

Arrest of mammalian fibroblasts in G1 in response to actin inhibition is dependent on retinoblastoma pocket proteins but not on p53

Olivier D. Lohez, Caroline Reynaud, Franck Borel, Paul R. Andreassen, and Robert L. Margolis

Institut de Biologie Structurale Jean Pierre Ebel (Commissariat à l'Energie Atomique–Centre National de la Recherche Scientifique–Université Joseph Fourier), 38027 Grenoble cedex 1, France

p53 and the retinoblastoma (RB) pocket proteins are central to the control of progression through the G1 phase of the cell cycle. The RB pocket protein family is downstream of p53 and controls S-phase entry. Disruption of actin assembly arrests nontransformed mammalian fibroblasts in G1. We show that this arrest requires intact RB pocket protein function, but surprisingly does not require p53. Thus, mammalian fibroblasts with normal pocket protein function reversibly arrest in G1 on exposure to actin inhibitors regardless of their p53 status. By contrast, pocket protein triple knockout mouse embryo fibroblasts and T antigen-transformed rat embryo fibroblasts lacking both p53 and

RB pocket protein function do not arrest in G1. Fibroblasts are very sensitive to actin inhibition in G1 and arrest at drug concentrations that do not affect cell adhesion or cell cleavage. Interestingly, G1 arrest is accompanied by inhibition of surface ruffling and by induction of NF2/merlin. The combination of failure of G1 control and of tetraploid checkpoint control can cause RB pocket protein-suppressed cells to rapidly become aneuploid and die after exposure to actin inhibitors, whereas pocket protein-competent cells are spared. Our results thus establish that RB pocket proteins can be uniquely targeted for tumor chemotherapy.

Introduction

Both p53 and the retinoblastoma (RB)* pocket protein family are central to the control of G1 progression in mammalian cells. Between them, they can impose G1 arrest in response to a multitude of stresses or signals, including DNA damage (Kastan et al., 1991; Di Leonardo et al., 1994), polyploidization (Cross et al., 1995; Minn et al., 1996; Lanni and Jacks, 1998; Andreassen et al., 2001b), depletion of nucleotide pools (Linke et al., 1996), and TGF- β (Polyak et al., 1994a; Zhang et al., 1999). During G1 progression, p53 mediates cell cycle arrest by transactivating specific targets. Among these, the Cdk inhibitor (CKI) p21^{WAF1} acts to arrest the cell cycle by inhibiting Cdk2/cyclin E and Cdk2/cyclin A (Dulic et al., 1994). Suppression of Cdk activity blocks G1 progression

by regulating the phosphorylation status of the RB pocket proteins (RB, p107, and p130), tumor suppressors of central importance to the control of S-phase entry (Weinberg, 1995; Mittnacht, 1998; Mulligan and Jacks, 1998). Pocket proteins are substrates for phosphorylation by G1 Cdks, and once fully phosphorylated, they release E2F transcription factors that permit progression from G1 to S phase (Dyson, 1998). The clear link between p53- and RB-dependent suppression of the cell cycle in G1 is underlined by the fact that abrogation of either RB pocket protein function or p53 function permits the cell cycle to continue after DNA damage (Levine, 1997; Dannenberg et al., 2000; Sage et al., 2000). RB pocket proteins appear to act downstream of p53 because a normal p53 and p21^{WAF1} response occurs in cells deficient for RB pocket protein function (Dannenberg et al., 2000; Sage et al., 2000). In addition, p53 and the RB pocket protein family cooperate in mediating G1 arrest in response to polyploidization or exposure to inhibitors of mitotic spindle assembly (Di Leonardo et al., 1997; Andreassen et al., 2001b; Borel et al., 2002b). In contrast, as exemplified by G1 arrest of fibroblasts in response to the growth factor TGF- β , RB pocket proteins can act independently of p53 (Zhang et al., 1999).

In addition to the intersection of the p53 and RB pathways, pocket protein suppression of G1 progression can be

Address correspondence to Robert L. Margolis, Institut de Biologie Structurale, 41 rue Jules Horowitz, 38027 Grenoble cedex 1, France. Tel.: (33) 4-38-78-9616. Fax: (33) 4-38-78-5494. E-mail: margolis@ibs.fr
P.R. Andreassen's present address is Dana-Farber Cancer Institute, M640, 44 Binney St., Boston, MA 02115.

*Abbreviations used in this paper: ARF, alternative reading frame; CKI, Cdk inhibitor; DCB, dihydrocytochalasin B; ERK, extracellular signal-regulated kinase; HU, hydroxyurea; MEF, mouse embryo fibroblast; RB, retinoblastoma; REF, rat embryo fibroblast.

Key words: aneuploidy; tetraploidy; cytoskeleton; dihydrocytochalasin B; cell cycle

activated by CKIs, such as p27^{Kip1} and p16^{INK4A}, that are independent of p53 control (Sherr and Roberts, 1995, 1999). Given that the RB pocket protein pathway is believed to be defective in nearly all human tumors (Weinberg, 1995; Sherr, 1996; Hanahan and Weinberg, 2000), whereas p53 mutations are present in approximately half of human tumors (Vogelstein et al., 2000), it is important to distinguish RB-dependent suppression of the cell cycle that is clearly independent of the p53 pathway. The recent development of mouse embryo fibroblast (MEF) cells with a knockout for all three RB pocket proteins (RB, p107, and p130) in a background of normal p53 response now permits dissection of such pocket protein-specific events.

Among the metabolic signals that the cell reads to enable G1 progression is the status of the cytoskeleton. We have established that stabilization of the microtubule array by taxol, a drug inhibitor of microtubule dynamics, arrests nontransformed fibroblasts in G1 in a p53-dependent manner (Trielli et al., 1996; unpublished data). In addition, numerous studies have shown that inhibition of actin assembly by drugs of the cytochalasin family also suppresses G1 progression (Maness and Walsh, 1982; Iwig et al., 1995; Bohmer et al., 1996; Huang et al., 1998; Reshetnikova et al., 2000), and that arrest is abrogated by SV-40 transformation (Maness and Walsh, 1982).

As the SV-40 T antigen suppresses both p53 and RB pocket protein function (Bargonetti et al., 1992; Zalvide et al., 1998), we have here addressed whether the actin inhibition-dependent suppression of G1 progression is due to p53- or RB pocket protein-specific signaling events. For this, we have used mammalian cells containing either specific suppression of p53 or triple knockout of the RB pocket proteins. We have also tested MEFs with a knockout of RB alone or knockout of the alternative reading frame (ARF) tumor suppressor that regulates p53 accumulation (Sherr and Weber, 2000).

Strikingly, we demonstrate here that suppression of G1 progression in response to dihydrocytochalasin B (DCB) exposure is dependent on the function of the RB pocket protein family, but not on p53 status. Moreover, RB pocket protein-deficient cells also fail to undergo tetraploid checkpoint arrest at concentrations of DCB that induce cleavage failure (Andreassen et al., 2001b; Borel et al., 2002b). As a result, RB pocket protein-deficient cells become highly aneuploid after transient DCB exposure and rapidly die, whereas p53-deficient cells efficiently arrest in G1. Our work thus demonstrates that the RB pocket protein control pathway has a unique capacity to control G1 progression after actin disruption that is clearly independent of the p53 pathway. Because there are functions specific to RB pocket proteins that are independent of p53, our results show it should be possible to selectively kill cells with RB pathway defects. This may be important for therapeutic considerations, given the nearly universal alterations of function of the RB family pathway in tumor cells.

In addition, we have found in this work that pocket protein-competent G1 cells are extraordinarily sensitive to actin inhibitors, arresting at concentrations that do not affect substrate adhesion, cell spreading, or cell cleavage, but leave the actin array largely undisturbed. This was a surprising result,

as the effect of actin assembly inhibition on G1 progression had been linked to loss of cytoskeletal integrity and substrate adhesion (Bohmer et al., 1996; Assoian and Zhu, 1997; Reshetnikova et al., 2000). The question arises concerning the control system in G1 that might be directly affected by actin status. We have found that cell surface ruffling is specifically suppressed in cells arrested with low DCB concentrations. As the actin-associated ERM (ezrin, radixin, and moesin) protein NF2/merlin has been implicated in both cell surface ruffling (Bashour et al., 2002) and in suppression of cell cycle progression in G1 (Shaw et al., 1998; Morrison et al., 2001), we tested its status in arrested cells and found that NF2/merlin is up-regulated in DCB-induced G1 arrest. This result suggests that subtle cues from the actin cytoskeleton induce NF2/merlin-dependent arrest.

Results

DCB reversibly arrests mammalian fibroblasts in G1 at concentrations that do not suppress cleavage

Actin inhibitors in the cytochalasin and latrunculin families interfere with actin assembly and dynamics in mammalian cells. At concentrations of DCB approaching 10 μ M, the suppression of actin assembly during late mitosis causes failure of cell cleavage (Aubin et al., 1981; Martineau et al., 1995; Andreassen et al., 2001b), creating tetraploid cells.

Cytochalasins, at concentrations sufficient to fully disrupt actin structures and block cytokinesis, also arrest nontransformed cells in G1 (Bohmer et al., 1996; Assoian and Zhu, 1997; Reshetnikova et al., 2000). To establish the sensitivity of cell cycle progression to actin disruption, we exposed nontransformed REF-52 cells (primary rat embryo fibroblasts) in random cycle to different concentrations of DCB. We found that cell proliferation was significantly suppressed at 0.5 μ M DCB and completely suppressed at concentrations at or above 2 μ M DCB (Fig. 1 A).

Surprisingly, we found that the lower drug concentrations that suppressed cell cycle progression were significantly below those that blocked cell cleavage, as demonstrated by analysis of the DCB concentration-dependent induction of binucleated cells (Fig. 1 B). Binucleated cells, indicative of cleavage failure, did not appear at drug concentrations below 4 μ M. The combined effect of these different sensitivities on the status of cells after 24 h of drug exposure was to create a population of 2N cells blocked in G1 at 2 μ M DCB, whereas higher DCB concentrations yielded cell populations approximately equally divided between 2N and 4N status (Fig. 1 C). The 4N cells arise from cleavage failure and subsequent arrest in tetraploid G1, as we have previously shown (Andreassen et al., 2001b). These concentration-dependent profiles of cell cycle arrest were stable and persisted for days (unpublished data). As a control, we show that a concentration of nocodazole (0.5 μ g/ml) sufficient to fully disrupt microtubules did not arrest cells in G1 (Fig. 1 C). Cells instead progressed normally to mitotic arrest in the same time course.

Arrest of the cell cycle in G1 is reversible. After 25 h exposure to DCB, there is almost no DNA replication, as assayed by BrdU incorporation. In contrast, after 2 h release from either 2 or 10 μ M DCB, mononucleate cells incorporated

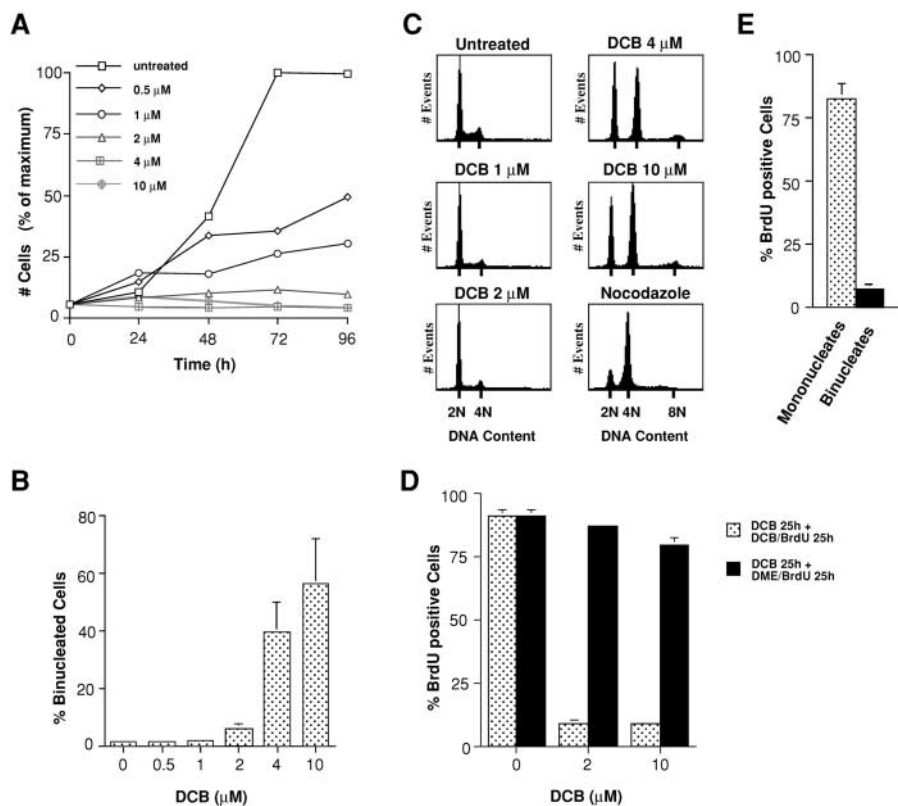


Figure 1. DCB induces reversible G1 arrest of rat embryo fibroblasts (REF-52) at concentrations that do not block cell cleavage. (A) Increasing concentrations of DCB progressively suppress REF-52 proliferation. Cells were exposed to the indicated DCB concentrations, and cell counts were taken at the indicated times. The curve is representative of three independent experiments. (B) DCB induces cytokinetic failure at concentrations $>2 \mu\text{M}$. REF-52 cells were exposed to the indicated DCB concentrations for 25 h. Binucleate cells, indicative of cleavage failure, were counted. Bar graph incorporates data from three independent experiments (300–500 cells counted each time). (C) DCB induces 2N arrest at concentrations $<4 \mu\text{M}$, whereas at higher concentrations, it yields both 2N and 4N arrest. REF-52 cells were exposed to the indicated concentrations of DCB for 25 h and assayed by flow cytometry. Nocodazole exposure ($0.5 \mu\text{g/ml}$) confirms that cells not treated with DCB were cycling in the same time course. (D) 2N G1 arrest imposed by DCB is reversible. REF-52 cells were exposed to 2 or $10 \mu\text{M}$ DCB for 25 h. Cells were then either maintained in DCB (spotted bars) or released from DCB (black bars) and exposed for

another 25 h to $10 \mu\text{M}$ BrdU to assay S-phase entry. BrdU incorporation was evaluated only in mononucleate cells. Values shown are the means of three independent counts of at least 300 cells each. (E) Binucleated cells do not recover from exposure to $10 \mu\text{M}$ DCB. REF-52 cells were exposed to $10 \mu\text{M}$ DCB for 25 h. DCB was then washed out, and cells were exposed to $10 \mu\text{M}$ BrdU for 25 h. Mononucleate and binucleate BrdU-positive cells were independently counted. Values shown are means from three independent counts of 300 cells each.

BrdU at levels comparable to controls that had not been exposed to DCB (Fig. 1 D). Reentry into the cell cycle is restricted to the mononucleate cells. As we have previously shown (Andreassen et al., 2001b), nontransformed cells do not recover from tetraploid G1 arrest. Thus, mononucleate cells recover from 25 h exposure to $10 \mu\text{M}$ DCB and incorporate BrdU, whereas binucleate cells do not (Fig. 1 E).

Arrest of randomly cycling cells in G1 by low concentrations of DCB is not restricted to REF-52 cells. Human nontransformed IMR-90 fibroblasts show the same concentration-dependent sensitivity to DCB and arrest in 2N G1 when exposed to $2 \mu\text{M}$ DCB, but arrest with approximately equal 2N and 4N status at higher DCB concentrations (Fig. 2 A). It is important to note that the arrest in 2N G1 can be distinguished from the arrest of tetraploid cells with 4N DNA content after DCB exposure (Andreassen et al., 2001b).

Loss of p53 function does not affect G1 arrest

Previous work has established that SV-40 abrogates G1 arrest by actin inhibition (Maness and Walsh, 1982). We also found (Fig. 2 A) that T antigen-transformed REF-52 (TAG) showed no euploid cell cycle arrest during exposure to DCB, nor did they arrest as tetraploid cells after cleavage failure at $10 \mu\text{M}$ DCB (Fig. 2 A). SV-40 large T antigen has multiple effects on the G1 cell cycle machinery and suppresses both p53- and RB pocket protein family-dependent G1 controls (Bargonetti et al., 1992; Zhu et al., 1992; Zal-

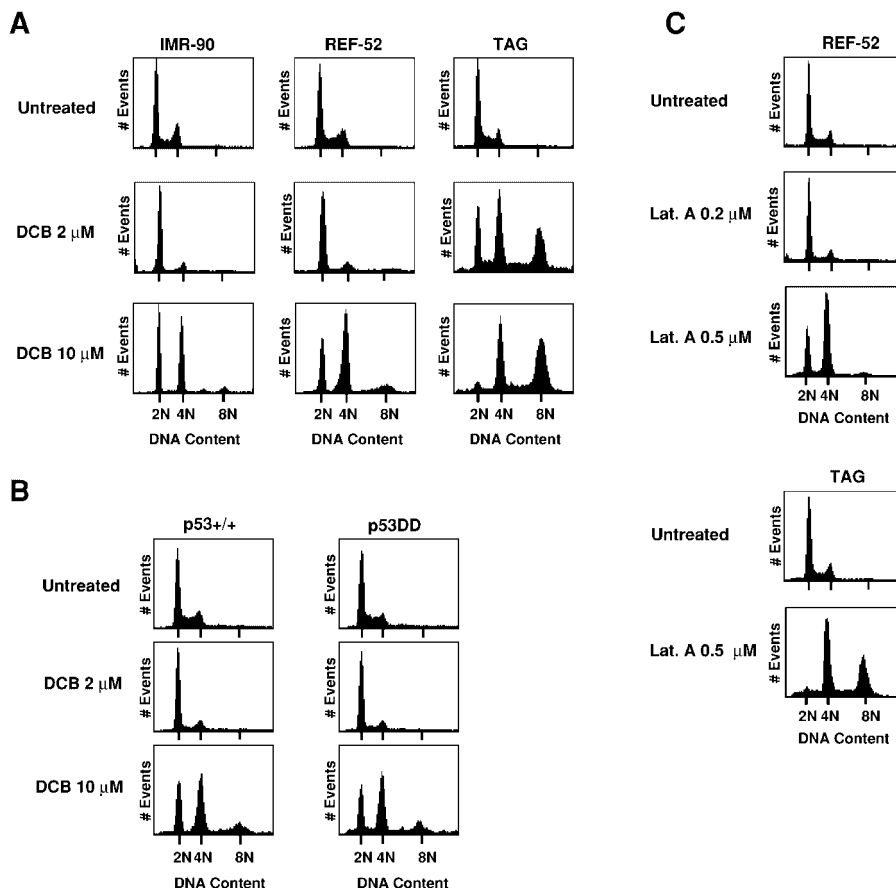
vide et al., 1998). We next asked whether G1 arrest was dependent on p53 or RB pocket protein status.

p53-dependent controls can be suppressed by expression of a dominant-negative truncation mutant of p53 (p53DD) (Shaulian et al., 1992). We have established a REF-52 cell line (p53DD REF-52) that expresses dominant-negative p53 (Andreassen et al., 2001b). To distinguish if p53 was involved in DCB suppression of G1 progression, we exposed p53DD REF-52 cells to both 2 and $10 \mu\text{M}$ DCB. Results (Fig. 2 B) clearly demonstrate that DCB-dependent arrest in G1 with 2N DNA content is independent of p53 function as cells arrest equally in G1, regardless of p53 status. We have obtained similar results with p53^{-/-} MEF cells (Fig. 3). We conclude that p53 is not involved in the DCB-dependent arrest of nontransformed fibroblasts in G1.

To confirm that the observed G1 arrest was specifically due to suppression of actin dynamics, we exposed REF-52 cells to latrunculin A, a compound that interferes with actin assembly by sequestering actin subunits (Spector et al., 1989). Results show that a low concentration of latrunculin A ($0.2 \mu\text{M}$) blocks cells in G1, as evidenced by a decrease in S-phase cells (Fig. 2 C), but does not interfere with cell cleavage, and thus fails to augment the 4N population. By contrast, a higher concentration ($0.5 \mu\text{M}$) yields approximately equal 2N and 4N populations (Fig. 2 C). As shown for DCB, TAG cells do not arrest when exposed to latrunculin A. Similar results have been obtained with cytochalasin D (unpublished data).

Figure 2. Actin inhibitors induce p53-independent 2N G1 arrest in nontransformed fibroblasts but not in transformed variants.

(A) Nontransformed fibroblasts REF-52 and IMR-90, but not transformed TAG variants, arrest in the presence of DCB. REF-52, IMR-90, and TAG cells were exposed to the indicated concentrations of DCB for 25 h. Both REF-52 and IMR-90 arrest as 2N populations in the presence of DCB, whereas TAG cells do not arrest and progress to higher ploidy. Note that TAG cell cleavage is partially sensitive to 2 μ M DCB. (B) p53 is not required for 2N G1 arrest in the presence of DCB. p53 was inactivated in REF-52 (p53DD) by expression of a dominant-negative mutant (Shaulian et al., 1992; Andreassen et al., 2001b). p53^{+/+} REF-52 and p53DD REF-52 cells were exposed to either 2 or 10 μ M DCB for 25 h. Flow cytometry shows that p53DD REF-52 and control (p53^{+/+}) REF-52 exhibit comparable 2N G1 arrest in the presence of DCB. (C) Latrunculin A imposes 2N G1 arrest in REF-52 cells but not in their transformed variants. REF-52 and TAG cells were exposed to the indicated concentrations of latrunculin A for 25 h. Flow cytometry shows that REF-52 cells exhibit 2N G1 arrest both at 0.2 and 0.5 μ M latrunculin A, whereas TAG cells do not arrest but progress to higher ploidy at 0.5 μ M latrunculin A.



Blockage by DCB in G1 is dependent on RB pocket proteins

REF-52 cells arrest in G1 in response to suppression of actin dynamics even when p53 function is suppressed, but no ar-

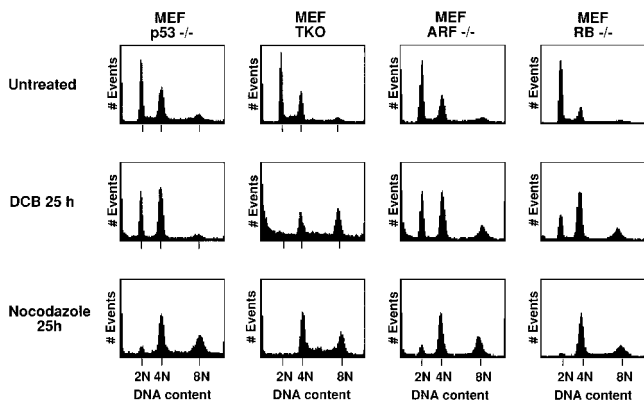


Figure 3. DCB-induced G1 blockage is dependent on the RB pocket protein family. p53^{-/-} MEFs, MEFs deleted for the three RB pocket proteins (TKO), ARF^{-/-} MEFs, and RB^{-/-} MEFs were exposed to 10 μ M DCB or to nocodazole (0.5 μ g/ml) for 25 h and analyzed for cell cycle distribution. Flow cytometric analysis shows that p53^{-/-} MEFs exhibit 2N G1 arrest, whereas TKO MEFs do not and instead progress to higher ploidy. ARF^{-/-} MEFs exhibit 2N G1 arrest like that of p53^{-/-} MEFs, whereas RB^{-/-} MEFs exhibit partial 2N G1 arrest unlike TKO MEFs, which do not arrest. In all cases, nocodazole exposure confirms that control cells, not exposed to DCB, were cycling in the same time course.

rest occurs in REF-52 transformed with SV-40 large T antigen (Fig. 2). As large T antigen suppresses the function of both p53 and the RB family of pocket proteins, RB, p107, and p130 (Bargonetti et al., 1992; Zalvide et al., 1998), the implication is that suppression of actin dynamics may act through the pocket proteins to block cells in G1.

To directly test the role of the RB pocket proteins in actin-dependent arrest in G1, we have compared the effect of 10 μ M DCB on cell cycle progression in p53^{-/-} MEFs with that in TKO MEFs, in which all three RB pocket proteins have been deleted (Sage et al., 2000). As the three RB pocket proteins have overlapping functions, deletion of all three is necessary for immortalization of MEFs (Mulligan and Jacks, 1998; Dannenberg et al., 2000; Sage et al., 2000). After 24 h exposure to DCB, randomly cycling p53^{-/-} cells arrest both in 2N G1 and in 4N G1 after cleavage failure (Fig. 3). In contrast, TKO MEFs fail to arrest in 2N G1 but proceed to a mixed 4N and 8N status (Fig. 3). We conclude that suppression of actin assembly arrests MEFs in G1 through the mediation of the RB pocket proteins, but not of p53.

In contrast to the differential effect of DCB on the cell cycle in these MEF cell lines, nocodazole, which interferes with microtubule dynamics and prevents completion of mitosis (Jordan et al., 1992), has the same effect on both p53^{-/-} and TKO MEFs. In both p53^{-/-} and TKO cell lines, 4N and 8N populations predominate after 24 h of nocodazole exposure (Fig. 3).

We obtained substantiating evidence for the role of the RB pocket protein family in DCB-dependent arrest in G1

by also examining the response of RB^{-/-} and ARF^{-/-} MEFs. Knockout of RB alone does not immortalize cells or block contact inhibition arrest (Dannenberg et al., 2000; Sage et al., 2000), as the RB protein family has overlapping and redundant functions. RB^{-/-} cells, however, showed a partial loss of arrest in G1 in response to DCB (Fig. 3). ARF stabilizes p53 and is required for p53-dependent cell cycle arrest, but is not required for the RB pocket protein control in G1 (Sherr and Weber, 2000). As expected for a protein that stabilizes p53 but does not influence the RB pathway, knockout of ARF had no effect on the capacity of MEFs to arrest in G1 when exposed to DCB (Fig. 3).

G1-arrested fibroblasts remain adherent but suppress ruffling and up-regulate NF2/merlin in 2 μM DCB

There is substantial evidence that the capacity of nontransformed fibroblasts to progress in the cell cycle is coupled to substratum adherence (Stoker et al., 1968; Assoian, 1997; Assoian and Schwartz, 2001). However, even at 10 μM DCB, we have found that cells that are no longer capable of undergoing cleavage remain adherent (unpublished data). At 2 μM DCB, there is little overt evidence of disturbance of the actin cytoskeleton in randomly cycling interphase cells and no evidence of disturbance of adherence (Fig. 4). Compared with controls, cells retain abundant actin cables, and focal adhesion plaques appear intact, as assayed by both vinculin antibody (Fig. 4 A) and antiphosphotyrosine antibody (Fig. 4 B). One notable change during arrest is that, in comparison to controls, cells exposed to 2 μM DCB have smooth margins and do not appear to undergo ruffling (Fig. 4 C). The absence of ruffling is rapidly reversible on release from DCB, and ruffling is equally evident at 30 min and a full day after DCB release (Fig. 4 C).

The absence of ruffling (Fig. 4) and the RB pocket protein dependence of DCB G1 arrest induced by DCB (Fig. 3) suggested a possible involvement of NF2/merlin as a mediator of DCB-dependent arrest. Knockout of the RB pocket protein family abolishes contact inhibition-dependent arrest (Dannenberg et al., 2000; Sage et al., 2000). The tumor suppressor NF2/merlin, an actin-binding protein (Gautreau et al., 2002) that suppresses cell surface ruffling (Bashour et al., 2002), is specifically up-regulated during, and is required for, contact inhibition arrest in G1 (Shaw et al., 1998; Morrison et al., 2001). We therefore assayed for the status of NF2/merlin in G1 arrest induced by 2 μM DCB. Strikingly, we found that NF2/merlin is strongly up-regulated and dephosphorylated in DCB-dependent arrest in comparison to randomly cycling cells (Fig. 4 D). This result suggests that NF2/merlin up-regulation may mediate the actin-dependent arrest.

Adherence to the substratum is intact in 2 μM DCB-treated cells by the criterion of the activation of extracellular signal-regulated kinase (ERK) on serum stimulation in the presence of 2 μM DCB. Fibroblast cell lines maintained in suspension fail to activate the ERK pathway in response to growth factor stimulation (Lin et al., 1997; Renshaw et al., 1997; Aplin et al., 1999). REF-52 cells arrest in G0 after serum starvation, and 2 μM or 10 μM DCB, but not nocodazole, inhibits progression past G1 upon serum add-back (Fig. 5 A). In accord with previous findings (Reshetnikova et al., 2000), we have found that REF-52 cells released from

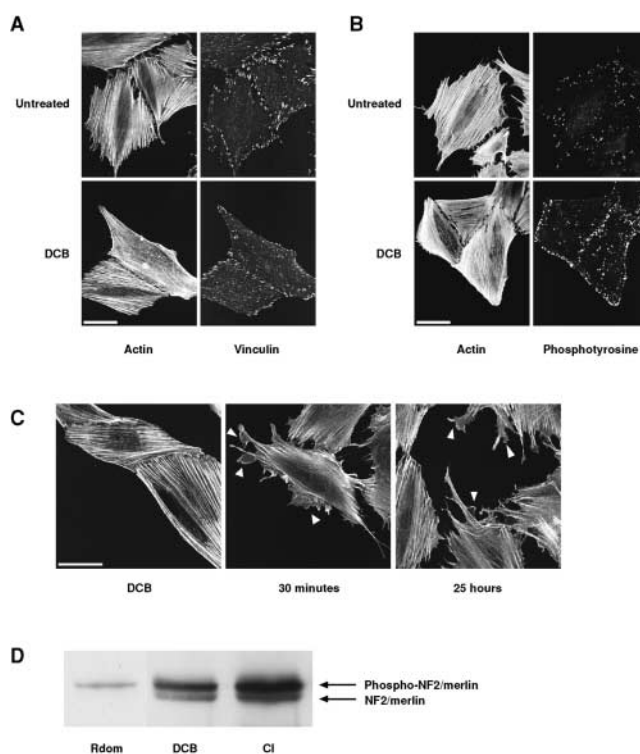


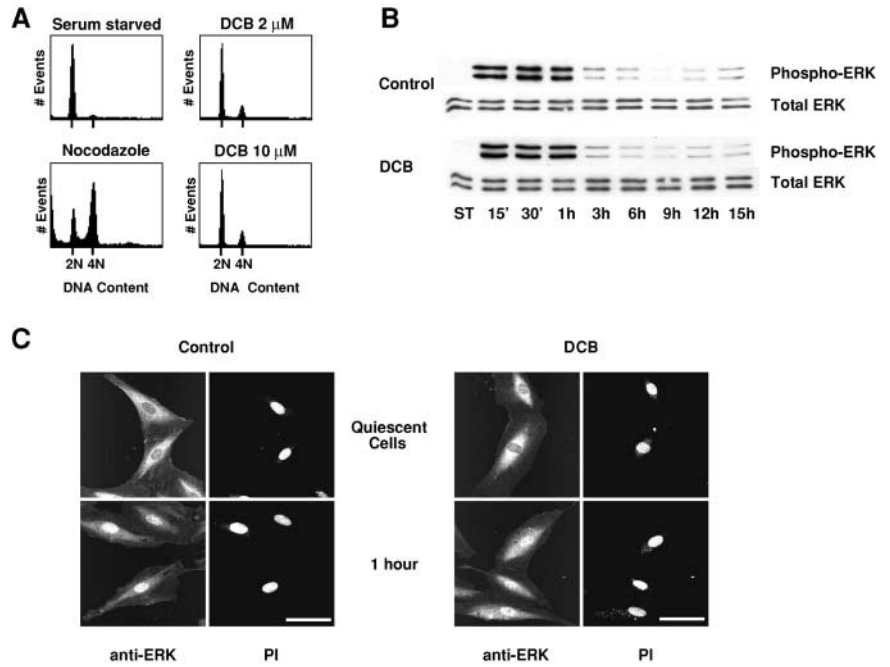
Figure 4. Actin stress fibers and substratum adherence are not perturbed but NF2/merlin is up-regulated after exposure of REF-52 cells to 2 μM DCB. REF-52 cells were exposed to 2 μM DCB for 25 h. Cells were then analyzed for actin distribution with TRITC-phalloidin (A and B, left), and vinculin (A, right) and phosphotyrosine (B, right) distribution were determined by immunolabeling. For each pair (untreated/DCB) in both A and B, the intensity settings used for confocal microscopy were kept constant. Bars, 40 μm. (C) Higher magnification images show that ruffling was suppressed in cells exposed to 2 μM DCB for 25 h (DCB), but reappeared rapidly (arrowheads) in cells released from DCB (30 min) and persisted at 25 h of release. Cells were imaged for actin with TRITC-phalloidin. Bar, 40 μm. (D) NF2/merlin is up-regulated and dephosphorylated in DCB-arrested cells. REF-52 cells, either random cycling (Rdom), contact inhibited (CI), or random cycling while exposed to 2 μM DCB for 25 h (DCB), were harvested and subjected to Western blotting procedures using anti-NF2/merlin antibodies. An equivalent amount of protein was loaded for each sample.

G0 serum starvation in the presence of low concentrations of DCB showed time-dependent ERK activation after serum add-back that is indistinguishable from controls (Fig. 5 B). Further, after serum stimulation, ERK migrates to the nuclei of DCB-blocked cells (Fig. 5 C).

Our results indicating up-regulation of NF2/merlin during DCB-induced arrest and the requirement for RB proteins both suggested that arrest would be in G1. Further, Bohmer et al. (1996) found, in timed experiments with synchronous cells, that arrest of fibroblasts with high concentrations of cytochalasin occurred in mid to late G1. To assess the physiological status of randomly cycling cells arrested in G1 by 25 h exposure to 2 μM DCB, we assayed for the presence and activity of different cell cycle markers, compared with the presence of these markers in contact-inhibited cells in G0/G1, in control cycling cells, or in cycling cells arrested in S phase (Fig. 6). S-phase cells were obtained by exposure for 25 h to 2 mM hydroxyurea (HU), which blocks cells at the G1/S

Figure 5. DCB does not interfere with ERK kinase activation and nuclear translocation upon serum stimulation of quiescent cells.

(A) Serum-starved REF-52 cells were reactivated with 10% FCS in the presence of 2 or 10 μM DCB. Flow cytometry shows that cells remain arrested in G1 25 h after release. Nocodazole exposure of cells in the absence of DCB indicates that control cells were cycling in the same time course. (B) ERK1/2 is normally activated in the presence of 2 μM DCB. At time intervals after add-back of serum to serum-starved REF-52, cells were harvested and subjected to Western blotting using either antibodies to total ERK or to activated phospho-ERK. Experiments were run either in the presence of 2 μM DCB (DCB, bottom two strips) or absence of DCB (Control, top two strips). ST designates quiescent serum-starved controls. (C) ERK1/2 is normally translocated to the nucleus in the presence of 2 μM DCB. 1 h after serum add-back to serum-starved REF-52 cells, ERK1/2 kinases were immunolocalized using anti-pan ERK antibodies. Experiments were run either in the presence of 2 μM DCB (DCB, right) or in the absence of DCB (Control, left). Nuclei were counterstained with propidium iodide (PI). Bars, 50 μm .



boundary with active Cdk2 and suppressed p27 (Borel et al., 2002a). The levels of different markers in DCB-arrested cells resembled those found in contact-inhibited cells. Cyclin A was absent in both cases, and p27^{Kip1}, a CKI (Polyak et al., 1994a,b), was elevated. p21^{WAF1}, a CKI transactivated by p53 (el-Deiry et al., 1993), was not elevated, consistent with the insensitivity of arrest to p53 status. RB was hypophosphorylated both in contact-inhibited cells and in DCB-treated cells. This profile is consistent with suppression of Cdk2 activity in both contact-inhibited and DCB-blocked status (Fig. 6 B). In contrast to contact-inhibited cells, drug-arrested cells showed reduced Cdk4 activity (Fig. 6 B). The induction of p27^{Kip1} was not incidental to DCB treatment, as

it did not occur in cells that were first arrested in S phase with HU and then treated with DCB (Fig. 6 C).

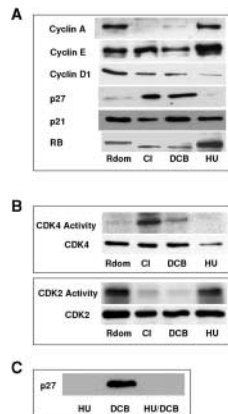
Cells with suppressed RB pocket protein function, but not with suppressed p53 function, die rapidly after DCB exposure

We, and others, have previously shown that a p53-dependent G1 checkpoint prevents progression of mammalian fibroblasts to S phase when they have exited mitosis without completing chromosome segregation or cell cleavage (Minn et al., 1996; Lanni and Jacks, 1998; Andreassen et al., 2001b). Failure to arrest in tetraploid G1 rapidly leads to aneuploidy and is lethal for the majority of cells that do not arrest. It is clear that TKO MEFs, in contrast to p53^{-/-} MEFs, fail to arrest in either euploid or tetraploid G1 after exposure to DCB (Fig. 3). Thus, as found after suppression of p53 activity (Andreassen et al., 2001b), suppression of RB pocket proteins can lead to aneuploidy and death after induction of tetraploidy.

In the presence of DCB, p53 suppression does not abrogate G1 arrest, whereas pocket protein suppression does. Thus, in a randomly cycling population exposed to DCB, lethality will differ depending on whether the cells are suppressed for p53 activity or for RB pocket protein activity. RB pocket protein-suppressed cells can progress past G1 and past tetraploid G1, potentially leading to aneuploidy and death. In contrast, p53-incompetent cells arrest in euploid G1 in DCB and can thus recover normally upon drug release. In accord with this hypothesis, we have found that both REF-52 and p53DD REF-52 cells recovered from DCB arrest, seeded by those cells that had been arrested in euploid G1 (Fig. 7, A and B). In contrast, TAG cells that additionally suppress RB pocket protein activity rapidly became aneuploid, as shown by a decrease in DNA content

Figure 6. DCB-arrested REF-52 cells have markers characteristic of G1.

(A) Asynchronous cells (Rdom), contact-inhibited cells (CI), and cells exposed for 25 h to either 2 μM DCB (DCB) or 2 mM HU (HU) were harvested and subjected to Western blotting procedures using the indicated antibodies. (B) The kinase activities of Cdk2 and Cdk4 were determined after immunoprecipitation of the kinases and incubation with histone H1 (Cdk2) or RB peptide (ckd4) as substrates. Autoradiographs of ³²P incorporation are shown (top strips) and compared with the kinase expression levels as determined by Western blotting (bottom strips). (C) p27^{Kip1} accumulation is not incidental to DCB treatment. REF-52 cells were exposed for 25 h to either 2 mM HU or 2 μM DCB. Alternatively, cells were exposed to 2 mM HU for 25 h before addition of 2 μM DCB for 25 h in the continuous presence of HU. Samples were subjected to Western blotting analysis using anti-p27^{Kip1} antibodies.



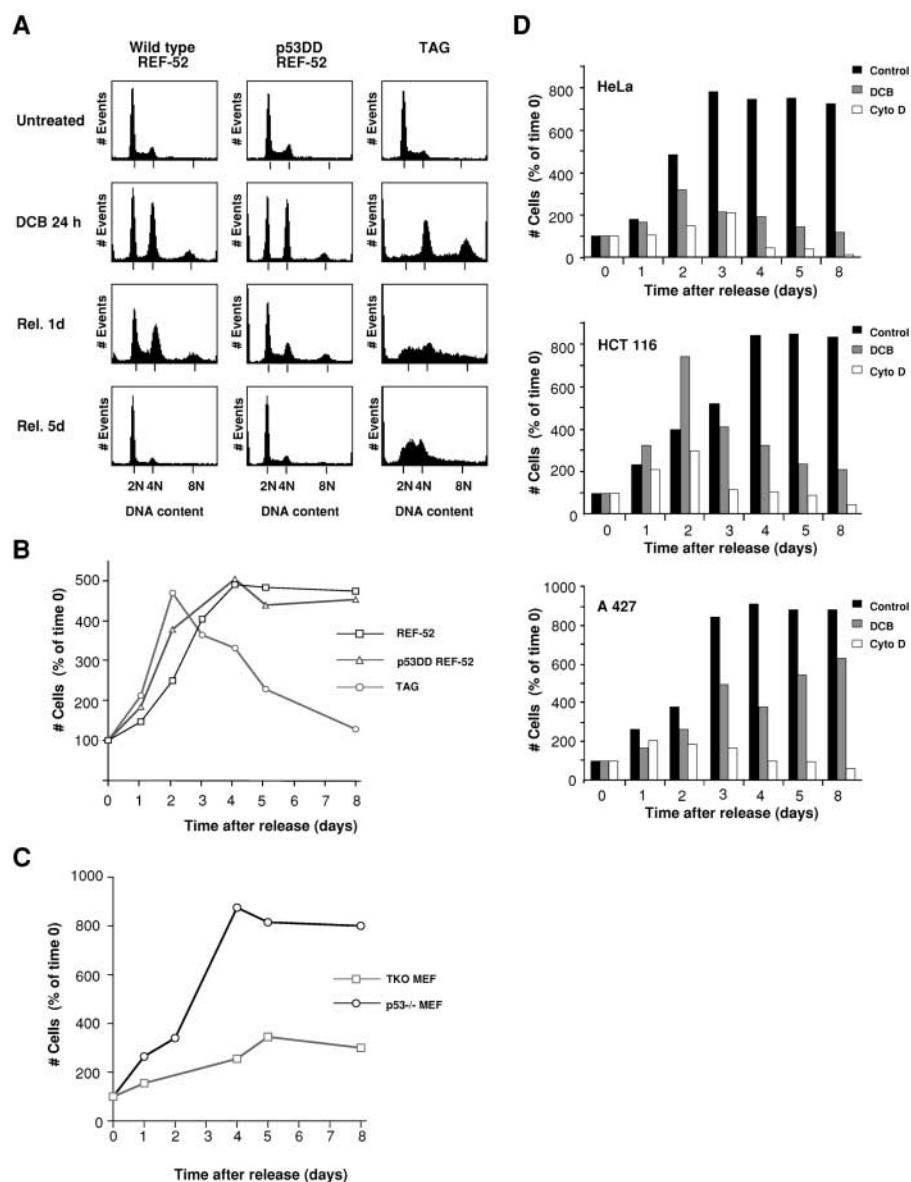


Figure 7. Failure of DCB-induced G1 blockage is lethal in RB pocket protein-suppressed cells. (A) Asynchronous REF-52, p53DD REF-52, and TAG cells were exposed to 5 μ M DCB for 24 h. Flow cytometric analysis of DNA content at the indicated times shows that TAG cells rapidly become aneuploid upon release from DCB, whereas REF-52 and p53DD REF-52 cells recover from DCB arrest and reach contact inhibition after a few days. (B) Asynchronous REF-52, p53DD REF-52, and TAG cells were exposed to 5 μ M DCB for 24 h. DCB was then washed out and cell counts were taken at the times indicated. (C) Asynchronous p53^{-/-} and TKO MEFs were exposed to 10 μ M DCB for 25 h. DCB was then washed out and cell counts taken at the indicated times. (D) Asynchronous HeLa, HCT 116, and A427 cells were exposed to either 10 μ M DCB or 5 μ M cytochalasin D (Cyto D) for 24 h. The drug was then washed out and cell counts taken at the times indicated. Control refers to untreated cells. In all cases, plateaus at late times indicate approach to confluency except for TKO MEFs, which do not reach high density.

and loss of identifiable 2N and 4N peaks in DNA histograms, and died after release from DCB (Fig. 7, A and B). Similarly, RB pocket protein-suppressed MEFs (TKO) showed a highly reduced capacity to proliferate after transient exposure to DCB compared with control p53^{-/-} MEFs (Fig. 7 C).

As our results suggested that short term exposure to actin inhibitors might kill tumor cells, we directly tested this possibility by exposing the human tumor cell lines HeLa (cervical adenocarcinoma), HCT116 (colon carcinoma), and A427 (lung carcinoma) to DCB or cytochalasin D. Cells were exposed for 24 h and then released from drug. Results (Fig. 7 D) show that all three tumor cell lines fail to proliferate after drug exposure, and that they are uniformly more sensitive to cytochalasin D than to DCB.

Discussion

Nontransformed mammalian fibroblasts arrest reversibly in G1 when exposed to low concentrations of actin assembly

inhibitors. We have found that G1 arrest is not sensitive to p53 status, but only occurs when RB pocket protein activity is intact. This observation demonstrates RB pocket protein control that is distinct from p53 mechanisms. Further, as it is believed that RB function must be suppressed in one way or another in all human tumors (Weinberg, 1995; Sherr, 1996; Hanahan and Weinberg, 2000), our results raise the possibility that a therapeutic approach could exploit the continued cycling of tumor cells when the actin cytoskeleton, or elements of an associated pathway, is disrupted, causing directed death by selective response of pocket protein-suppressed cells to toxic agents. Independence of this G1 arrest from p53 is critical to the potential therapeutic effectiveness of such an approach in tumors with deficiencies in the RB pathway, but with normal p53 function.

The effective concentrations of actin inhibitors that induce G1 arrest are well below the concentrations that globally suppress actin assembly, substrate adhesion, or cell cleavage. Suppression is accompanied by expression of p27^{Kip1}, hypophosphorylation of RB, and inhibition of surface ruffling.

However, there appears to be no inhibition of ERK activation under arrest conditions (Reshetnikova et al., 2000; Fig. 4).

G1 arrest due to RB pocket proteins but not p53

We have demonstrated that G1 arrest due to actin inhibition is dependent on the presence of the RB pocket proteins but is independent of p53. Previous work had demonstrated that SV-40–transformed fibroblasts do not arrest in G1 upon actin inhibition (Maness and Walsh, 1982). As SV-40 large T antigen suppresses both p53 and RB (Bargonetti et al., 1992; Zalvide et al., 1998), the relative involvement of p53 and the pocket proteins in the G1 suppression remained unresolved. p53 activation arrests cells in G1 in response to DNA damage, as well as a variety of cell cycle disturbances. For example, it is involved in G1 arrest induced by taxol (Trielli et al., 1996; Wahl et al., 1996) or by creation of tetraploidy after failure of mitosis or cell cleavage (Cross et al., 1995; Minn et al., 1996; Lanni and Jacks, 1998; Andreassen et al., 2001b). p53 thus clearly mediates G1 arrest independent of DNA damage.

The interconnections between p53- and RB pocket protein–dependent controls in G1, coupled with the fact that the RB pocket proteins are overlapping in function (Mulligan and Jacks, 1998), have made it difficult to distinguish events in G1 that are dependent on the RB pocket proteins but are independent of p53. The generation of triple knockouts of the three RB pocket proteins in MEF cells has, for the first time, permitted an analysis of pocket protein functions responsive to the actin assembly state that are independent of p53.

G1 arrest and the actin cytoskeleton

It has been evident for a long time that mammalian fibroblasts arrest in G1 when exposed to concentrations of cytochalasin sufficient to fully dismantle the actin network (Maness and Walsh, 1982; Iwig et al., 1995; Bohmer et al., 1996; Huang et al., 1998; Reshetnikova et al., 2000). The interpretation has been that G1 arrest due to cytochalasin derives from loss of cytoskeletal integrity, triggering a G1 arrest similar to that observed after loss of substrate adhesion (Assoian, 1997; Assoian and Schwartz, 2001). We have here demonstrated that arrest of cells in G1 after inhibition of actin assembly surprisingly occurs at inhibitor concentrations that do not interfere with cell spreading, formation of focal adhesions, or normal cell cleavage. Thus, although it is evident that actin-dependent substrate adhesion is required for cell cycle progression, our work clearly shows that this is a precondition for the cytoskeleton-dependent signal processing that permits G1 progression, and that arrest occurs after much more subtle perturbation of the actin cytoskeleton than previously appreciated.

The progression of fibroblasts through G1 is regulated by the combination of growth factor activation and integrin-mediated adhesion to the extracellular matrix (Howe et al., 1998; Assoian and Schwartz, 2001). The activation pathways converge on ERK1/2, and adhesion-dependent G1 progression thus appears to require the joint regulation of p42/p44 MAP kinase by integrins and growth factors. We find that ERK has sustained activity and correctly translocates to the nucleus despite stable G1 arrest in DCB, indicating that the receptor tyrosine kinase and integrin path-

ways are both active in the blocked cells (Lin et al., 1997; Renshaw et al., 1997). Interestingly, our results show that ERK1/2 is indistinguishably activated in DCB-treated cells compared with G1 controls, and thus indicate that sustained ERK1/2 activation is not sufficient for G1 progression. These results agree with prior results that suggested ERK activation in cytochalasin-arrested cells (Reshetnikova et al., 2000), and are interesting in light of evidence that sustained ERK1/2 activity alone is not sufficient to drive G1 progression in suspended fibroblasts and that other attachment-dependent factors are involved (Le Gall et al., 1998).

The activation of ERK is a central feature of anchorage-dependent cell growth, and its activity may be related to RB pocket protein control that is independent of p53. The induction of cyclin D1 expression and the down-regulation of the CKIs p21^{WAF1} and p27^{Kip1} are major targets of anchorage-dependent signaling in G1 (Assoian, 1997). ERK1/2 is critical to induction of cyclin D1 expression (Lavoie et al., 1996; Weber et al., 1997) and has been shown to be involved in p27^{Kip1} down-regulation (Rivard et al., 1999; Delmas et al., 2001). These events are, in turn, requisite for activation of Cdk4/6 and Cdk2 cyclin-dependent kinases, which, in turn, hyperphosphorylate RB (for review see Mittnacht, 1998), ultimately permitting release of the E2F transcription factors that activate S phase. Interestingly, we have found that, whereas ERK1/2 are activated by phosphorylation and are properly translocated to the nucleus, cyclin D1 expression and Cdk4 activation are both reduced. In keeping with mid-G1 arrest (Bohmer et al., 1996), Cdk2 is inactive and p27^{Kip1} is up-regulated. Perhaps as a result, RB remains hypophosphorylated in DCB-blocked cells. It will be interesting to pursue these observations further, to understand how actin suppression can block the pathway that permits RB hyperphosphorylation after ERK1/2 activation.

It is of substantial interest that NF2/merlin up-regulation and hypophosphorylation accompany DCB-dependent G1 arrest (Fig. 3 D). NF2/merlin is a member of the ERM (ezrin, radixin, and moesin) family of actin-binding proteins that link the actin cytoskeleton to the cell cortex (Gautreau et al., 2002). It is up-regulated by, and required for, contact inhibition arrest in G1 (Shaw et al., 1998; Morrison et al., 2001). Further, increased levels of expression correlate well with suppressed cell surface ruffling (Bashour et al., 2002). Our results thus suggest that NF2/merlin is involved in the pathway that leads to G1 arrest in DCB. It is possible that suppression of ruffling by DCB gives a false signal of cell–cell contact, leading to the contact inhibition response. In accord with this suggestion, the RB pocket protein family, which is required for DCB arrest, has been directly implicated in the contact inhibition response (Dannenberg et al., 2000; Sage et al., 2000).

Comparison to other cytoskeleton-dependent arrest in G1

The state of the cytoskeleton influences G1 progression through mechanisms other than actin-dependent suppression. We have previously shown that taxol, an inhibitor of microtubule dynamics, reversibly arrests nontransformed fibroblasts in G1 (Trielli et al., 1996). Further, we have established that the taxol-dependent arrest is suppressed in T an-

tigen-transformed cells (Trielli et al., 1996). Unlike DCB-dependent G1 arrest, taxol-dependent arrest in G1 is p53 dependent (unpublished data).

Arrest with DCB in G1 can also be distinguished from the G1 arrest that arises from creation of a tetraploid state by DCB-dependent suppression of cell cleavage. Tetraploid G1 arrest is permanent, persisting after removal of DCB (Andreassen et al., 2001b), and unlike the DCB G1 block, is dependent on p53 status (Andreassen et al., 2001b). Tetraploid G1 arrest occurs regardless of the cause of tetraploidy (Andreassen et al., 2001b) and appears to be the predominant cause of p53-dependent 4N arrest in colon carcinoma cells after DNA damage (Andreassen et al., 2001a).

Implications for tumor therapy

A remarkable aspect of the DCB-dependent G1 arrest reported here is that it is insensitive to p53 status, but sensitive to the status of the RB pocket proteins. Only MEF cells that have a triple knockout for the three RB pocket proteins fail to arrest in G1 in response to DCB exposure. The result is duplicated in TAG cells, which are large T antigen-transformed REF-52. We have shown that p53DD REF-52 cells respond like wild-type cells to DCB, but that TAG cells fail to arrest. The major effects of large T antigen are to suppress p53 and the RB pocket protein functions (Bargonetti et al., 1992; Zalvide et al., 1998). It is therefore likely that TAG cells fail to arrest, as in the case of TKO MEF cells, because the RB pocket proteins are suppressed.

To our knowledge, this is the first evidence that a pharmacological approach can both selectively block cells with intact RB function and permit induction of death in cells with compromised function. The result has been striking. The failure of RB pocket protein-suppressed cells to arrest in G1 in the presence of high concentrations of DCB causes them to selectively continue to cycle to aneuploid status and die. We have further demonstrated that DCB and cytochalasin D efficiently kill human tumor cells of three different tissue origins. Latrunculin and other actin inhibitors are being used in clinical chemotherapy trials (Jordan and Wilson, 1998). It will now be of primary importance to establish whether latrunculin or other drugs will have a selective lethal effect on RB-suppressed cells. As the RB pocket protein pathway is suppressed in virtually all human tumors, this approach may hold substantial promise for selective tumor therapy.

We have demonstrated the effect of RB suppression on progression to cell death after exposure to DCB alone. An alternative approach that might prove highly effective would be to use low doses of actin inhibitors accompanied by exposure to ionizing radiation or taxol, to which cycling cells are particularly sensitive (Iliakis, 1991; Trielli et al., 1996; Blajeski et al., 2001). In this case, only RB-suppressed cells would bypass G1 arrest and would be selectively vulnerable to death induced by conventional tumor therapy.

Materials and methods

Cell culture and infection

REF-52 cells and their SV-40 large T antigen-transformed derivatives (TAG) (Perry et al., 1992) were a gift from G.R. Stark (Cleveland Clinic, Cleveland, OH). REF-52 primary cells were used at <35 passages. IMR-90 cells were obtained from Coriell Cell Repositories. p53DD REF-52 were

prepared as previously described (Andreassen et al., 2001b). p53^{-/-} MEFs were generated as described by Borel et al. (2002b). TKO and RB^{-/-} MEFs were a gift from J. Sage and T. Jacks (Massachusetts Institute of Technology, Cambridge, MA). ARF^{-/-} MEFs were obtained from C. Sherr and M. Roussel (St. Jude's Children's Hospital, Memphis, TN). MEFs were used at no more than four passages. HeLa and A427 cells were obtained from the American Type Culture Collection. HCT 116 cells were the gift of B. Vogelstein (Johns Hopkins University, Baltimore, MD). Cell doubling times at mid-log phase were 24 h for REF-52, p53DD REF-52, IMR-90, TKO MEFs, p53^{-/-} MEFs, RB^{-/-} MEFs, and Arf^{-/-} MEFs and 18 h for TAG. All cells were cultured as monolayers in DME (GIBCO BRL) supplemented with 10% FCS (Biological Industries). Cells were maintained in a humid incubator at 37°C in a 5% CO₂ environment.

Cell treatment and synchronization

Randomly cycling cells were exposed to the indicated doses of DCB for 24 h. To assay for cell cycle progression during or after exposure to DCB, cells were incubated with 10 μM BrdU for 24 h. To synchronize REF-52 cells in G0, cells were cultured in medium containing 0.1% FCS for 24 h. Serum-starved cells were then released from G0 by the addition of 10% FCS, and DCB, as appropriate, was added 2 h before serum addition. To induce contact inhibition, REF-52 cells were kept at confluency for 36 h.

To synchronize REF-52 cells in early S phase, randomly cycling cells were incubated with 2 mM HU for 24 h. In all cases, drugs were added to randomly cycling cells no earlier than 36 h after replating to allow for full spreading before drug treatment. HU, DCB, and nocodazole were obtained from Sigma-Aldrich. Latrunculin A was obtained from Molecular Probes. HU was prepared as a 200 mM stock in DME containing 10% FCS. DCB, latrunculin A, and nocodazole were prepared in DMSO as 10 mM, 2 mM, and 1 mg/ml stocks respectively. BrdU was prepared from a 10 mM stock in DME.

Cell counts and flow cytometry

Randomly cycling REF-52 cells were incubated with the indicated doses of DCB. Every 24 h, cells were harvested by trypsinization and resuspended in 1 × PBS, and cell counts were taken using a Neubauer hemacytometer. To assess the impact of DCB or cytochalasin D on cell survival, cells were exposed to drug for 24 h and then released, and counts were then taken every 24 h. For flow cytometry, cells were trypsinized, collected by centrifugation, and then fixed, stored, and analyzed as previously described (Andreassen et al., 2001b).

Assay of Cdk activity

To assay for Cdk2 and Cdk4 activities, cell lysates were prepared from random cycling REF-52, contact-inhibited REF-52, or random cycling REF-52 exposed to 2 μM DCB or 2 mM HU for 24 h. Cells were collected by trypsinization, and preparation of lysates, immunoprecipitation, and radioimmuno assay for Cdk4 and Cdk2 were as described by Trielli et al. (1996) and Andreassen and Margolis (1994), respectively.

Immunoblotting

Cell lysates were prepared for Western blots as described above for Cdk activity assay. 10 μg of whole cell lysates (25 μg for cyclin D1 and RB) was then resolved by PAGE, transferred to nitrocellulose or Immobilon (RB) sheets, blocked with 5% nonfat milk in TNT buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), incubated overnight with primary antibodies, and then incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies for 1 h. Protein-antibody complexes were detected by ECL (Pierce Chemical Co.). The primary antibodies used were anti-cyclin A (1:5,000), anti-cyclin E (1:3,000), anti-Cdk2 (1:3,000) (Brenot-Bosc et al., 1995), anti-cyclin D1 (HD11; 1:2,000), anti-Cdk4 (1:1,000), anti-p21^{WAF1} (C-19; 1:2,000), anti-NF2/merlin (C-18; 1:200) (all from Santa Cruz Biotechnology, Inc.), anti-p27^{Kip1} (1:2,500; Transduction Laboratories), anti-RB (1:500; BD Biosciences), anti-pan ERK (1:12,000; Zymed Laboratories), and anti-dual phosphorylated ERK (V8031; 1:2,000; New England Biolabs, Inc.). All primary antibodies, except for p21^{WAF1}, were diluted in TNT buffer containing nonfat milk (1% for RB; 2% for p27^{Kip1} and ERK antibodies; 2.5% for cyclin A, cyclin E, cyclin D1, and Cdk2; 5% for Cdk4; and 10% for NF2/merlin). All secondary antibodies were diluted 1:5,000 (except for detection of RB [1:300] and cyclin D1 [1:2,500]) in TNT buffer (also containing 2.5% nonfat milk for detection of cyclin A, cyclin E, cyclin D1, and ERK).

Immunofluorescence microscopy

For immunofluorescence microscopy, cells were grown on poly-D-lysine-coated glass coverslips. To assay for cytokinetic failure in the presence of

DCB, cells were fixed with methanol at -20°C for 10 min, microtubules were labeled as previously described (Trielli et al., 1996), and nuclei were stained by the addition of 0.5 $\mu\text{g}/\text{ml}$ propidium iodide. For quantification of BrdU incorporation, cells were fixed and prepared with FITC-conjugated anti-BrdU antibody as previously described (Andreassen et al., 2001b).

To assay for actin assembly and cell adhesion, randomly cycling cells were exposed to 2 μM DCB for 24 h as described above, fixed with 2% paraformaldehyde in PBS, and incubated with antibodies as previously described (Andreassen and Margolis, 1994). Monoclonal anti-vinculin and anti-phosphotyrosine antibodies (Sigma-Aldrich) were diluted 500-fold. F-actin was detected by a 30-min incubation at 37°C with TRITC-conjugated phalloidin (1:1,000) (Sigma-Aldrich). To assay for ERK localization, cells were fixed and prepared as above, except for pretreatment for 1 h with PBS containing 5% FCS and 1% BSA and then incubation with anti-pan ERK for 1 h at 37°C .

After appropriate staining and washes in PBS, cells were observed using an Optiphot II microscope (Nikon) attached to an MRC-600 laser scanning confocal apparatus (Bio-Rad Laboratories). Images were treated with Adobe Photoshop[®].

We thank G.R. Stark for providing REF-52 and TAG cell lines, J. Sage and T. Jacks for TKO and RB^{-/-} MEFs, C. Sherr and M. Roussel for ARF^{-/-} MEFs, M. Oren (Weizmann Institute of Science, Rehovot, Israel) for p53DD retrovirus-producing cells, and B. Vogelstein for HCT 116 cells.

This work was supported by grants to R.L. Margolis from La Ligue Nationale Contre le Cancer (Equipe Labellisée) and l'Association pour la Recherche sur le Cancer (ARC). O.D. Lohez was supported by the Commissariat à l'Énergie Atomique, ARC, and Centre National de la Recherche Scientifique.

Submitted: 22 August 2002
Revised: 28 February 2003
Accepted: 28 February 2003

References

- Andreassen, P.R., and R.L. Margolis. 1994. Microtubule dependency of p34cdc2 inactivation and mitotic exit in mammalian cells. *J. Cell Biol.* 127:789–802.
- Andreassen, P.R., F.B. Lacroix, O.D. Lohez, and R.L. Margolis. 2001a. Neither p21WAF1 nor 14-3-3 σ prevents G2 progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21WAF1 induces stable G1 arrest in resulting tetraploid cells. *Cancer Res.* 61:7660–7668.
- Andreassen, P.R., O.D. Lohez, F.B. Lacroix, and R.L. Margolis. 2001b. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Mol. Biol. Cell.* 12:1315–1328.
- Aplin, A.E., S.M. Short, and R.L. Juliano. 1999. Anchorage-dependent regulation of the mitogen-activated protein kinase cascade by growth factors is supported by a variety of integrin α chains. *J. Biol. Chem.* 274:31223–31228.
- Assoian, R.K. 1997. Anchorage-dependent cell cycle progression. *J. Cell Biol.* 136:1–4.
- Assoian, R.K., and M.A. Schwartz. 2001. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. *Curr. Opin. Genet. Dev.* 11:48–53.
- Assoian, R.K., and X. Zhu. 1997. Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. *Curr. Opin. Cell Biol.* 9:93–98.
- Aubin, J.E., M. Osborn, and K. Weber. 1981. Inhibition of cytokinesis and altered contractile ring morphology induced by cytochalasins in synchronized PtK2 cells. *Exp. Cell Res.* 136:63–79.
- Bargonetti, J., I. Reynisdottir, P.N. Friedman, and C. Prives. 1992. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. *Genes Dev.* 6:1886–1898.
- Bashour, A.M., J.J. Meng, W. Ip, M. MacCollin, and N. Ratner. 2002. The neurofibromatosis type 2 gene product, merlin, reverses the F-actin cytoskeletal defects in primary human Schwannoma cells. *Mol. Cell. Biol.* 22:1150–1157.
- Blajeski, A.L., T.J. Kottke, and S.H. Kaufmann. 2001. A multistep model for paclitaxel-induced apoptosis in human breast cancer cell lines. *Exp. Cell Res.* 270:277–288.
- Bohmer, R.M., E. Scharf, and R.K. Assoian. 1996. Cytoskeletal integrity is required throughout the mitogen stimulation phase of the cell cycle and mediates the anchorage-dependent expression of cyclin D1. *Mol. Biol. Cell.* 7:101–111.
- Borel, F., F.B. Lacroix, and R.L. Margolis. 2002a. Prolonged arrest of mammalian cells at the G1/S boundary results in permanent S phase stasis. *J. Cell Sci.* 115:2829–2838.
- Borel, F., O.D. Lohez, F.B. Lacroix, and R.L. Margolis. 2002b. Multiple centrosomes arise from tetraploidy checkpoint failure and mitotic centrosome clusters in p53 and RB pocket protein-compromised cells. *Proc. Natl. Acad. Sci. USA.* 99:9819–9824.
- Brenot-Bosc, F., S. Gupta, R.L. Margolis, and R. Fotadar. 1995. Changes in the subcellular localization of replication initiation proteins and cell cycle proteins during G1- to S-phase transition in mammalian cells. *Chromosoma.* 103:517–527.
- Cross, S.M., C.A. Sanchez, C.A. Morgan, M.K. Schimke, S. Ramel, R.L. Idzerda, W.H. Raskind, and B.J. Reid. 1995. A p53-dependent mouse spindle checkpoint. *Science.* 267:1353–1356.
- Dannenberg, J.H., A. van Rossum, L. Schuijff, and H. te Riele. 2000. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev.* 14:3051–3064.
- Delmas, C., S. Manenti, A. Boudjelal, C. Peyssonnaud, A. Eychene, and J.M. Darbon. 2001. The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells. *J. Biol. Chem.* 276:34958–34965.
- Di Leonardo, A., S.P. Linke, K. Clarkin, and G.M. Wahl. 1994. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* 8:2540–2551.
- Di Leonardo, A., S.H. Khan, S.P. Linke, V. Greco, G. Seidita, and G.M. Wahl. 1997. DNA rereplication in the presence of mitotic spindle inhibitors in human and mouse fibroblasts lacking either p53 or pRb function. *Cancer Res.* 57:1013–1019.
- Dulic, V., W.K. Kaufmann, S.J. Wilson, T.D. Tlsty, E. Lees, J.W. Harper, S.J. Elledge, and S.I. Reed. 1994. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell.* 76:1013–1023.
- Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes Dev.* 12:2245–2262.
- el-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 75:817–825.
- Gautreau, A., D. Louvard, and M. Arpin. 2002. ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling. *Curr. Opin. Cell Biol.* 14:104–109.
- Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57–70.
- Howe, A., A.E. Aplin, S.K. Alahari, and R.L. Juliano. 1998. Integrin signaling and cell growth control. *Curr. Opin. Cell Biol.* 10:220–231.
- Huang, S., C.S. Chen, and D.E. Ingber. 1998. Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol. Cell. Biol.* 9:3179–3193.
- Iliakis, G. 1991. The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *Bioessays.* 13:641–648.
- Iwig, M., E. Czeslick, A. Muller, M. Gruner, M. Spindler, and D. Glaesler. 1995. Growth regulation by cell shape alteration and organization of the cytoskeleton. *Eur. J. Cell Biol.* 67:145–157.
- Jordan, M.A., and L. Wilson. 1998. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr. Opin. Cell Biol.* 10:123–130.
- Jordan, M.A., D. Thrower, and L. Wilson. 1992. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *J. Cell Sci.* 102:401–416.
- Kastan, M.B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R.W. Craig. 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* 51:6304–6311.
- Lanni, J.S., and T. Jacks. 1998. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol. Cell. Biol.* 18:1055–1064.
- Lavoie, J.N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur. 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem.* 271:20608–20616.
- Le Gall, M., D. Grall, J.C. Chambard, J. Pouyssegur, and E. Van Obberghen-Schilling. 1998. An anchorage-dependent signal distinct from p42/44 MAP kinase activation is required for cell cycle progression. *Oncogene.* 17:1271–1277.
- Levine, A.J. 1997. p53, the cellular gatekeeper for growth and division. *Cell.* 88:323–331.
- Lin, T.H., Q. Chen, A. Howe, and R.L. Juliano. 1997. Cell anchorage permits efficient signal transduction between ras and its downstream kinases. *J. Biol. Chem.* 272:8849–8852.

- Linke, S.P., K.C. Clarkin, A. Di Leonardo, A. Tsou, and G.M. Wahl. 1996. A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev.* 10:934–947.
- Maness, P.F., and R.C. Walsh, Jr. 1982. Dihydrocytochalasin B disorganizes actin cytoarchitecture and inhibits initiation of DNA synthesis in 3T3 cells. *Cell.* 30:253–262.
- Martineau, S.N., P.R. Andreassen, and R.L. Margolis. 1995. Delay of HeLa cell cleavage into interphase using dihydrocytochalasin B: retention of a postmitotic spindle and telophase disc correlates with synchronous cleavage recovery. *J. Cell Biol.* 131:191–205.
- Minn, A.J., L.H. Boise, and C.B. Thompson. 1996. Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. *Genes Dev.* 10:2621–2631.
- Mittnacht, S. 1998. Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.* 8:21–27.
- Morrison, H., L.S. Sherman, J. Legg, F. Banine, C. Isacke, C.A. Haipek, D.H. Gutmann, H. Ponta, and P. Herrlich. 2001. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev.* 15:968–980.
- Mulligan, G., and T. Jacks. 1998. The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet.* 14:223–229.
- Perry, M.E., M. Commare, and G.R. Stark. 1992. Simian virus 40 large tumor antigen alone or two cooperating oncogenes convert REF52 cells to a state permissive for gene amplification. *Proc. Natl. Acad. Sci. USA.* 89:8112–8116.
- Polyak, K., J.Y. Kato, M.J. Solomon, C.J. Sherr, J. Massague, J.M. Roberts, and A. Koff. 1994a. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor- β and contact inhibition to cell cycle arrest. *Genes Dev.* 8:9–22.
- Polyak, K., M.H. Lee, H. Erdjument-Bromage, A. Koff, J.M. Roberts, P. Tempst, and J. Massague. 1994b. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimutagenic signals. *Cell.* 78:59–66.
- Renshaw, M.W., X.D. Ren, and M.A. Schwartz. 1997. Growth factor activation of MAP kinase requires cell adhesion. *EMBO J.* 16:5592–5599.
- Reshetnikova, G., R. Barkan, B. Popov, N. Nikolsky, and L.S. Chang. 2000. Disruption of the actin cytoskeleton leads to inhibition of mitogen-induced cyclin E expression, Cdk2 phosphorylation, and nuclear accumulation of the retinoblastoma protein-related p107 protein. *Exp. Cell Res.* 259:35–53.
- Rivard, N., M.J. Boucher, C. Asselin, and G. L'Allemain. 1999. MAP kinase cascade is required for p27 downregulation and S phase entry in fibroblasts and epithelial cells. *Am. J. Physiol.* 277:C652–C664.
- Sage, J., G.J. Mulligan, L.D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou, and T. Jacks. 2000. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev.* 14:3037–3050.
- Shaulian, E., A. Zauberman, D. Ginsberg, and M. Oren. 1992. Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. *Mol. Cell. Biol.* 12:5581–5592.
- Shaw, R.J., A.I. McClatchey, and T. Jacks. 1998. Regulation of the neurofibromatosis type 2 tumor suppressor protein, merlin, by adhesion and growth arrest stimuli. *J. Biol. Chem.* 273:7757–7764.
- Sherr, C.J. 1996. Cancer cell cycles. *Science.* 274:1672–1677.
- Sherr, C.J., and J.M. Roberts. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* 9:1149–1163.
- Sherr, C.J., and J.M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 13:1501–1512.
- Sherr, C.J., and J.D. Weber. 2000. The ARF/p53 pathway. *Curr. Opin. Genet. Dev.* 10:94–99.
- Spector, I., N.R. Shochet, D. Blasberger, and Y. Kashman. 1989. Latrunculin–novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D. *Cell Motil. Cytoskeleton.* 13:127–144.
- Stoker, M., C. O'Neill, S. Berryman, and V. Waxman. 1968. Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer.* 3:683–693.
- Trielli, M.O., P.R. Andreassen, F.B. Lacroix, and R.L. Margolis. 1996. Differential Taxol-dependent arrest of transformed and nontransformed cells in the G1 phase of the cell cycle, and specific-related mortality of transformed cells. *J. Cell Biol.* 135:689–700.
- Vogelstein, B., D. Lane, and A.J. Levine. 2000. Surfing the p53 network. *Nature.* 408:307–310.
- Wahl, A.F., K.L. Donaldson, C. Fairchild, F.Y. Lee, S.A. Foster, G.W. Demers, and D.A. Galloway. 1996. Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. *Nat. Med.* 2:72–79.
- Weber, J.D., D.M. Raben, P.J. Phillips, and J.J. Baldassare. 1997. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem. J.* 326:61–68.
- Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell.* 81:323–330.
- Zalvide, J., H. Stubdal, and J.A. DeCaprio. 1998. The J domain of simian virus 40 large T antigen is required to functionally inactivate RB family proteins. *Mol. Cell. Biol.* 18:1408–1415.
- Zhang, H.S., A.A. Postigo, and D.C. Dean. 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGF β , and contact inhibition. *Cell.* 97:53–61.
- Zhu, J., P.W. Rice, L. Gorsch, M. Abate, and C.N. Cole. 1992. Transformation of a continuous rat embryo fibroblast cell line requires three separate domains of simian virus 40 large T antigen. *J. Virol.* 66:2780–2791.