Assays—Viability assays were performed according to the manufacturer's recommendations (Promega) as described previously (26). Briefly, we used 10⁵ cells, and 20 μ l of MTS and phenazine methosulfate solutions per well. Cells were incubated at 37 °C for the indicated times, and absorbance was measured at 496 nm on a plate reader.

Polymerase Chain Reaction—For real-time PCR, total RNA was collected using RNeasy (Qiagen), cDNA was generated using SuperScript First Strand Synthesis (Invitrogen), and amplification was performed using Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) according to the manufacturer's instructions in a Bio-Rad iCycler. LUX Fluorogenic primers were as follows: GAPDH forward, 5'-Fam-GACG-TATGCTGGCGCTGAGTAACG; GAPDH reverse, 3'-GTGGCAGGAGG-CATTGCTGAT; PTPN11 forward, 5'-Fam-GAACTGACCACCAACGT-CGTATTTTCAG; PTPN11 reverse, 3'-GCGCACTGGTGTATGACAAAG;

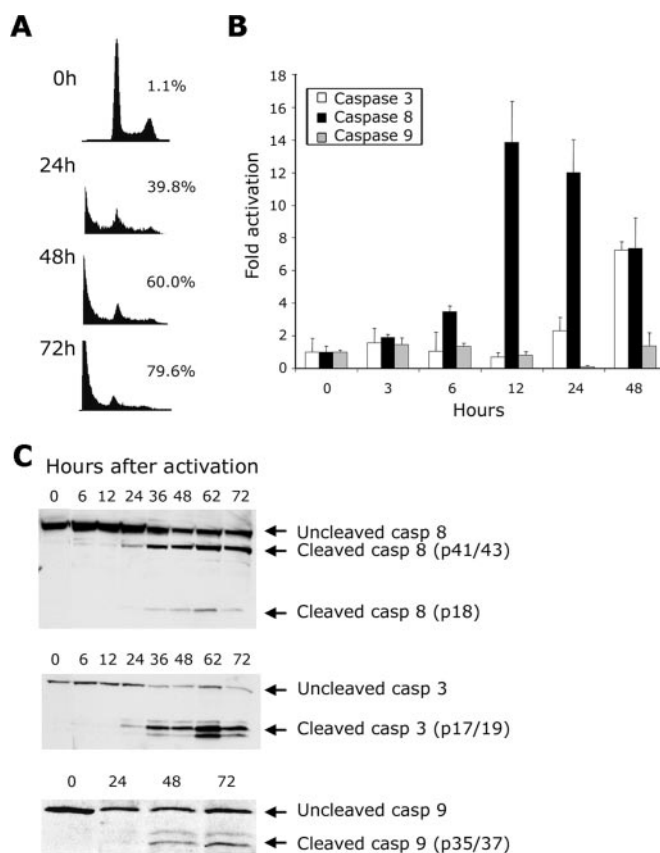


FIG. 1. Derepression of genes inhibited by Rb-E2F results in caspase-8-mediated apoptosis. A, activation of ER-dnE2F cells by addition of 4-OHT induces an accumulation of cells with $<2N$ DNA content by flow cytometry, consistent with apoptosis. B, activity of caspases-3, -8, and -9 following activation of ER-dnE2F cells, as measured by colorimetric substrate assays. C, immunoblots showing cleavage of procaspase-3, -8, and -9 to the smaller active forms following activation of ER-dnE2F cells.

PTP1B forward, 5'-Fam-GACGAGACTCAGTGCATGGTCCTCG; PTP1B reverse, 3'-GGCATCGAAAGCATGAGTCAA; PTEN forward, 5'-Fam-CAGACCAGTAACTGTCTTCCCGTCG; and PTEN reverse, 3'-TGGTCTGCCAGTAAAGGTGAA. The ΔC_T values were generated by iCycler software, and the logarithms of the sample gene ΔC_T values were normalized to that of GAPDH (control).

RESULTS

Displacement of Rb-E2F Complexes from Promoters Induces Apoptosis—To analyze how loss of transcriptional repression by Rb-E2F complexes leads to apoptosis, we used U2OS cells that conditionally express an ER-fused dnE2F that retains the DNA binding domain but lacks the transactivation domain (21). Activation of dnE2F in these cells displaces endogenous Rb-E2F complexes from promoters, allowing expression of repressed genes (7, 21). This cell model and several others using similar dnE2F mutants have been shown to induce apoptosis (16, 27). Consistent with prior studies, we found that activation of dnE2F resulted in prompt onset of apoptosis, as measured by flow cytometric accumulation of cells with $<2N$ DNA content, with most cell death occurring within 48 h (Fig. 1A).

Activation of Caspase-8—To explore the molecular mechanisms regulating this apoptosis, we started by examining caspases-8 and -9, which are the principal initiator caspases of the extrinsic and intrinsic apoptotic pathways, respectively, as well as caspase-3, a major downstream executioner of the death program (1). At multiple time points after activation of ER-dnE2F, caspase activity was monitored by colorimetric assay, and cleavage of procaspases to their active forms was monitored with immunoblots. Surprisingly, caspase-8 activity began

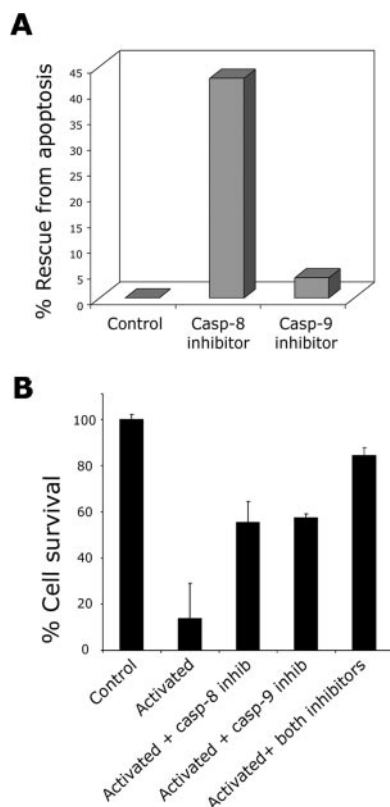


FIG. 2. Inhibition of caspase-8 rescues apoptosis caused by derepression of E2F sites. A, representative results from flow cytometric experiments showing percent rescue of apoptosis by caspase-8 and -9 inhibitors in cells after activation of ER-dnE2F. Apoptosis rescue is measured as the reduction in cells with $<2N$ DNA content compared with activated ER-dnE2F cells not treated with caspase inhibitors. B, cell survival at 24 h following activation of ER-dnE2F cells and treatment with the indicated caspase inhibitors, as measured by colorimetric viability assay.

to increase within 6 h and peaked at 12 h, whereas caspase-9 activity remained unchanged (Fig. 1B). Similarly, cleavage of caspase-8 was detectable as early as 6 h, whereas caspase-9 cleavage was only detected later at 48 h (Fig. 1C). As expected, caspase-3, which acts downstream of both caspase-8 and caspase-9, was activated somewhat later than caspase-8 (Fig. 1, B and C).

To further analyze the roles of caspase-8 and -9, we attempted to block the apoptosis using cell-permeable inhibitors of each caspase. We confirmed that the inhibitory peptides were specific by testing them with recombinant caspases *in vitro* (data not shown). By flow cytometric analysis of cells with $<2N$ DNA content, inhibition of caspase-8 rescued almost half of the apoptosis at 24 h, whereas inhibition of caspase-9 rescued only about 5% of the apoptosis (Fig. 2A). We repeated these experiments using colorimetric detection of viable cells. As with flow cytometry, the caspase-8 inhibitory peptide rescued about half of the apoptosis at 24 h (Fig. 2B). Interestingly, however, the caspase-9 inhibitor also rescued about half of the apoptosis, and there was an additive effect with almost complete rescue when both inhibitors were used. Taken together, these results suggest that the apoptosis induced by displacement of Rb-E2F repressor complexes is mediated largely through caspase-8, which has been associated primarily with the extrinsic apoptotic pathway. The fact that caspase-9 appeared to play some role suggests either that it is activated secondarily as apoptosis proceeds or that some of the apoptosis in this system is in fact mediated through the intrinsic pathway.

Inactivation of FAK—Recently, it was shown that loss of FAK leads to caspase-8-dependent apoptosis in both anchorage-dependent and -independent cell systems (28). To explore whether FAK may play a role in the apoptosis in our system, we first examined the protein levels of FAK at multiple time points after activation of dnE2F. As evidenced by immunoblot analysis, the overall protein levels of FAK did not change over 72 h after dnE2F activation (Fig. 3A). We then examined the phosphorylation status of FAK using phosphorylation site-specific FAK antibodies. FAK activity is regulated by phosphorylation, predominantly on tyrosine residues. Tyr³⁹⁷ is a major autophosphorylation site that is required for FAK activity such as binding to Src kinases and the p85 subunit of phosphatidylinositol 3-kinase, whereas Tyr⁸⁶¹ is phosphorylated by Src family kinases and regulates the interaction of FAK with integrins (29). Interestingly, activation of dnE2F and onset of apoptosis were associated with dephosphorylation of FAK at both Tyr³⁹⁷ and Tyr⁸⁶¹ (Fig. 3A). In contrast, two other tyrosine residues, Tyr⁴⁰⁷ and Tyr⁵⁷⁶, remained phosphorylated throughout the time course. To investigate whether loss of FAK activity could induce caspase-8-mediated apoptosis in these cells, we expressed FRNK, a dominant-negative form of FAK lacking the kinase domain, and inspected cells for activation of caspase-8 by immunofluorescence, as described previously (30). Indeed, expression of FRNK was associated with rounding of cells and the appearance of activated (cleaved) caspase-8 by 24 h (Fig. 3B). In contrast, the morphologic changes and activation of caspase-8 were not observed in cells expressing an empty control vector or wild type FAK. To determine whether overexpression of wild type FAK in the activated ER-dnE2F cells could rescue apoptosis, we expressed CD2-FAK, a chimeric protein containing the CD2 transmembrane domain and fused to the FAK protein, which anchors FAK to the cell membrane and causes it to be constitutively active (25, 31). Activated cells expressing CD2-FAK exhibited significantly less apoptosis (as measured by flow cytometric DNA content) than control cells expressing an empty vector, with over half of the apoptosis rescued at 48 h (Fig. 3C). Furthermore, expression of CD2-FAK in cells treated with camptothecin, which activates both the intrinsic and extrinsic pathways (32), preferentially blocked the activation of caspase-8 compared with caspase-9 (Fig. 3D).

Given the novelty of these findings, we wished to reproduce them in an independent cell system. For these experiments, we expressed in wild type REFs two proteins that have been shown to inhibit FAK through a dominant-negative mechanism: FRNK (lacking the N-terminal catalytic domain of FAK) (33) and FAT (the focal adhesion targeting domain of FAK that localizes the protein to focal adhesions) (34). Expression of either of these dominant-negative FAK proteins induced apoptosis with a time course similar to the ER-dnE2F cells (Fig. 4A). Furthermore, a caspase-8 inhibitor, but not a caspase-9 inhibitor, efficiently rescued FRNK-induced apoptosis (Fig. 4B), indicating that FRNK is relatively specific for activating the extrinsic death pathway. We then examined FAK protein status in REF-derived cells that rapidly undergo apoptosis upon induction of full-length E2F1 and DP1 by a tetracycline-regulated system (22). Importantly, these cells overexpress full-length E2F1, which induces apoptosis through transactivation and derepression (displacement of Rb-E2F complexes) of E2F-responsive apoptotic genes (7). Interestingly, in this system overall FAK protein levels diminished rapidly upon induction of the cells (Fig. 4C). This loss of FAK was not accompanied by the appearance of FAK degradation products, suggesting that the reduced protein expression was because of down-regulation. Similar to the previous experiments, CD2-FAK efficiently rescued this apoptosis (Fig. 4D). As expected, both

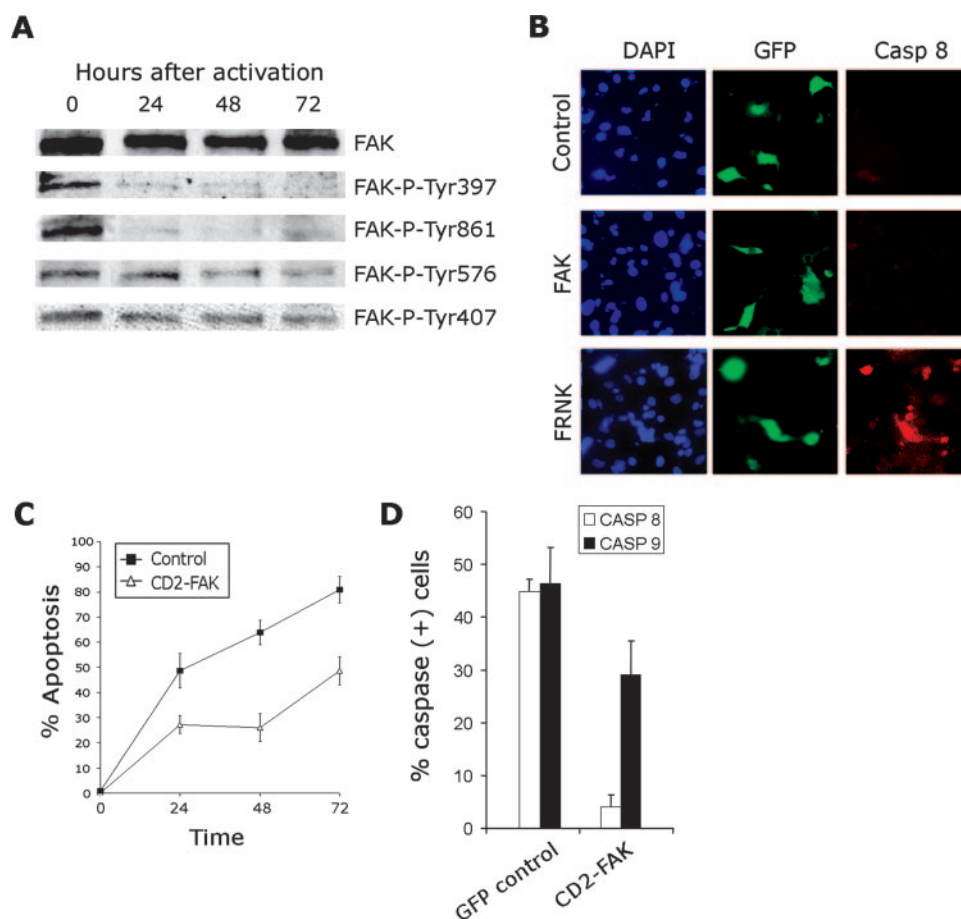


FIG. 3. Caspase-8-mediated apoptosis caused by derepression of E2F sites involves dephosphorylation and inactivation of FAK. *A*, immunoblots of ER-dnE2F cells at the indicated time points after activation using antibodies against FAK (all phosphorylated forms) and FAK phosphorylated at the indicated tyrosine residues. *B*, immunofluorescence studies in parental EH1 cells at 36 h after transfection of FAK, FRNK, or control empty vector, along with green fluorescent protein (GFP). Cell nuclei were stained with DAPI (blue). Transfected cells are indicated by positive GFP signal (green). Red staining indicates cleaved (active) caspase-8. *C*, rescue of apoptosis in activated ER-dnE2F cells by the constitutively active form of FAK (CD2-FAK). Cells were transfected with CD2-FAK or control empty vector, then ER-dnE2F was activated 48 h later. Apoptosis was measured at various time points after addition of 4-OHT as the percent of cells with flow cytometric DNA content $<2N$. *D*, activity of caspases-8 and -9, as measured by colorimetric substrate assays, in EH1 cells transfected with vectors expressing GFP (control) or CD2-FAK. Error bars represent standard error.

caspase-8 and caspase-9 are activated in this system, but the greater amplitude of caspase-8 activation suggests that the extrinsic pathway is a major mechanism of apoptosis (Fig. 4E). Taken together, these experiments support the existence of a novel link between FAK, caspase-8, and Rb-E2F-associated apoptosis.

Role of PTP1B—To explore potential mechanisms for the dephosphorylation of FAK in the ER-dnE2F cells, we investigated whether one or more tyrosine phosphatases may be up-regulated by displacement of Rb-E2F complexes from promoters. Several phosphatases have been shown to promote FAK dephosphorylation, including PTEN, PTP1B, and PTPN11 (35–37). Because PTEN is a well characterized tumor suppressor, we examined this gene first. Following activation of the ER-dnE2F cells, PTEN protein levels did not change appreciably, and the phosphorylation status of AKT-Ser⁴⁷³, a downstream target of PTEN signaling, also remained unchanged (Supplemental Materials Fig. S1), suggesting that PTEN does not play a role in dnE2F-induced apoptosis. Consistent with this finding, PTEN mRNA levels (as measured by real-time PCR) did not change upon activation of the ER-dnE2F cells (Fig. 5A). Likewise, PTPN11 mRNA levels remained unchanged in the activated dnE2F cells. In contrast, PTP1B mRNA levels increased by ~20-fold by real-time PCR at 24 h (Fig. 5A). A tyrosine phosphatase inhibitor that strongly inhibits PTP1B

(DHMV) efficiently blocked ER-dnE2F-induced apoptosis, with almost 100% rescue at 50 μM concentration (Fig. 5B). Because DHMV is not specific to PTP1B but also inhibits other members of the protein-tyrosine phosphatase family (23), (24), we depleted cells of PTP1B using RNA interference, which impaired the cleavage of caspase-8 in activated ER-dnE2F cells (Fig. 5C). Furthermore, ectopic expression of PTP1B in parental EH1 cells efficiently induced cleavage of caspase-8 by immunoblot and immunofluorescence (Fig. 5, D–F). Intriguingly, the upstream regulatory region of PTP1B contains likely E2F binding sites using E2Fsite Scan software (38), suggesting that this gene may be directly repressed by Rb-E2F and up-regulated as a consequence of Rb-E2F disruption.

DISCUSSION

Herein, we provide evidence for a novel mechanism linking Rb-E2F to the extrinsic apoptotic pathway through inactivation of FAK and activation of caspase-8. These results contribute to the growing body of evidence suggesting that loss of Rb induces apoptosis through multiple mechanisms involving both the intrinsic and extrinsic pathways.

Role of FAK Inhibition in Intrinsic and Extrinsic Death Pathways—We provide several lines of evidence indicating that inactivation of FAK plays an important role in Rb-E2F-associated apoptosis. First, we show that displacement of Rb-E2F

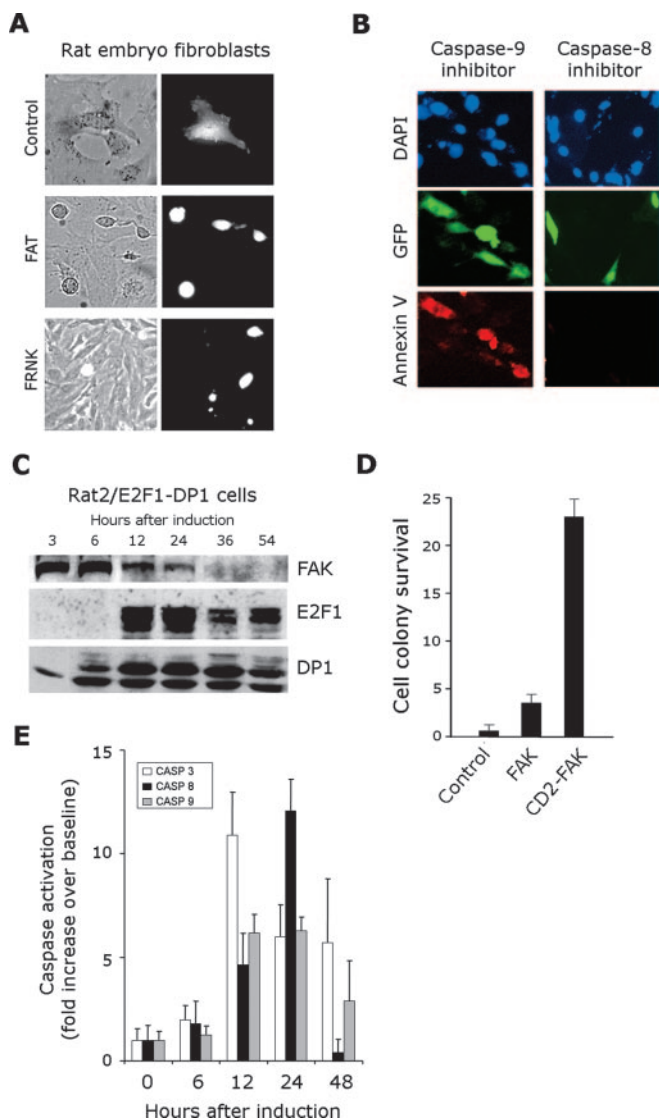


FIG. 4. Inactivation of FAK induces apoptosis in rat embryo fibroblasts overexpressing full-length E2F1. *A*, the effect of FAK inhibition was studied in REFs expressing dominant negative forms of FAK (FAT and FRNK) or a control empty vector. REFs were transfected with the indicated vectors along with GFP. *Left panels* are phase-contrast micrographs, and *right panels* are corresponding fluorescence micrographs showing transfected GFP-positive cells (white cells). Cells expressing FAT and FRNK are rounded and exhibit nuclear condensation, consistent with apoptosis. *B*, immunofluorescence studies in parental EH1 cells at 24 h after transfection of GFP-FRNK. Cell nuclei were stained with DAPI (*blue*). Transfected cells are indicated by positive GFP signal (*green*). *Red* staining indicates annexin V staining of apoptotic cells. *C*, immunoblots of Rat2-E2F1/DP1 cells at various time points after induction, showing progressive loss of FAK protein paralleling the increased expression of E2F1 and DP1. *D*, FAK rescue of apoptosis in Rat2-E2F1/DP1 cells. Cells were transfected with control empty vector, FAK, or a constitutively active FAK (CD2-FAK) and induced 24 h after. Cell survival was measured as the ability of cells to form colonies at 2 weeks. *E*, activation of caspases-3, -8, and -9, as measured by colorimetric substrate assays, at the indicated time points following tetracycline removal. *Error bars* represent S.E.

complexes from promoters results in apoptosis that is mediated largely through caspase-8-dependent mechanisms. Second, we show that this apoptosis is associated with dephosphorylation of FAK and can be blocked by constitutive activation of FAK. FAK inhibits caspase-8/extrinsic pathway apoptosis (28) through mechanisms that are still incompletely understood. FAK binds the receptor-interacting protein, which is a serine/threonine kinase that contains a death domain and is a major component of the death receptor complex (39). Receptor-inter-

acting protein interacts with the death domains of cell surface receptors of the tumor necrosis factor superfamily and death domain adaptor proteins, whereby it provides proapoptotic signals that are suppressed by its interaction with FAK, which appears to directly interfere with the interaction between receptor-interacting protein and the death receptor complex (40). FAK is located at sites of focal adhesions, where it binds and phosphorylates many proteins including the p85 subunit of phosphatidylinositol 3-kinase, members of the Src kinase family, Shc, and p130^{Cas} (29), and it is possible that these FAK-associated proteins may mediate effects on caspase-8 as well (41). Additionally, we have found that pro-caspase-8 contains potential FAK phosphorylation motifs, and that FAK may directly phosphorylate and inactivate caspase-8.² Hence, FAK may serve as a point of regulation for multiple death and survival signals (42), and it is frequently deregulated in cancer (43, 44).

In ER-dnE2F cells, where caspase-8-mediated apoptosis is induced by derepression of Rb-E2F inhibited genes, FAK is inactivated by dephosphorylation. As a potential explanation for this dephosphorylation, we found that *PTP1B* contains E2F sites in its promoter (data not shown) and is potently induced by dnE2F. Ectopic expression of *PTP1B* induces caspase-8 cleavage, whereas inhibition of *PTP1B* blocks caspase-8 cleavage. *PTP1B* is a plausible candidate for mediating this apoptotic response because it localizes to focal adhesions, promotes FAK dephosphorylation, causes cell rounding, and suppresses tumorigenesis through inhibition of FAK (36). Consistent with this potentially anti-oncogenic role for *PTP1B*, there is growing evidence that *PTP1B* may act as a tumor suppressor (45). *PTP1B* can suppress transformation by Bcr-Abl (46), and it is down-regulated in esophageal cancers (47). These findings do not rule out the possibility that additional phosphatases may also be involved in this process.

In contrast to the ER-dnE2F cells in which the dnE2F lacks a transactivation domain, the Rat2-E2F1/DP1 cells overexpress full-length E2F1 that can both activate transcription and displace endogenous Rb-E2F complexes. Interestingly, FAK is inactivated in the Rat2-E2F1/DP1 cells by down-regulation (rather than dephosphorylation) of the protein. Two potential explanations are that excess E2F1/DP1 promotes degradation of FAK, or that it transcriptionally inhibits *FAK*. Distinguishing between these possibilities is beyond the scope of this study, but it is intriguing that the *FAK* promoter contains p53 sites and is transcriptionally repressed by p53 (48), which is a component of the intrinsic death pathway and is activated by full-length E2F1. Hence, both extrinsic and intrinsic Rb-E2F-associated apoptotic mechanisms appear to target FAK for inactivation, possibly as a means of coordinating apoptosis with cytoskeletal/adhesion functions.

Rb-E2F Deregulation Triggers a Multifaceted Apoptotic Response—Rb-E2F deregulation triggers a complex and redundant apoptotic response involving both the intrinsic and extrinsic death pathways through transactivation and derepression of apoptotic genes to ensure the efficient elimination of abnormally proliferative cells (Fig. 6). Nahle *et al.* (10) recently showed that caspases involved in both the intrinsic and extrinsic pathways are directly transactivated by E2Fs in the apoptotic responses induced by adenovirus E1A, loss of Rb, or E2F1 overexpression. Liedtke *et al.* (49) showed that p53, an intrinsic pathway component that is activated by the E2F-inducible ARF protein, positively regulates caspase-8, a component of the extrinsic pathway. Some E2F responsive apoptotic genes may be regulated through both repression and

² J. H. Lieman, L. A. Worley, and J. W. Harbour, unpublished results.

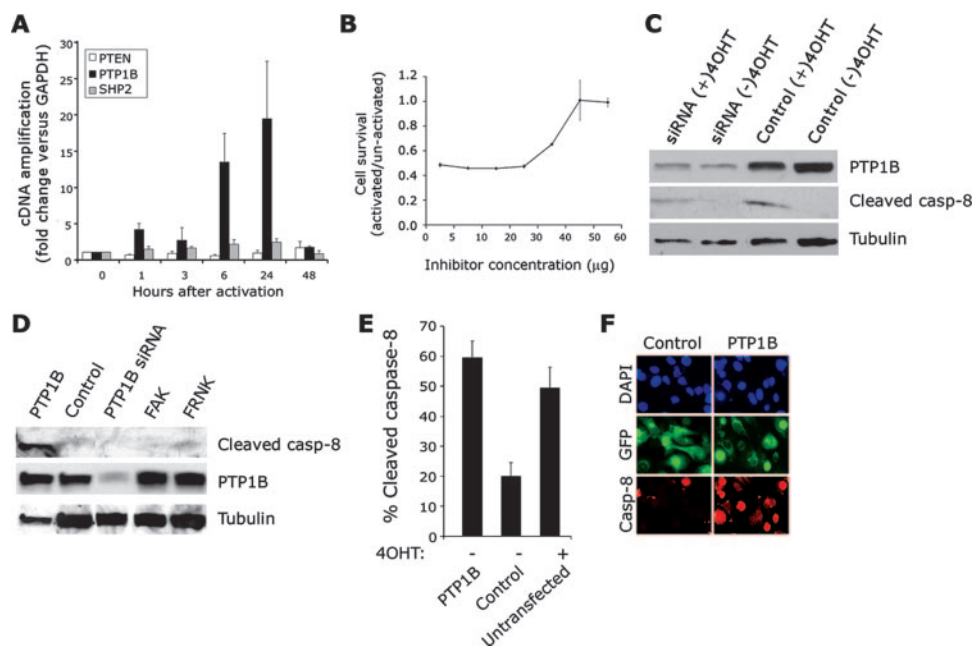


FIG. 5. *PTP1B* is up-regulated by loss of Rb-E2F and activates caspase-8. *A*, comparison of mRNA levels of the three indicated phosphatases at various time points after activation of ER-dnE2F cells, measured as cDNA amplification of mRNA product by real-time PCR and expressed as -fold increase compared with GAPDH. *B*, rescue of activated ER-dnE2F cells by the phosphatase inhibitor bis(*N,N*-dimethylhydroxamido)hydroxooxovanadate, measured as a ratio of cell viability of activated *versus* un-activated cells at 24 h after addition of 4-OHT. *C*, immunoblots from activated ER-dnE2F cells expressing PTP1B small interfering RNA (*siRNA*) or control scrambled *siRNA*, and analyzed with antibodies against PTP1B, cleaved (activated) caspase-8, and tubulin. *D*, immunoblots from parental EH1 cells transfected with PTP1B, PTP1B *siRNA*, FAK, FRNK (a dominant-negative FAK mutant), or control empty vector, and analyzed with the indicated antibodies. *E*, summary of immunofluorescence experiments in which ER-dnE2F cells were transfected with PTP1B, a control empty vector, or untransfected. Untransfected ER-dnE2F cells were activated with 4-OHT. The bars indicate the percent of transfected (GFP-positive) cells that stain positively with an antibody against cleaved caspase-8. Standard error is indicated. *F*, representative example of immunofluorescence experiments showing cells transfected with empty control vector or PTP1B. Transfected cells are indicated by positive GFP signal (green). Red staining indicates cleaved (active) caspase-8.

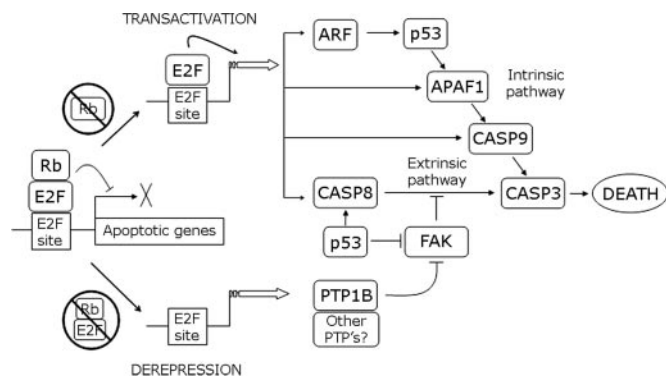


FIG. 6. Overview of apoptotic pathways activated by deregulation of Rb-E2F. Deregulation of E2F activity, which occurs frequently in human cancers because of Rb inactivation, leads to increased transactivation of E2F-responsive apoptotic genes such as *ARF*, *APAF1*, and *CASP9*, stimulating the intrinsic apoptotic pathway through activation of caspase-9. Loss of Rb leads to derepression of other apoptotic genes. We show here that loss of Rb-E2F repression leads to up-regulation of *PTP1B* and potentially other phosphatases, inactivation of FAK, and stimulation of the extrinsic apoptotic pathway through activation of caspase-8. The intrinsic pathway also suppresses FAK activity through p53-mediate transcriptional repression, suggesting that inactivation of FAK may play an important role in regulating and amplifying death signals through the intrinsic and extrinsic pathways. The complexity and redundancy of these pathways may serve to ensure a robust apoptotic response in abnormally proliferative cells.

transactivation. Aslanian and co-workers (50) showed that *ARF* is basally repressed by E2F3b and becomes derepressed by E2F3 loss; under oncogenic conditions, however, E2F1 and E2F3a are recruited to the *ARF* promoter to transcriptionally activate the gene. Additionally, the liberation of free E2F in this model system may interfere with E2F-independent anti-

apoptotic functions of Rb, such as inhibition of c-Abl and other pro-apoptotic proteins (51). Our results corroborate and extend these findings by linking Rb-E2F-associated apoptosis to FAK and caspase-8. This relationship may serve to coordinate apoptotic and cytoskeletal signaling, and may explain at least in part the rounding and detachment of adherent cells that occurs during with apoptosis (52).

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