Cell Death and Differentiation (2006) 13, 324–334

www.nature.com/cdd

Cooperation between p53 and p130(Rb2) in induction of cellular senescence

A Kapić[1,2], H Helmbold[1,2], R Reimer[1], O Klotzsche[1], W Deppert[1] and W Bohn*[,1]

[1] Heinrich-Pette-Institute for Experimental Virology and Immunology at the University of Hamburg, Martinistr. 52, 20251 Hamburg, Germany
[2] These authors contributed to this work equally
* Corresponding author: W Bohn, Heinrich-Pette-Institute for Experimental Virology and Immunology at the University of Hamburg, Martinistrasse 52, D-20251 Hamburg, Germany. Tel: +49 40 48051 231; Fax: +49 40 48051 117; E-mail: wolfgang.bohn@hpi.uni-hamburg.de

Received 23.12.04; revised 21.6.05; accepted 22.6.05; published online 26.8.05
Edited by M Oren

Abstract

To determine pathways cooperating with p53 in cellular senescence when the retinoblastoma protein (pRb)/p16INK4a pathway is defunct, we stably transfected the p16INK4a-negative C6 rat glioma cell line with a temperature-sensitive mutant p53. Activation of $p53^{Val-135}$ induces a switch in pocket protein expression from pRb and p107 to p130(Rb2) and stalls the cells in late G1, early S-phase at high levels of cyclin E. Maintenance of the arrest depends on the functions of p130(Rb2) repressing cyclin A. Inactivation of p53 in senescent cultures restores the pocket proteins to initial levels and initiates progression into S-phase, but the cells fail to resume proliferation, likely due to DNA damage becoming apparent in the arrest and activating apoptosis subsequent to the release from p53-dependent growth suppression. The data indicate that p53 can cooperate selectively with p130(Rb2) to induce cellular senescence, a pathway that may be relevant when the pRb/p16INK4a pathway is defunct.
Cell Death and Differentiation (2006) **13,** 324–334.
doi:10.1038/sj.cdd.4401756; published online 26 August 2005

Keywords: p53; p130(Rb2); cellular senescence; cell death

Abbreviations: arr., arrested cells; BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; FACS, flow cytometry; FCS, fetal calf serum; IP, immunoprecipitation; mAb, monoclonal antibody; pRb, retinoblastoma protein; prol., proliferating cells; SA-β-gal, senescence-associated β-galactosidase; TUNEL, TdT-mediated dUTP-X nick end labeling; WB, Western blot, immunoblotting

Introduction

Loss of growth control due to inactivation of p53 is a critical step towards unlimited proliferation and cell transformation. Ectopic expression of wild-type (wt) p53 has proven as an efficient approach to restore growth control in p53-null tumor cells to a normal level. For instance, overexpression of wtp53 in p53-null human EJ bladder cells triggered a rapid onset of G1 and G2/M growth arrest associated with upregulation of the cyclin-dependent kinase (CDK) inhibitor p21CIP1, repression of cyclin A and B, and development of a senescence phenotype.[1] Expression of wtp53 in human H1299 lung cancer cells resulted in a sustained growth arrest associated with expression of senescence-associated β-galactosidase (SA-β-gal).[2] When p53 expression was suppressed again, the cells were unable to resume proliferation, indicating that the arrest had become irreversible. The data suggest that signaling pathways used by p53 to induce cellular senescence may still be intact in p53-deficient tumor cells and can be reactivated by an exogenous p53. The way p53 activates cellular senescence in these cells and the specific settings in signaling pathways required for p53 to induce this response are not yet clearly defined.

Aside from p53, retinoblastoma protein (pRb) and p16INK4a are pivotal regulators of cellular senescence and some data suggest that p53 and pRb crosstalk in this response. When p53 and pRb were restored concomitantly to normal levels in human cervix carcinoma cells, cellular senescence was induced in nearly all of the cells. When only p53 was expressed, the proportion of senescent cells was reduced significantly.[3] The activity of pRb is primarily determined by p16INK4a, an inhibitor of cyclin D-dependent kinases.[4] Loss of p16INK4a, as is frequently found in tumor cells, is sufficient to inactivate the Rb pathway. Accordingly, ectopic p16INK4a is a potent inducer of cellular senescence, provided pRb is present.[5–8] However, ectopic p53 activated cellular senescence in H1299 lung tumor cells despite the absence of p16INK4a.[2] It suggests that the inhibitor is dispensible for p53-mediated cellular senescence. This is consistent with the note that p16INK4a$-/-$; pRb$+/+$ mouse embryo fibroblasts can enter cellular senescence upon transfection with an activated oncogene quite as efficient as the wt cells.[9,10] In contrast, loss of p53 blocked the response despite the presence of p16INK4a.[11,12] It not necessarily implies that functions of pRb are also negligible in the p53-mediated response. pRb may be activated via pathways different from those controlled by p16INK4a. Furthermore, the growth arrest may not only rely on pRb, but may also require complementary functions of or crosstalk with the other pocket proteins, p107 and p130(Rb2).

To identify pathways cooperating with p53 in the absence of p16INK4a, we stably transfected C6 rat glioma cells with the conditional mutant (mt) $p53^{Val-135}$.[13] C6 cells do not express p53 and lack endogenous p16INK4a due to a homozygous deletion in the *ink4a* locus.[14] Ectopic expression of p16INK4a was shown to induce cellular senescence in this cell line, indicating that the pRb pathway can be reactivated.[7] When $p53^{Val-135}$ was activated by a shift to the permissive temperature, the C6 cells entered a sustained growth arrest and developed a senescence phenotype. The arrest was characterized by a shift in the pocket protein expression from pRb and p107 to p130(Rb2). Our data indicate that p53

cooperates specifically with p130(Rb2) in maintenance of the cell cycle arrest.

Results

C6D10-p53$^{Val-135}$ cells enter a sustained growth arrest at the restrictive temperature

C6D10 cells used for transfection with the temperature-sensitive p53$^{Val-135}$ do not express endogenous rat p53 as determined by Northern and Western blotting (WB), and by immunofluorescence.[15] In accordance with observations in other cell systems,[16] p53$^{Val-135}$ in C6D10-p53$^{Val-135}$ cells exhibited an mt conformation at the restrictive temperature (Figure 1a; pAb240), and adopted the wt conformation upon shift to the permissive temperature (Figure 1a; pAb246). After shift to the permissive temperature, the steady-state level of p53$^{Val-135}$ was reduced to a low, nearly physiological level, as shown by immunofluorescence (Figure 1b) and WB (Figure 1c). In contrast to other cell systems, p53$^{Val-135}$ was constitutively present in the nucleus both at the restrictive (39°C) as well as at the permissive temperatures (30°C)

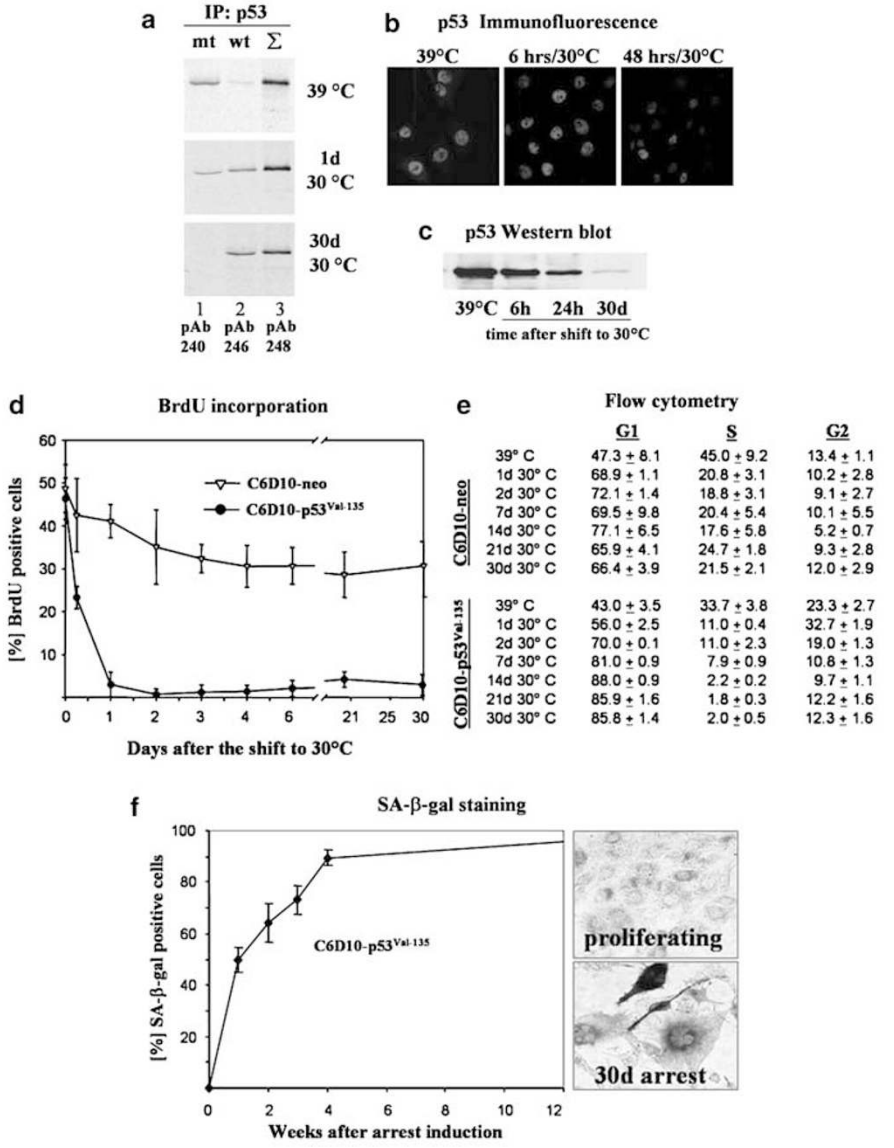

Figure 1 Induction of a senescence phenotype by p53$^{Val-135}$. (**a**) SDS-PAGE and autoradiography of ^{35}S-methionine-labeled p53$^{Val-135}$, immunoprecipitated (IP: p53) from C6D10-p53$^{Val-135}$ cell lysates with antibodies detecting the mt (pAb240), the wt (pAb246) or both conformations (pAb248) of p53; p53$^{Val-135}$ changes from the mt (**a**, lane 1) to the wt conformation (**a**, lane 2) when shifted to 30°C; the wt conformation of p53$^{Val-135}$ is degraded rapidly and reaches a low level, as shown by immunofluorescence (**b**) and WB (**c**). (**d**) C6D10-p53$^{Val-135}$ and control cultures (C6D10-neo) assayed for BrdU incorporation by immunofluorescence microscopy; the percentage of BrdU-incorporating C6D10-p53$^{Val-135}$ cells decreases to less than 5% after the shift to the permissive temperature; shown are the mean values and SDs of a representative experiment; at each time point, 10 microscopic areas containing at least 50 cells per area were selected randomly and assayed for BrdU incorporation. (**e**) FACS analysis; C6D10-p53$^{Val-135}$ cultures held at permissive temperature (30°C) show a predominant accumulation of cells in G1; shown are the mean values and S.D.'s of three independent experiments, analyzing 10^4 cells in each experiment at each time point. (**f**) C6D10-p53$^{Val-135}$ cultures become positive for SA-β-gal, while staying in the arrest; shown are the mean values and S.D. of two experiments; 2000–3000 cells were totally assayed at each time point

(Figure 1b). Thus, growth-regulatory functions of p53$^{Val-135}$ were not impaired by nuclear exclusion of the protein, as was reported for other systems.[17]

At restrictive temperature, the percentage of C6D10-p53$^{Val-135}$ cells incorporating bromodeoxyuridine (BrdU) was similar to that of the control cell line (C6D10-neo) (Figure 1d). At 24 h after the shift to the permissive temperature, less than 5% of the p53$^{Val-135}$-expressing cells, but still about 30% of the control cells, incorporated BrdU (Figure 1d). As shown by flow cytometry (FACS) (Figure 1e), more than 80% of the p53$^{Val-135}$-expressing cells showed a G1 DNA content and about 2% were in S-phase 4 weeks after arrest induction. The data indicate that p53$^{Val-135}$ induced a sustained growth arrest in C6D10 cells at the permissive temperature, with cells predominantly accumulating in G1.

Arrested cells adopt a senescence-like phenotype

Arrested cells adopted a flat morphology, partially became multinucleated, and expressed the SA-β-gal (Figure 1f), features that are characteristic of senescent cells.[18] Individual β-gal-positive cells first appeared at day 2 after the temperature shift, and the number of positive cells increased gradually, reaching 64, 80 and 89% after 2, 3 and 4 weeks, respectively (Figure 1f). p53$^{Val-135}$-deficient C6 cells cultivated at the permissive temperature remained SA-β-gal negative.

We also probed whether arrested cells showed any markers of differentiation, typically found in mature glial cells,[19] but could neither detect glial acidic fibrillary protein (GFAP), a characteristic marker of astrocytic differentiation, nor galactocerebroside, a marker of differentiating oligodendroglial cells. The data indicate that C6D10-p53$^{Val-135}$ adopt a senescence-like phenotype while staying in the arrest.

Cells are stalled in between cyclin E- to A-mediated steps in the cell cycle

To determine the cell cycle status of arrested C6D10-p53$^{Val-135}$ cells, we first monitored the expression of cyclins by WB and immunofluorescence (Figure 2a and b). Lysates from C6D10 cells, lacking p53$^{Val-135}$, were enclosed as control.

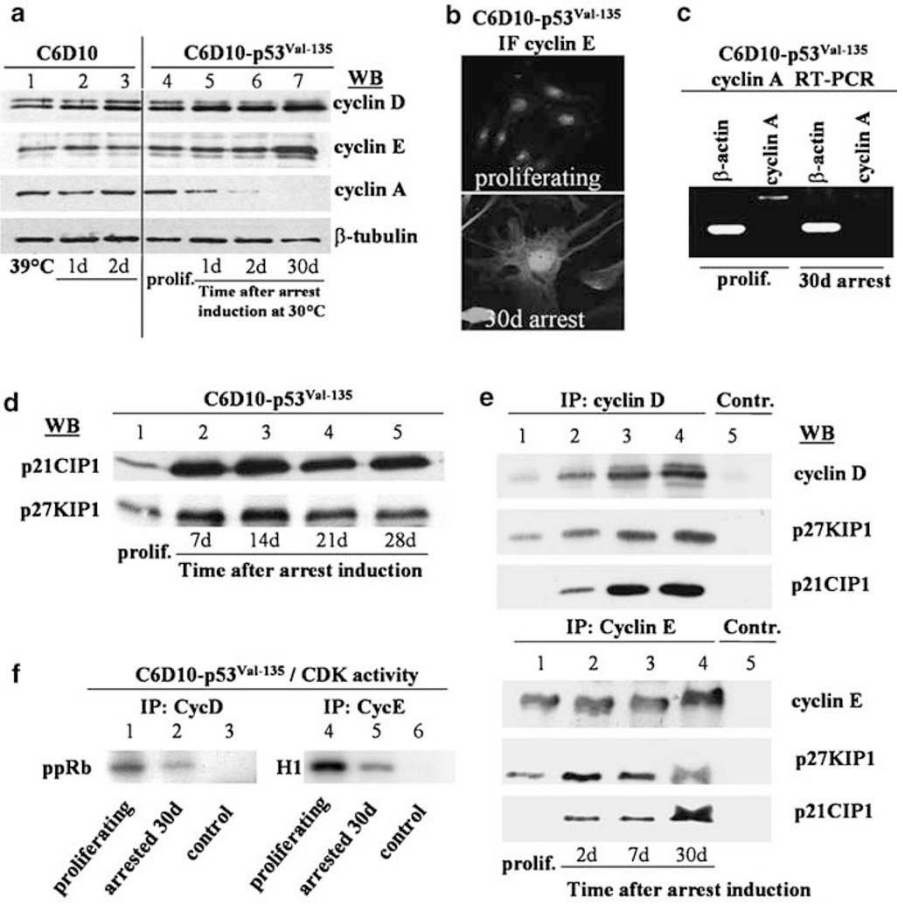

Figure 2 Changes in the cyclin pattern and suppression of CDK activity in arrested cells. (**a**) The level of cyclin A declines in C6D10-p53$^{Val-135}$ cells after the shift to the permissive temperature (30°C), whereas that of cyclin E increases (**a**, lanes 4–7, WB of total cell lysates); loading control: β-tubulin. (**b**) Immunofluorescence of cyclin E. (**c**) Cyclin A mRNA levels, assayed by semiquantitative RT-PCR, are reduced in arrested C6D10-p53$^{Val-135}$ cells; control: β-actin RT-PCR. (**d**) The steady-state levels of the CDK inhibitors p21CIP1 and p27KIP1 in C6D10-p53$^{Val-135}$ cells rise in arrested cells (**d**, lanes 2–5, WB of total cell lysates). (**e**) p21CIP1 and p27KIP1 co-immunoprecipitate with cyclin D (IP: cyclin D) and cyclin E (IP: cyclin E); lysates were collected from prolif. (**e**, lane 1) or arr. (**e**, lanes 2–4) C6D10-p53$^{Val-135}$ cells. (**e**) Lane 5: control-IP with a nonrelevant antibody. (**f**) *In vitro* assay for cyclin D- and E-associated kinase activity; autoradiography of ^{32}P-labeled Rb and histone H1; the kinase activity associated with immunoprecipitated cyclin D (IP: CycD) and cyclin E (IP: CycE) is reduced in arrested C6D10-p53$^{Val-135}$ cells

The steady-state level of cyclin D did not change significantly subsequent to arrest induction. In contrast, the level of cyclin E increased continuously throughout the arrest (Figure 2a and b), whereas cyclin A declined to a nearly undetectable level (Figure 2a). Semiquantitative RT-PCR showed that cyclin A mRNA was reduced, indicating that cyclin A was repressed at the transcriptional level (Figure 2c). Thus, p53$^{Val\text{-}135}$ stalled the cells precisely in between cyclin E- to cyclin A-mediated steps in the cell cycle. As an accumulation of cyclin E requires passage of the cell through the restriction point,[20,21] we conclude that C6D10-p53$^{Val\text{-}135}$ cells were arrested beyond this cell cycle checkpoint.

CDK activity is decreased in arrested cells

Normally, growth arrest functions by p53 are based on transactivation of the pleitropic CDK inhibitor p21CIP1.[22] Accordingly, the p21CIP1 level, which was negligible at the restrictive temperature, increased significantly when p53$^{Val\text{-}135}$ adopted its wt conformation (Figure 2d). Significant amounts of the protein were already detectable at 6 h after the temperature shift (data not shown). Not only p21CIP1 but also p27KIP1 increased (Figure 2d), a CDK inhibitor that is not transcriptionally regulated by p53.

Both of these inhibitors were functional in terms of binding to cyclin–CDK complexes, as they coimmunoprecipitated with cyclin D and E from lysates of arrested C6D10-p53$^{Val\text{-}135}$ cells (Figure 2e). Immunoprecipitates obtained with antibodies to cyclin D and E were assayed for the activities of CDK4/6 and CDK2, respectively, using pRb and histone H1 as substrates. As shown in Figure 2f, the activity of both of these kinases was reduced in arrested cells.

pRb is replaced by p130(Rb2) in arrested cells

pRb is considered to play a key role in cellular senescence, acting as a repressor of S-phase genes in its active, hypophosphorylated form and thereby stalling the cells in G1/S transition.[23–25] Therefore, we asked whether pRb showed any mobility changes in SDS-PAGE when C6D10-p53$^{Val\text{-}135}$ cells entered the arrest. For comparison, we monitored the pRb status in normal rat kidney (NRK) cells, when they were arrested by serum withdrawal (Figure 3a).

As shown with lysates of C6D10-p53$^{Val\text{-}135}$ cells, pRb shifted to the hypophosphorylated form shortly after arrest induction (Figure 3a, lanes 3 and 4). Surprisingly, the level of pRb then declined almost totally, while the cells stayed in the arrest (Figure 3a, lane 6; Figure 3b, lanes 2–6).

To determine if pRb was substituted by other members of the pocket protein family, we assayed the cell lysates for the presence of p107 and p130(Rb2). Similar to pRb, the pocket protein p107 was reduced to a nearly undetectable level in arrested cells (compare lanes 1 and 2 in Figure 5b). In contrast, p130(Rb2), which was hardly detectable in cycling cells (Figure 3b, lane 1), reached a prominent level 7 days after arrest induction (Figure 3b, lane 4), and stayed at this level throughout further maintenance of the arrest (Figure 3b, lanes 4–6). p130(Rb2) accumulating in arrested cells was present in a hypophosphorylated form, as is indicated by the change in mobility seen when the cells were released from the

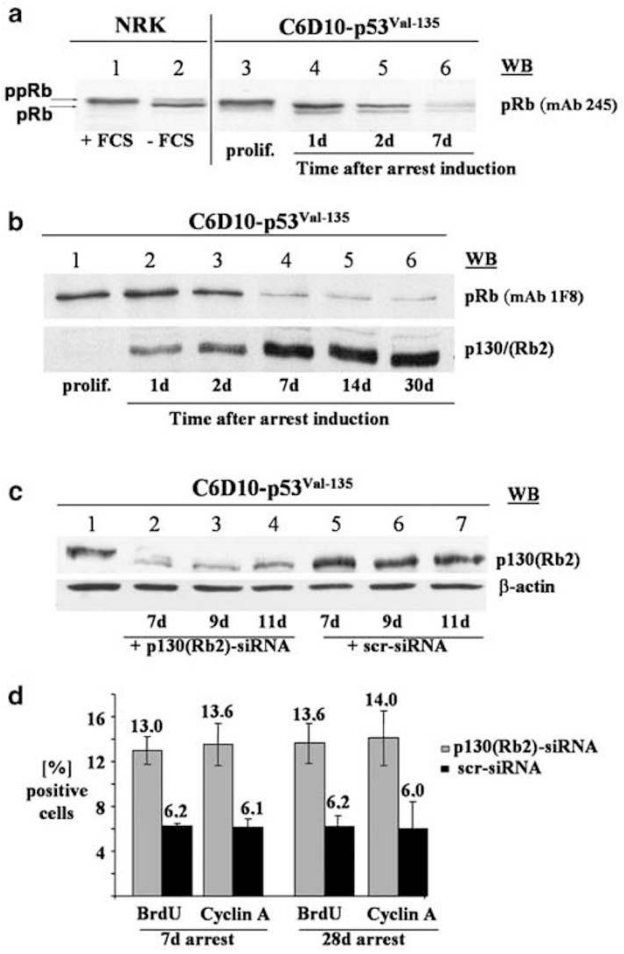

Figure 3 Replacement of pRb and p107 by p130(Rb2) in arrested cells. Detection of pRb and p130(Rb2) by WB of C6D10-p53$^{Val\text{-}135}$ cell lysates. (**a**) pRb shifts from the hyperphosphorylated form (ppRb) in proliferating cells (**a**, lane 3) to a hypophosphorylated form (pRb) in arrested cells (**a**, lane 4). NRK cells depleted of FCS show an identical shift in pRb mobility (**a**, lanes 1 and 2). pRb declines in arrested C6D10-p53$^{Val\text{-}135}$ cells (**a**, lane 6; **b**, lanes 4–6), whereas the level of p130(Rb2) increases (**b**, lanes 4–6). (**c**) Transfection of C6D10-p53$^{Val\text{-}135}$ cells with p130(Rb2)-siRNA reduces the p130(Rb2) level in cells cultivated at the permissive temperature for 7, 9, or 11 days (WB of total cell lysates); control: transfection with scrambled siRNA (scr-siRNA). (**c**) Lane 1: p130(Rb2) level detectable prior to siRNA treatment; β-actin as loading control. (**d**) The proportion of S-phase cells assayed by BrdU incorporation or detection of cyclin A increases in C6D10-p53$^{Val\text{-}135}$ cultures arrested for 7 or 28 days upon treatment with p130(Rb2)-siRNA, but not with scrambled siRNA (scr-siRNA); shown are the mean values and SDs of three independent experiments; 3000 cells were assayed in each experiment at each time point; in each of the experiments, the difference between p130(Rb2)-siRNA- and scr-siRNA-treated cells is statistically significant ($P < 0.005$)

arrest (compare lanes 1–3 in Figure 5a). The data indicate that p130(Rb2) replaced the pocket proteins pRb and p107, while the C6D10-p53$^{Val\text{-}135}$ cells stayed in the arrest.

p130(Rb2) is required to maintain the arrest

To determine if p130(Rb2) was functional in arrest maintenance, we asked whether we might be able to initiate S-phase activity in senescent C6D10-p53$^{Val\text{-}135}$ cells by

ectopic expression of the pocket protein inhibitor E1A-12S, an adenovirus E1A splice product.[26,27] At 48 h after transfection of arrested C6D10-p53$^{Val\text{-}135}$ cells, 16.2$\pm$3.2% of the E1A-positive cells, but only 3.3$\pm$1.7% of the E1A-negative cells, showed incorporation of BrdU as assayed by immunofluorescence. The mean value and S.D. are based on three

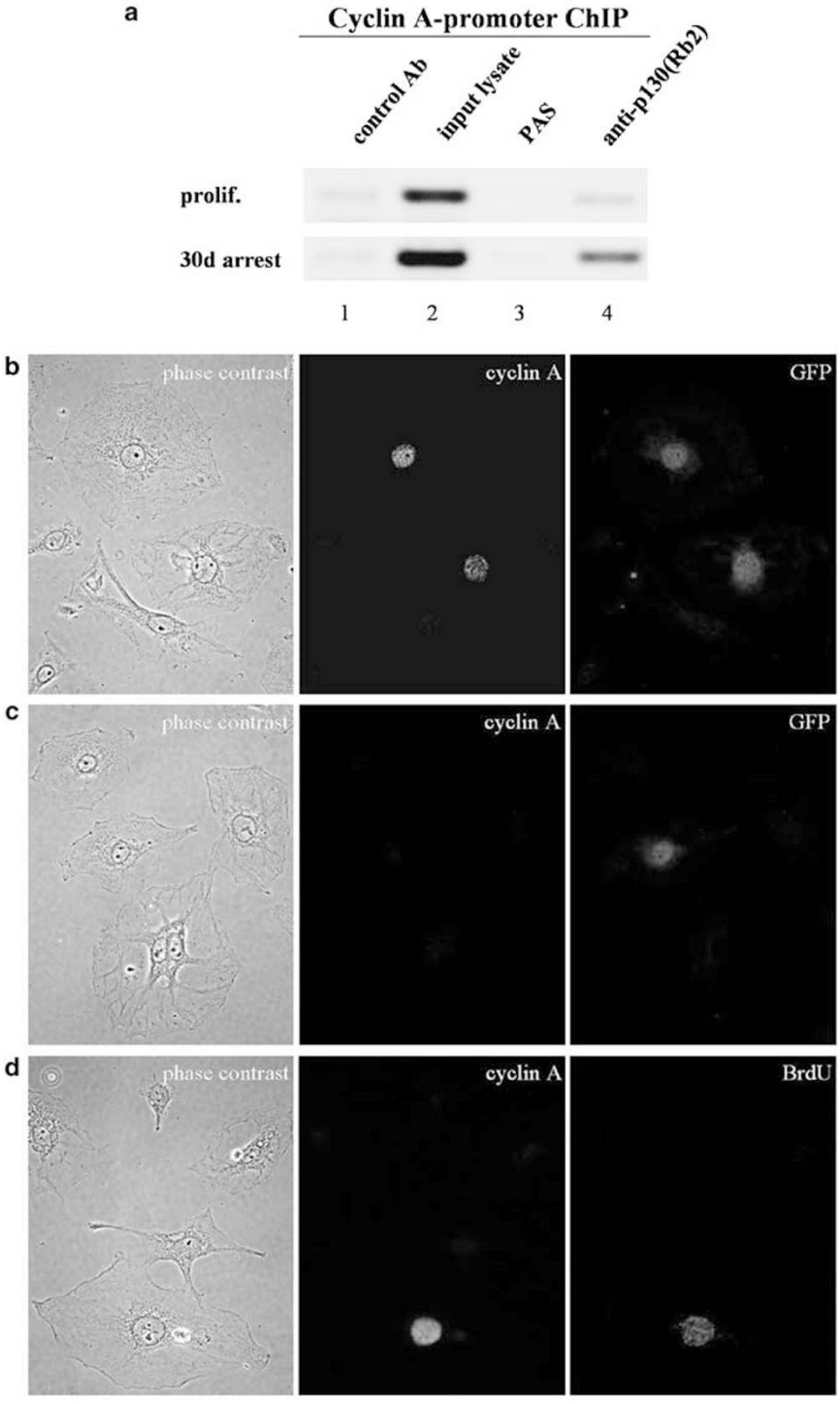

Figure 4 Association of p130(Rb2) with the cyclin A promoter and initiation of S-phase activity in arrested cells by ectopic expression of cyclin A. (**a**) Rat cyclin A promoter-specific PCR products obtained with chromatin fragments from prolif. or arr. (30 days arrest) C6D10-p53$^{Val\text{-}135}$ cells; chromatin fragments obtained with control antibodies (IP: control Ab; **a**, lane 1), in the absence of antibodies (purified PAS only; IP: PAS; **a**, lane 3), or with the p130(Rb2) antibody (IP: p130(Rb2); **a**, lane 4: control: input lysates used for IP. (**b**) Arrested cells transfected with a pIRES2-AcGFP1-cycA expression vector; double labeling with antibodies to cyclin A and GFP 24 h after transfection. Cyclin A signal is only seen in transfected cells, as demonstrated by colocalization of GFP. (**c**) Arrested cells transfected with the pIRES2-AcGFP1 control vector; double labeling as in (**b**). (**d**) Arrested cells transfected with pIRES2-AcGFP1-cycA expression vector and incubated with BrdU 24 h after transfection; cyclin A-expressing cells are BrdU positive

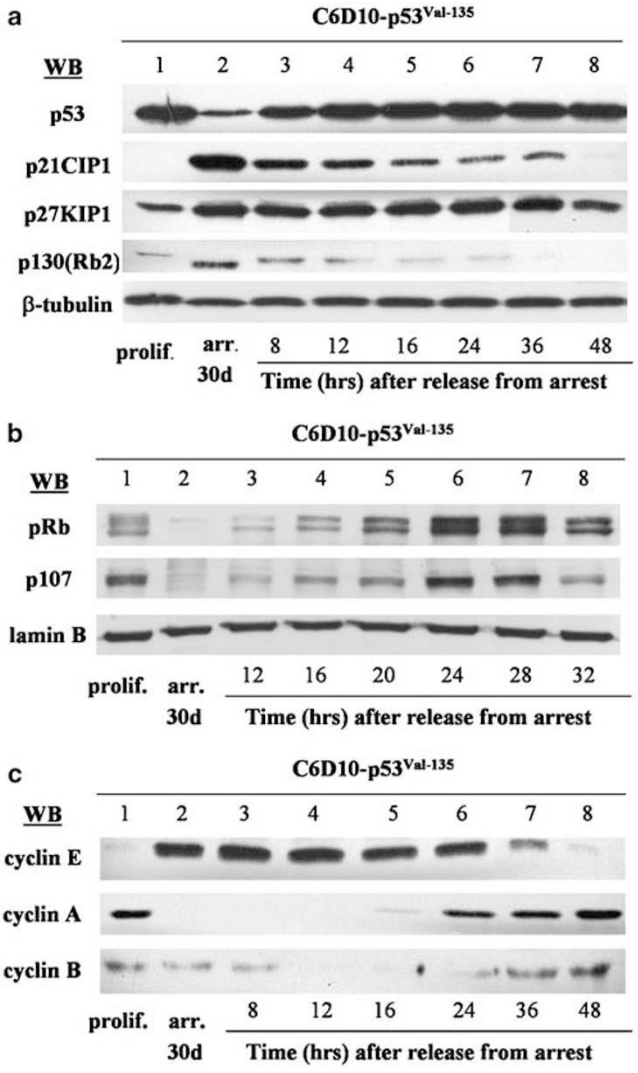

Figure 5 Inactivation of p53$^{Val-135}$ in senescent cultures and reversal of the cyclin, CDKI, and Rb expression pattern. Cells arrested for 30 days (arr. 30d) were released from p53$^{Val-135}$-dependent growth suppression by a shift to the restrictive temperature; total lysates of released and prolif. cells were probed by WB; the shift to restrictive temperature (lanes 3–9 in **a** and **b**) stabilizes p53 (**a**), decreases the level of p21CIP1 and p130(Rb2) (**a**), raises the level of pRb and p107 (**b**), induces a switch from cyclin E to cyclin A (**c**), and leads to the appearance of cyclin B (**c**)

independent experiments, counting 1200 cells in an experiment. In each of the experiments, the difference between E1A-positive and -negative cells was statistically significant ($P<0.005$). The data indicate that ectopic expression of E1A-12S promoted S-phase activity in arrested cells, supporting the suggestion that p130(Rb2) was active as a growth suppressor.

In a more stringent approach, we aimed at reducing the p130(Rb2) level by RNA interference. Of the various siRNAs tested, siRNA(Rb2)-3 proved to efficiently reduce the p130(Rb2) level in arrested C6D10-p53$^{Val-135}$ cells over a period of at least 7 days (Figure 3c). We then transfected C6D10-p53$^{Val-135}$ cells arrested for 7 or 28 days with p130(Rb2)-siRNA and assayed the cultures for incorporation of BrdU and expression of cyclin A by immunofluorescence. As shown in Figure 3d, p130(Rb2)-siRNA increased the number of S-phase cells significantly. This let us conclude that p130(Rb2) is functional in arrest maintenance in the system.

p130(Rb2) is known to be the predominant pocket protein bound to E2F target gene promoters in quiescent (G0) and early G1 cells,[28] and acts as an efficient repressor of cell cycle genes,[29] among those cyclin A.[30] To determine if p130(Rb2) was involved in repression of cyclin A in the arrested C6D10-p53$^{Val-135}$ cells, we performed a chromatin immunoprecipitation (ChIP) assay. As shown in Figure 4a, lane 4, the ChIP analysis revealed binding of p130(Rb2) to the cyclin A promoter in lysates of arrested, but not of proliferating, cells.

Ectopic expression of cyclin A in arrested cells

As repression of cyclin A by p130(Rb2) seemed to be critical for arrest maintenance in the senescent cells, we asked whether we could overcome the arrest and initiate S-phase activity by ectopic expression of cyclin A. Arrested cells were transfected with an IRES bicistronic expression vector coding for cyclin A and GFP. At 24 h after transfection, all of the GFP-expressing cells also showed expression of cyclin A (Figure 4b). No cyclin A signal was found in cultures transfected with the control vector lacking the *cyclin A* insert (Figure 4c). Thus, the cyclin A signal is specific for transfected cells. After pulse labeling with BrdU, cyclin A-expressing cells were found to be BrdU positive (Figure 4d), whereas GFP-positive cells in cultures transfected with the control vector were absolutely BrdU negative (data not shown). The data indicate that ectopic expression of cyclin A is sufficient to trigger DNA synthesis in arrested C6D10-p53$^{Val-135}$ cells, suggesting that lack of cyclin A is limiting G1/S transition.

Inactivation of p53$^{Val-135}$ in senescent cultures induces progression into S-phase

The data suggested a functional link between activation of p53, appearance of p130(Rb2), and induction of the senescence-like arrest. We next asked if inactivation of p53 in senescent cultures restored the expression of p130(Rb2) to the normal low level seen in proliferating cells. When senescent cultures that had been maintained in the arrest for 4 weeks were shifted back to the nonpermissive temperature, the level of p53$^{Val-135}$ increased, corresponding to the extended half-life of the mt form (Figure 5a). Concomitantly, expression of p21CIP1 was downregulated. In contrast, the level of p27KIP1 stayed high. p130(Rb2) itself was first shifted to a slower migrating form and then declined (Figure 5a), whereas p107 and pRb reappeared (Figure 5b). The data confirmed the tight link between the appearance of p130(Rb2) and the presence of p53$^{Val-135}$ in the wt conformation.

We also checked if inactivation of p53 in senescent cultures changed the cyclin expression pattern. As shown by WB, cyclin E declined after inactivation of p53 and was exchanged for cyclin A (Figure 5c). In addition, cyclin B became detectable. The data suggest that a shift of p53$^{Val-135}$ to its mt form was sufficient to trigger progression of arrested cells into S-phase.

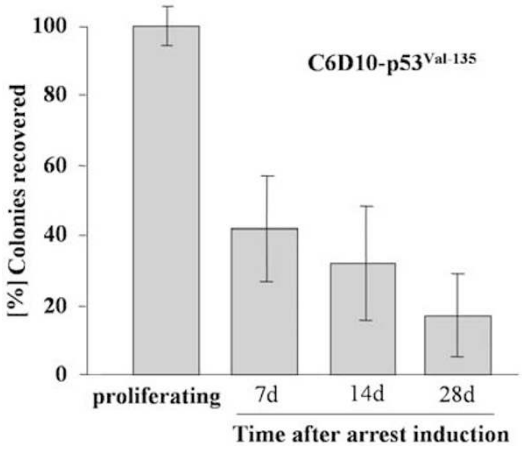

Figure 6 Colony formation assay. C6D10-p53$^{Val-135}$ cells were maintained in the arrest at permissive temperature for 7, 14, and 28 days. At each time point, the cells were detached from the substrate by trypsination and equal cell numbers were seeded on tissue culture plates and cultivated at restrictive temperature; colony numbers were assayed 7 days after plating; colony numbers of cultures proliferating at restrictive temperature (39°C) were set to 100%; error bars represent the standard deviation of the mean of three experiments

To determine whether these cells also resumed proliferation, we performed a colony formation assay. Equal numbers of viable cells, both from cycling and arrested cultures, were seeded on tissue culture plates and colony numbers were determined 7 days later. As shown in Figure 6, the number of colonies recovered from the cultures was reduced down to about 15% when the cultures had been arrested for 28 days. The data indicate that senescent C6D10-p53$^{Val-135}$ cells could overcome the G1/S arrest and proceed into S-phase upon inactivation of p53$^{Val-135}$, but only a minority of these cells could resume proliferation.

Senescent cells accumulate DNA strand breaks while staying in the arrest

FACS showed that C6D10-p53$^{Val-135}$ cells entered the S-phase 16 h after inactivation of p53$^{Val-135}$ (Figure 7a). Subsequently, cells with a sub-G1 DNA content appeared, suggesting that release from the arrest initiated cell death. Cell death could be due to a conflict between proliferation-inhibitory and proliferation-promoting signals, or could result from DNA damage. To assay the cells for DNA damage, we

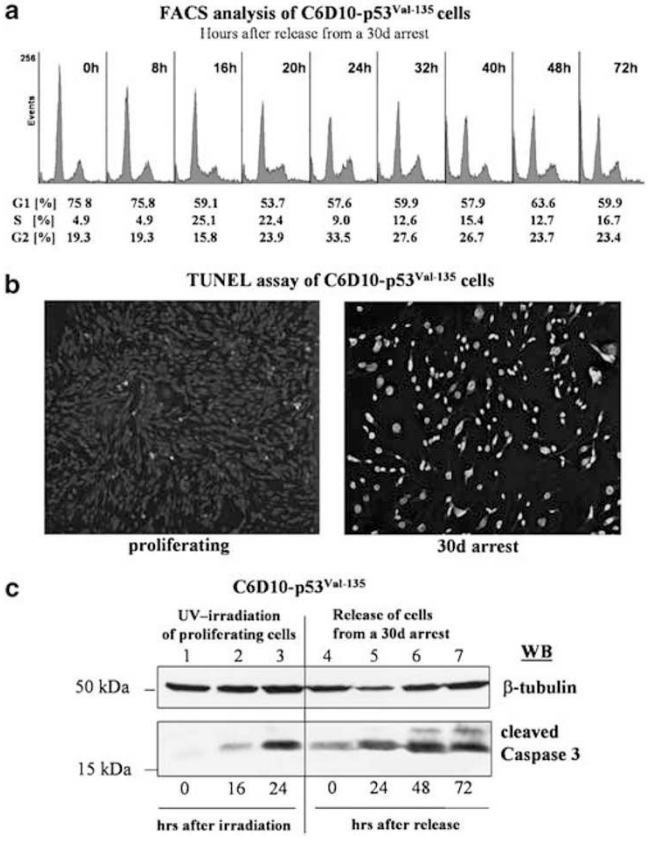

Figure 7 Induction of apoptosis upon release from the arrest. (**a**) Representative FACS profile of C6D10-p53$^{Val-135}$ cells released from p53$^{Val-135}$-dependent growth suppression after a 30-day arrest by a shift back to the restrictive temperature; 10^4 cells were analyzed at each time point; a sub-G1 population appears subsequent to S-phase entry at 16 h. (**b**) C6D10-p53$^{Val-135}$ cultures, assayed for DNA damage by the TUNEL assay, react positively after a 30-days arrest. (**c**) C6D10-p53$^{Val-135}$ cells released from p53-dependent growth suppression show expression of apoptosis-associated cleaved caspase 3 (WB of total cell lysates) (**c**, lanes 4–7); control: UV irradiation of proliferating cells (**c**, lanes 1–3); loading control: β-tubulin

looked for the presence of DNA strand breaks by TdT-mediated dUTP-X nick end labeling (TUNEL) staining. In cultures which had been arrested for 4 weeks, nearly all of the cells reacted positively in this assay, whereas proliferating cells were TUNEL negative (Figure 7b). A time course showed that the number of TUNEL-positive cells increased gradually after the shift to the permissive temperature (data not shown). Thus, the cells contained DNA strand breaks already prior to the release from the arrest and progression into the S-phase.

To further support the notion that cell death occurred by apoptosis, we analyzed cell lysates for the presence of cleaved caspase 3. For comparison, proliferating C6D10-p53$^{Val\text{-}135}$ cells were irradiated with UV at a dose of 100 J. As shown in Figure 7c, untreated, cycling cells were negative for cleaved caspase 3 (Figure 7c, lane 1), whereas arrested cells showed a weak signal (Figure 7c, lane 4). A strong signal was obtained with lysates of proliferating, UV-irradiated cells. The weak signal in arrested cells increased subsequent to inactivation of p53 and transition into the S-phase. The data demonstrate that arrested C6D10-p53$^{Val\text{-}135}$ cells accumulated DNA damage, which was without any obvious consequences as long as the arrest was maintained, but activated a cell death program subsequent to release from the arrest and entry into the S-phase.

Discussion

We show that C6 cells stably transfected with the temperature-sensitive mt p53$^{Val\text{-}135}$ entered a sustained G1/S arrest, when p53$^{Val\text{-}135}$ became activated by a shift to the permissive temperature, and subsequently acquired a senescence-like phenotype. Inactivation of p53$^{Val\text{-}135}$ by a shift back to the restrictive temperature readily abrogated the arrest, even in cultures, where 90% of the cells exhibited a senescence-like phenotype. The data suggest that functions of p53$^{Val\text{-}135}$ were required not only for arrest induction but also for its maintenance.

As expected, the shift of p53$^{Val\text{-}135}$ to the wt conformation induced expression of the pleiotropic CDK inhibitor p21CIP1, the primary p53-dependent target gene product in growth control.[22] p21CIP1 was present at an elevated level as long as the cells stayed in the arrest, suggesting that the inhibitor was functional both in arrest induction and arrest maintenance. However, not only p21CIP1 but also p27KIP1 accumulated in arrested cells, and, like p21CIP1, also coimmunoprecipitated with cyclin D and cyclin E. p27KIP1, in contrast to p21CIP1, was already present when p53$^{Val\text{-}135}$ was inactive, indicating that p27KIP1 levels were not directly controlled by p53.

The second major change that became apparent subsequent to activation of p53$^{Val\text{-}135}$ concerned the expression of pocket proteins. pRb, known to play a critical role in G1/S arrest[23–25] as well as in maintenance of the cell cycle arrest in cellular senescence, shifted to the hypophosphorylated form in accordance with the reduction in CDK activity. The level of pRb and that of p107 then became completely to the benefit of p130(Rb2) while the cells stayed in the arrest, leading to a pocket protein expression pattern, which is typical of a nonproliferative state, such as quiescence, rather than of a transient G1/S arrest. Thus, we assume that pRb is functional as a growth suppressor in C6 cells only in the initial arrest phase subsequent to the activation of p53, but less likely plays a role in arrest maintenance. The assumption is supported by the fact that cyclin E, which is normally repressed by pRb when stalling cells in G1/S,[31] accumulated to a high level in the arrested cells, while the level of pRb declined. p130(Rb2), which replaced pRb in arrested cells, does not act as a repressor of cyclin E.[30]

While pRb and p107 declined, the level of hypophosphorylated p130(Rb2) increased when p53$^{Val\text{-}135}$ was shifted to the active, wt form. *p130(rb2)* is not a target gene of p53. Like p27KIP1, p130/Rb2 levels are regulated by Cdk-dependent phosphorylation; the phosphorylated form is degraded by ubiquitin-dependent proteolysis.[32] In accordance, release of C6D10-p53$^{Val\text{-}135}$ cells from the G1/S arrest by inactivation of p53 induced a shift of p130(Rb2) to a slower migrating form in SDS-PAGE before it declined.

Our data provide evidence that the increase in p130(Rb2) is not an epiphenomenon, but that p130(Rb2) is actively involved in the growth arrest. Ectopic expression of the adenoviral protein E1A-12S, which disturbs pocket protein functions, as well as suppression of p130(Rb2) expression by siRNA initiated S-phase activity.

p130(Rb2) is known to impose a G1 block via two activities: First, p130(Rb2) forms stable complexes with cyclin E–CDK2 and cyclin A–CDK2 via a p21CIP1-like domain, and thereby impairs the activity of CDK2.[30,33,34] Accordingly, p130(Rb2) coimmunoprecipitated with cyclin E, but not with cyclin D from lysates of arrested C6D10-p53$^{Val\text{-}135}$ cells (data not shown). Secondly, p130(Rb2) is known to act as an efficient repressor of cyclin A.[29,30] In C6 cells arrested by p53$^{Val\text{-}135}$, cyclin A was repressed at the transcriptional level and ChIP showed that p130(Rb2) was bound to the repressed cyclin A promoter. Thus, repression of cyclin A by p130(Rb2) seems to be an essential step in this system to retain the cells in the arrest. The critical role of cyclin A repression in our system is further corroborated by the fact that ectopic expression of cyclin A was sufficient to induce S-phase activity in arrested cells. The increase in p27KIP1, p130/Rb2 and cyclin E levels, and the concomitant repression of cyclin A initiated by p53$^{Val\text{-}135}$ in C6 cells closely correspond to the growth-suppressive effects seen upon an ectopic expression of p130(Rb2) in a rodent tumor cell line,[30] indicating that p130(Rb2) can act as a master protein in this crosstalk.

Our data show that p53 can cooperate specifically with p130(Rb2) to induce a sustained arrest, suggesting that p130(Rb2) is an efficient inducer of cellular senescence when the major arrest pathway determined by pRb/p16INK4a is defunct. Appearance of the senescent phenotype was a late event. The number of cells capable of resuming proliferation when p53 was inactivated decreased continuously with maintenance of the arrest. At 4 weeks after arrest entry, only a minority of cells formed colonies, indicating that the arrest had largely become irreversible. This is consistent with observations of others on senescent embryonic mouse fibroblasts (MEFs) treated with p53-siRNA.[35] Only 0.5–1% of the senescent MEFs divided successfully. The data shown here suggest that not the growth-suppressive functions of p130(Rb2) and p53 *per se*, but secondary changes were responsible for the change to the irreversible status.

In search for secondary changes which may hamper the re-entry into the proliferative status, we found that the cells accumulated DNA damage while staying in the arrest. In cultures arrested for an extended period of time, nearly 100% of the cells displayed a senescent phenotype, and concomitantly were TUNEL positive. This accumulation of DNA damage shown here may represent an alternative mechanism to chromatin silencing by pRb[36] to hamper re-entry of senescent cells into the proliferative status. The damage was silent and was not recognized as long as the cells stayed in the arrest. It did even not prevent re-entry into and progression through the S-phase when p53 was inactivated. Thus, in agreement with the observations of others, senescent cells have the potential to escape the arrest and may even resume proliferation when any of the major control proteins such as p53 or pRb is inactivated.[1,2,35,37] Cells which lack p16INK4a, a frequent feature of tumor cells, seem to be prone to escape the arrest more efficiently.[38] The silent accumulation of DNA damage may increase the risk to select for spontaneous cell mt's, which can overcome the arrest. Thus, to make cellular senescence irreversible, the presence of a back-up fail-safe mechanism seems to be of advantage. As we show here, activation of apoptosis may provide such a mechanism.

Materials and Methods

Cell culture

C6D10 cells were subcloned from the C6 rat glioma cell line obtained from ATCC (CLL107).[39] C6D10-p53$^{Val\text{-}135}$ cells stably express the murine temperature-sensitive mt p53$^{Val\text{-}135}$. The cells were grown with Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum (FCS). For incorporation of BrdU, the cells were incubated with DMEM containing 10 μM BrdU for 30 min.

Antibodies and expression plasmids

The following antibodies were used: mouse monoclonal antibodies (mAbs) to p53 pAb240, pAb246, pAb248 and pAb421;[35–37] antibodies to cyclin D (M-20), cyclin E (M-20), cyclin A (H432), CDK2(D-12), p21(H164 and Sx118), p107 (sc-318), p130(Rb2) (sc-317), GFP (sc-8334 and sc-9996) from Santa Cruz Biotechnology (Santa Cruz, USA); CDK4 (H22) (Biomol, Hamburg, Germany); pRb(p105) (G3-245) (BD Biosciences, San Diego, USA), cleaved caspase 3 (Cell Signaling Technology), tubulin (Oncogene Research, San Diego, USA), cyclin A (C-4710) (Sigma, Munich, Germany), rabbit anti-lamin B. Incorporation of BrdU was monitored with a mouse mAb to BrdU (Boehringer Mannheim, Mannheim, Germany); normal goat serum and normal donkey serum were purchased from DAKO (Glostrup, Denmark); FITC- and Texas Red-conjugated secondary antibodies were from Jackson Immuno Research Laboratories (West Grove, USA). The plasmids used were: pcDNA3E1A12S (T Dobner, Regensburg, Germany) and pIRES2-AcGFP1-cycA.

Radioactive labeling of cell lysates

Cells were grown to 80% confluence in a 100-mm cell culture dish. The cells were pulse labeled for 30 min with 100 μCi ^{35}S-methionine and ^{35}S-cysteine in methionine- and cysteine-free DMEM, supplemented with 10% dialyzed FCS. p53 was immunoprecipitated, subjected to SDS polyacrylamide electrophoresis (PAGE) and visualized by autoradiography.

FACS analysis

Cells grown on a 100 mm petri dish were trypsinized and washed once with PBS, resuspended in 0.5 ml PBS and fixed in 80% cold ethanol. After rehydration in PBS, the cells were suspended in 1 ml of propidium iodide solution (5 μg propidium iodide; 1 mg RNase A per ml of PBS) for 30 min at 37°C. Labeled cells were run through a Coulter Counter (Beckman Coulter, USA) and cell cycle fractions were calculated with the Cylchred program.

Immunofluorescence

Cells were grown on glass coverslips to 50% confluence and fixed in cold (−20°C) acetone for 30 min. Fixed cells were rehydrated in PBS for 10 min and blocked with 2% normal serum for 20 min. For detection of BrdU, the cells were fixed in 70% cold ethanol in 50 mM glycine (pH 2.0). Goat anti-mouse IgG and goat anti-rabbit IgG conjugated with Texas Red or FITC were used as secondary antibodies. Images were taken with a Leica TCS confocal microscope (Leica, Bensheim, Germany).

Immunoprecipitation (IP) and WB

Subconfluent cultures were washed twice with cold (4°C) PBS. The cells were scraped from the culture dish and lysed with 1 ml of TEN buffer (50 mM Tris/HCl, pH 7.3; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% NP40; 1% aprotinin (Trasylol: Bayer; Leverkusen, Germany); 50 mM leupeptin), while shaking on ice for 30 min. The lysates were cleared in an Eppendorf microfuge at 13 000 rpm for 30 min. The protein concentration in the lysates was determined colorimetrically with the 'BCA Protein Assay' reagent (Pierce, Rockford, USA). An aliquot containing 100 μg of protein was adjusted to a total volume of 300 μl with lysis buffer. The probes were incubated with the appropriate antibodies and 100 μl of a 10% slurry of protein G-Sepharose for 2 h at 4°C The PGS with the bound immunocomplexes was washed three times with cold lysis buffer. Precipitated proteins were dissolved in SDS sample buffer, electrophoresed on 10% polyacrylamide gels, and transferred onto nitrocellulose (Hybond, Amersham, UK). The nitrocellulose sheets were blocked with 2% BSA in Tris-buffered salt solution (20 mM Tris/HCl, pH 7.6, 150 mM NaCl) containing 0.05% Tween-20. Proteins were detected with the appropriate primary antibodies and secondary antibodies conjugated with peroxidase. For detection of p53, the sheets were incubated with the appropriate mouse mAb, followed by a rabbit anti-mouse IgG (DAKO, Denmark) and a peroxidase-conjugated mouse antibody (DAKO, Denmark). The bound peroxidase was detected using the ECL WB detection kit (Amersham; Braunschweig, Germany) and chemiluminescence was visualized by exposing the membrane to X-ray films.

Chromatin immunoprecipitation

Cells cultivated on Petri dishes (2×10^6 cells per dish) were fixed with 1% formaldehyde in MEM without serum for 10 min at RT. Residual aldehyde groups were blocked with 125 mM glycine. After washing with ice-cold PBS containing protease inhibitors, the cells were scraped from the dish and sedimented in Eppendorf tubes for 5 min at 5000 rpm. The pellet was lysed in SDS-lysis buffer for 10 min and the lysate was sonificated on ice six times for 5 s each. Unsoluble material was pelleted for 25 min at 20 000 $\times$ *g*. The supernatant was diluted 1 : 10 and preabsorbed for 15 min at 4°C with 20 μl of a 10% protein A-Sepharose (PAS) slurry

coated with single-strand DNA (ssDNA). In all, 2 μg of the appropriate antibodies was added to the supernatant and incubated overnight. The immune complexes were precipitated with ssDNA-coated PAS. DNA isolated from these immune complexes was probed for the presence of rat cyclin A promoter sequences by PCR, using 5′CGCAGCAGAGG CTCAAGACTC and 5′CACAGCCAAATGCAGGGTCTC as primer pair.

Transfection and RNA interference

Proliferating cells were transfected with siRNA by use of Oligofectamine (Invitrogen) according to the manufacturer's protocol. Arrested cells were transfected by electroporation in an AMAXA apperature (AMAXA, Cologne, Germany) using the Nucleofactor Solution Kit V and program U09 (siRNA) or program U30 (pIRES vector). siRNAs was generated with the *Silencer*^{R} siRNA Construction Kit (Ambion, USA). The sequence selected for synthesis of p130/Rb2 si-RNA covered nucleotides 2548–2568 of the p130/Rb2 cDNA sequence (gi: 13592040).

Kinase Assay

Immunoprecipitates with antibodies to cyclin D or E were washed with 0.5 ml of CDK2-kinase buffer,[40] equilibrated in this buffer, sedimented, and resuspended in 30 μl kinase buffer. Histone H1 (1 μg; Biomol, Hamburg), 2 mg recombinant Rb (sc-411; Santa Cruz, USA), 10 μCi ^{32}P-γATP and 50 μM cold ATP were added and the probes were incubated at 37°C for 30 min. The reaction was stopped by adding 6 ml of six-fold concentrated SDS-sample buffer. The probes were electrophoresed on a polyacrylamide gel, transferred onto nitrocellulose sheets and subjected to autoradiography.

Acknowledgements

We are grateful to L Hengst, Munich, and T Dobner, Regensburg, for support with expression plasmids. This work was supported by Deutsche Krebshilfe, grant 10-1975-Bo II. A Kapic is a Werner Otto Stiftung scholarship holder. The Heinrich-Pette-Institut is financially supported by *Freie und Hansestadt Hamburg* and *Bundesministerium für Gesundheit*.

References

1. Sugrue MM, Shin DY, Lee SW and Aaronson SA (1997) Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. Proc. Natl. Acad. Sci. USA 94: 9648–9653
2. Wang Y, Blandino G, Oren M and Givol D (1998) Induced p53 expression in lung cancer cell line promotes cell senescence and differentially modifies the cytotoxicity of anti-cancer drugs. Oncogene 17: 1923–1930
3. Psyrri A, DeFilippis RA, Edwards AP, Yates KE, Manuelidis L and DiMaio D (2004) Role of the retinoblastoma pathway in senescence triggered by repression of the human papillomavirus E7 protein in cervical carcinoma cells. Cancer Res. 64: 3079–3086
4. Bruce JL, Hurford Jr RK, Classon M, Koh J and Dyson N (2000) Requirements for cell cycle arrest by p16INK4a. Mol. Cell 6: 737–742
5. Timmermann S, Hinds PW and Munger K (1998) Re-expression of endogenous p16ink4a in oral squamous cell carcinoma lines by 5-aza-2′-deoxycytidine treatment induces a senescence-like state. Oncogene 17: 3445–3453
6. Steiner MS, Wang Y, Zhang Y, Zhang X and Lu Y (2000) p16/MTS1/INK4A suppresses prostate cancer by both pRb dependent and independent pathways. Oncogene 19: 1297–1306
7. Strauss BE, Fontes RB, Lotfi CF, Skorupa A, Bartol I, Cipolla-Neto J and Costanzi-Strauss E (2002) Retroviral transfer of the p16INK4a cDNA inhibits C6 glioma formation in Wistar rats. Cancer Cell Int. 2: 2
8. Dai CY and Enders GH (2000) p16 INK4a can initiate an autonomous senescence program. Oncogene 19: 1613–1622
9. Krimpenfort P, Quon KC, Mooi WJ, Loonstra A and Berns A (2001) Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. Nature 413: 83–86
10. Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ, Wu EA, Horner JW and DePinho RA (2001) Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature 413: 86–91
11. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G and Sherr CJ (1997) Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell 91: 649–659
12. Serrano M, Lin AW, McCurrach ME, Beach D and Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88: 593–602
13. Martinez J, Georgoff I and Levine AJ (1991) Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev. 5: 151–159
14. Schlegel J, Piontek G, Kersting M, Schuermann M, Kappler R, Scherthan H, Weghorst C, Buzard G and Mennel H (1999) The p16/Cdkn2a/Ink4a gene is frequently deleted in nitrosourea-induced rat glial tumors. Pathobiology 67: 202–206
15. Klotzsche O, Etzrodt D, Hohenberg H, Bohn W and Deppert W (1998) Cytoplasmic retention of mutant tsp53 is dependent on an intermediate filament protein (Vimentin) scaffold. Oncogene 16: 3423–3434
16. Michalovitz D, Halevy O and Oren M (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell 62: 671–680
17. Knippschild U, Oren M and Deppert W (1996) Abrogation of wild-type p53 mediated growth-inhibition by nuclear exclusion. Oncogene 12: 1755–1765
18. Stein GH, Namba M and Corsaro CM (1985) Relationship of finite proliferative lifespan, senescence, and quiescence in human cells. J. Cell Physiol. 122: 343–349
19. Benda P, Someda K, Messer J and Sweet WH (1971) Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. J. Neurosurg. 34: 310–323
20. Dulic V, Lees E and Reed SI (1992) Association of human cyclin E with a periodic G1–S phase protein kinase. Science 257: 1958–1961
21. Ekholm SV, Zickert P, Reed SI and Zetterberg A (2001) Accumulation of cyclin E is not a prerequisite for passage through the restriction point. Mol. Cell. Biol. 21: 3256–3265
22. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817–825
23. Tiemann F and Hinds PW (1998) Induction of DNA synthesis and apoptosis by regulated inactivation of a temperature-sensitive retinoblastoma protein. EMBO J. 17: 1040–1052
24. Sellers WR, Rodgers JW and Kaelin Jr WG (1995) A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA 92: 11544–11548
25. Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S and Dean DC (1995) Mechanism of active transcriptional repression by the retinoblastoma protein. Nature 375: 812–815
26. Dyson N, Guida P, McCall C and Harlow E (1992) Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J. Virol. 66: 4606–4611
27. Bagchi S, Raychaudhuri P and Nevins JR (1990) Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell 62: 659–669
28. Ikeda MA, Jakoi L and Nevins JR (1996) A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA 93: 3215–3220
29. Hurford Jr RK, Cobrinik D, Lee MH and Dyson N (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev. 11: 1447–1463
30. Howard CM, Claudio PP, De Luca A, Stiegler P, Jori FP, Safdar NM, Caputi M, Khalili K and Giordano A (2000) Inducible pRb2/p130 expression and growth-suppressive mechanisms: evidence of a pRb2/p130, p27Kip1, and cyclin E negative feedback regulatory loop. Cancer Res. 60: 2737–2744
31. Herrera RE, Sah VP, Williams BO, Makela TP, Weinberg RA and Jacks T (1996) Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. Mol. Cell. Biol. 16: 2402–2407

32. Tedesco D, Lukas J and Reed SI (2002) The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev. 16: 2946–2957
33. Hannon GJ, Demetrick D and Beach D (1993) Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev. 7: 2378–2391
34. Claudio PP, De Luca A, Howard CM, Baldi A, Firpo EJ, Koff A, Paggi MG and Giordano A (1996) Functional analysis of pRb2/p130 interaction with cyclins. Cancer Res. 56: 2003–2008
35. Dirac AM and Bernards R (2003) Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. J. Biol. Chem. 278: 11731–11734
36. Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, Hannon GJ and Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113: 703–716
37. Gire V and Wynford-Thomas D (1998) Reinitiation of DNA synthesis and cell division in senescent human fibroblasts by microinjection of anti-p53 antibodies. Mol. Cell. Biol. 18: 1611–1621
38. Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P and Campisi J (2003) Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J. 22: 4212–4222
39. Roser K, Bohn W, Giese G and Mannweiler K (1991) Subclones of C6 rat glioma cells differing in intermediate filament protein expression. Exp. Cell Res. 197: 200–206
40. Schneider E, Montenarh M and Wagner P (1998) Regulation of CAK kinase activity by p53. Oncogene 17: 2733–2741