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# Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain

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**The proliferation rate of a cell population reflects a balance between cell division, cell cycle arrest, differentiation and apoptosis. The regulation of these processes is central to development and tissue homeostasis, whereas dysregulation may lead to overt pathological outcomes, notably cancer and neurodegenerative disorders. We report here the cloning of a novel zinc finger protein which regulates apoptosis and cell cycle arrest and was accordingly named Zac1. *In vitro* Zac1 inhibited proliferation of tumor cells, as evidenced by measuring colony formation, growth rate and cloning in soft agar. *In vivo* Zac1 abrogated tumor formation in nude mice. The antiproliferative activity of Zac1 was due to induction of extensive apoptosis and of $G_1$ arrest, which proceeded independently of retinoblastoma protein and of regulation of $p21^{WAF1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ and $p16^{INK4a}$ expression. Zac1-mediated apoptosis was unrelated to cell cycle phase and $G_1$ arrest was independent of apoptosis, indicating separate control of apoptosis and cell cycle arrest. *Zac1* is thus the first gene besides *p53* which concurrently induces apoptosis and cell cycle arrest.**

*Keywords*: apoptosis/cell cycle/PACAP/p53/zinc finger

## Introduction

The proliferation of a cell population is regulated by a balance between cell division, growth arrest, differentiation and programed cell death. A network of genes, including cell cycle regulatory genes (Hunter and Pines, 1994; Grana and Reddy, 1995), protooncogenes (Hoffman and Liebermann, 1995) and tumor suppressor genes (Levine, 1993) have emerged which play major roles in normal physiological processes, such as development (Raff *et al.*, 1993; Steller, 1995) and aging (Jazwinski, 1996), as well as various pathological states (Thompson, 1995), such as neoplasia (Hartwell and Kastan, 1994; Karp and Broder, 1995) and neurodegenerative disorders (Heintz, 1993; Ross, 1996).

All eukaryotic cells possess similar mechanisms to regulate progression of the cell cycle by sequential formation, activation and subsequent inactivation of a series of cyclin–cyclin-dependent kinase (Cdk) complexes (Grana and Reddy, 1995). Control of the protein kinase activity of these complexes is critical to the orchestrated development of multicellular organisms, as well as to the response and adaptation to various physiological or pathological stimuli in mature organisms. In addition to positive regulation by active cyclin–Cdk complexes, negative regulation of the cell cycle occurs at checkpoints, which are the transitions where feedback mechanisms operate to prevent premature entry of the cell into the next phase of the cycle prior to completion of the necessary macromolecular events. Growth suppressor genes play an important role in checkpoint function and loss or mutation of genes associated with checkpoint functions seem to have important implications in the development of cancer (Hartwell and Kastan, 1994).

Apoptosis, or programed cell death, is the process by which a cell will actively commit suicide under tightly controlled circumstances (Steller, 1995). Apoptosis is a morphologically distinct form of programmed cell death that plays a major role during development and in many pathological states, including cancer (Fisher, 1994; Karp and Broder, 1995), acquired immunodeficiency syndrome (Gougeon and Montagnier, 1993) and neurodegenerative disorders (Heintz, 1993; Ross, 1996). Apoptosis occurs through the activation of a cell-intrinsic suicide program. The basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells at all times, but activation of the suicide program is under the control of a network of interrelated signals that originate both from the intracellular and the extracellular milieu.

Therefore, genetic lesions in genes controlling the cell cycle and/or the apoptotic machinery are pivotal to the development of malignancy or neurodegenerative disorders. Among these genes, the tumor supressor gene *p53* deserves particular attention, due to its frequent loss of function in various human cancers (Hollstein *et al.*, 1991) and its impact on tumor progression, prognosis and treatment regimens (Lowe *et al.*, 1993, 1994). The tumor suppressor activity of *p53* resides in the so far unique ability to control concurrently two fundamental cellular mechanisms, namely cell cycle progression and induction of apoptosis (Bates and Vousden, 1996; Ko and Prives, 1996).

We report here cloning of a novel gene, designated *Zac1*, which encodes a protein with seven zinc fingers of the $C_2H_2$ type which is only distantly related to previously characterized zinc finger proteins. Unexpectedly, we isolated *Zac1* by a functional expression cloning technique, which resulted in addition in isolation of the wild-type form of *p53*. This technique is based on co-transfection of pools of an expression library with a cAMP-responsive

reporter gene (Spengler *et al.*, 1993). Zac1 and p53 were found to induce expression of the gene encoding the type I receptor ($PACAP_1$-R) for the peptide PACAP (pituitary adenylate cyclase activating polypeptide) (Miyata *et al.*, 1989) through mechanisms which remain not totally elucidated at present. Of note, though Zac1 and p53 are structurally unrelated, they caused an equivalent inhibition of tumor cell growth *in vitro* under constitutive and regulated expression through apparently different pathways. Interestingly again, Zac1-dependent growth inhibition relied on separate induction of apoptosis and $G_1$ arrest.

## Results

### *Functional expression cloning of Zac1*

We used a recently described expression cloning method (Spengler *et al.*, 1993) to screen simultaneously for different receptors positively coupled to adenylyl cyclase. This method is based on transcriptional induction of a cAMP-responsive luciferase reporter gene by stimulation of adenylyl cyclase through activated target receptors. Pools of clones from a corticotrophic tumor cell line (AtT-20) cDNA library and from a newborn rat colliculus cDNA library were co-transfected with the reporter construct pΔMC16LUC into the host cell line LLC-PK1. Separate aliquots of cells were incubated with various peptides, including PACAP, 12 h after electroporation. One pool of clones from the rat colliculus library consistently stimulated luciferase activity in the presence of PACAP, and a functional clone encoding $PACAP_1$-R was isolated by successive subdivisions (Spengler *et al.*, 1993). Several other pools displayed the same phenotype, namely PACAP-dependent stimulation of the reporter gene (data not shown), and the corresponding active clones were isolated by the same subdivision process. Sequencing of one of these PACAP-positive clones from the rat library revealed that this cDNA was identical to the wild-type form of the tumor suppressor gene *p53*. Partial sequencing of two PACAP-positive clones from the AtT-20 library also identified them as wild-type mouse *p53*. In addition, two clones (p2195, EMBL accession No. X95503, and p1270) inducing $PACAP_1$-R expression turned out to encode the same protein, which we named Zac1 for reasons detailed in the following sections of this manuscript.

### *Structural analysis and tissue distribution of Zac1*

The isolated cDNA clones, p2195 and p1270, contained a 2.8 and a 4.7 kb insert respectively. Entire sequencing of clone p2195 revealed a 2790 bp cDNA encoding an open reading frame of 667 amino acids giving rise to a protein with a predicted molecular weight of 75 kDa (Figure 1A). The ATG of AGGCCATGG was assigned as initiation codon on the basis of its close match to the CC(A/G)CCATGG Kozak consensus sequence for favored initiation of translation and the presence of an in-frame TGA stop codon 12 nt upstream (data not shown). Examination of the protein sequence revealed the presence of seven zinc finger motifs of the $C_2H_2$ type (Klug and Schwabe, 1995) in the N-terminal region of Zac1. However, homology to other members of the zinc finger protein family was low (30% at best), with the closest group being the GLI-Kruppel family, whose members have been implicated in normal development and tumor formation

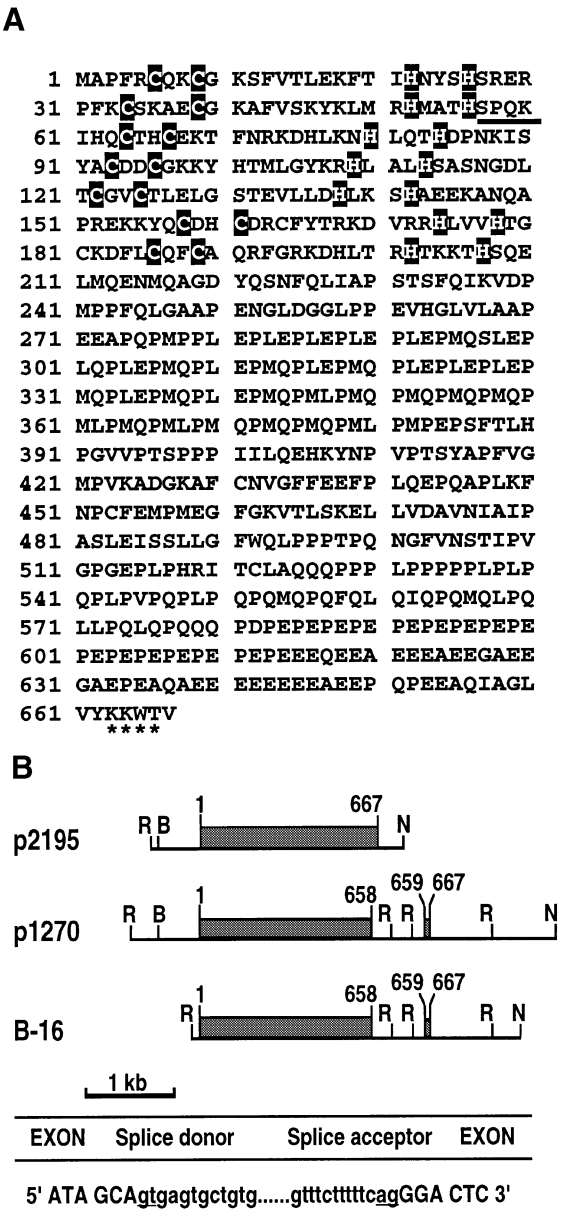



**Fig. 1.** (**A**) Sequence of Zac1 protein. Cysteine and histidine residues of the seven zinc finger motifs of the $C_2H_2$ type are boxed. A putative phosphorylation site for Cdks, corresponding to the consensus motif (b/p)(S/T)Pxb located at residues 56–60, is underlined. A putative phosphorylation site for protein kinase A (PKA) at residue 666 is indicated (*). (**B**) Schematic representation of the *Zac1* clones. Clones p2195 and p1270 were derived from the AtT-20 corticotrophic tumor cell line. Clone B-16 was isolated from a BALB/c pituitary library and encodes the same protein identified in p2195 and p1270. The coding region of p1270 and B-16 is interrupted at residue 658 by a 630 bp insertion. The sequences at the boundaries of this insertion are displayed in the lower part of the figure, are in excellent agreement with consensus exon–intron junctions and preserve the reading frame. Restriction sites for *Eco*RI (R), *Bam*HI (B) and *Not*I (N) are indicated.

(Ruppert *et al.*, 1993). In particular, the first H/C link (HSRERPFKC) is in good agreement with the consensus motif for the GLI-Kruppel family (H(S/T)GEKP(F/Y)XC) (Schuh *et al.*, 1986). On the other hand, the remaining 459 C-terminal amino acids displayed no significant homology to sequences in the Swissprot and NBRF-PIR databases. The central region of the protein (275–383) is characterized by 34 PLE, PMQ or PML repeats, suggestive

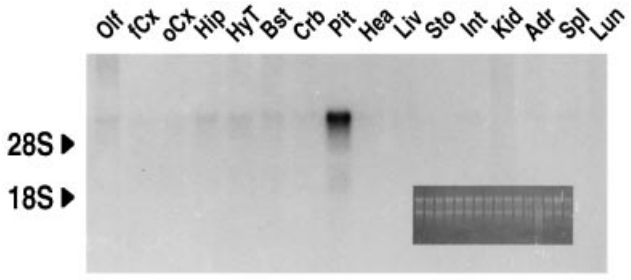



**Fig. 2.** Distribution of *Zac1* mRNA in mouse tissues. *Zac1* distribution was assessed by Northern blot analysis of total RNA prepared from different brain regions [olfactory bulb (Olf), frontal cortex (fCx), occipital cortex (oCx), hippocampus (Hip), hypothalamus–thalamus (HyT), brain stem (BSt), cerebellum (Crb)] and peripheral tissues [anterior pituitary gland (Pit), heart (Hea), liver (Liv), stomach (Sto), intestine (Int), kidney (Kid), adrenal gland (Adr), spleen (Spl), lung (Lun)]. Ethidium bromide staining of the gel is shown in the insert to document equal and intact amounts of each RNA preparation.

of a structure known as a poly(proline) type II helix, which is considered to be critically involved in protein–protein interactions (Williamson, 1994). The C-terminal region is particularly P, Q and E rich, a feature often displayed by transactivation domains of transcription factors. In addition, the presence of a putative phosphorylation site (HSPQK) for Cdks located between the second and third zinc finger motifs (residues 56–60), as well as a putative protein kinase A phosphorylation site (KKWT) at the very C-terminus (residues 663–666), suggests possible regulation by protein kinases.

Since the cDNAs p2195/p1270 were isolated from the AtT-20 tumor cell line, there was a potential risk that they harbored mutations which may have resulted in loss or gain of functions not associated with the wild-type form. To rule out this possibility, we recloned Zac1 from a plasmid library constructed from whole pituitary tissue from BALB/c mice. Screening of ~$0.5\times10^6$ clones with the p2195 cDNA probe allowed isolation of one full-length cDNA clone designated B-16 (EMBL accession No. X95504), which contained a 3.7 kb insert. Transfection of B-16 into LLC-PK1 cells successfully substituted for p2195, p1270 or wild-type *p53* with respect to regulation of $PACAP_1$-R expression (data not shown). Entire sequencing of clone B-16 showed an 86 bp 5′-untranslated region and an extended 3′-untranslated region of 0.7 kb (Figure 1B). The coding region of B-16 was identical to p2195 except that the reading frame was interrupted at residue 658 by a 630 bp insertion. The sequences at the boundaries of this insertion are in excellent agreement with consensus exon–intron junction sequences and preserve the reading frame (Figure 1B). We observed this insertion at exactly the same position in clone p1270 derived from the AtT-20 library (Figure 1B). This finding argues against a cloning artefact in clone B-16 and suggests the presence of an unspliced intron region. In support of this hypothesis, a PCR fragment corresponding to the intron region failed to hybridize to a poly(A)$^+$ blot from AtT-20 cells (data not shown).

The distribution of *Zac1* was assessed by Northern blot of total RNA prepared from different mouse tissues. Interestingly, in adult mice the anterior pituitary gland displayed by far the highest level of expression of *Zac1* mRNA (Figure 2). *Zac1* was expressed at lower levels in various brain areas, including olfactory bulb, cortex, hippocampus, hypothalamus–thalamus, brain stem and cerebellum, and faintly in peripheral tissues such as stomach, kidney, adrenal gland, heart and lung (Figure 2 and data not shown).

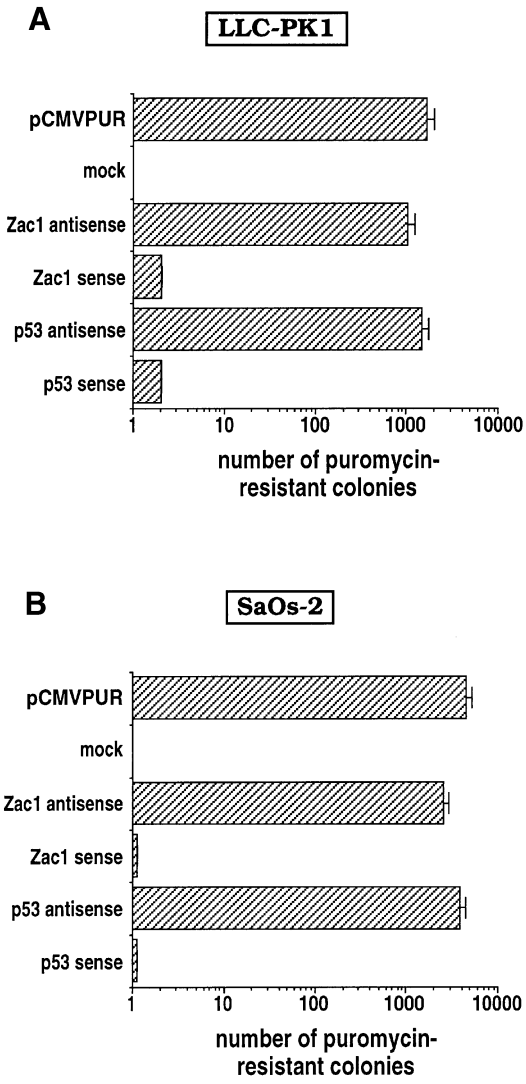



**Fig. 3.** Zac1 and p53 inhibit colony formation. LLC-PK1 (**A**) or SaOs-2 (**B**) cells were transfected with plasmids encoding puromycin resistance alone (pCMVPUR) or containing *Zac1* or *p53* cDNAs in sense or antisense orientation. Puromycin was added for 10 days and viable colonies were scored following staining with MTT.

### *Constitutive expression of Zac1 and p53 abates growth of tumor cells*

To test whether Zac1 shares with p53 additional properties besides regulation of the $PACAP_1$-R gene, we examined cell proliferation in a colony formation assay (Baker *et al.*, 1990; Diller *et al.*, 1990). We transfected *Zac1* and *p53* cDNAs in sense and antisense orientation in a vector carrying the puromycin resistance gene and selected for puromycin-resistant clones in LLC-PK1 and SaOs-2 cells. The *Zac1* sense cDNA caused a substantial reduction in the number of colonies, comparable with *p53*, whereas similar numbers were noted for the parent vector and the antisense constructs (Figure 3). Abrogation of cell growth by Zac1 and p53 was more prominent in the SaOs-2 cell

line. In addition, the clones that did appear after transfection of *Zac1* or *p53* sense constructs into the LLC-PK1 cell line died when re-exposed to selection after passaging and grew at a slow rate if further selection was omitted (data not shown). In contrast, expression of the human glucocorticoid receptor, which encodes a strong transactivation domain and profoundly alters cell homeostasis, revealed a <10-fold difference in the number of colonies in the absence and presence of dexamethasone, ruling out the possibility that differences under Zac1 and p53 are solely due to protein overexpression or squelching (data not shown).

### Regulated expression of Zac1 and p53 impairs proliferation rate, colony formation in soft agar and tumor formation in nude mice

*Zac1 and p53 suppress growth of tumor cells.* For further analysis, we used a tetracycline-regulated expression system (Gossen and Bujard, 1992, 1993) to control expression by a transactivator (tTA) which is blocked in the presence of tetracycline (Tc) or anhydrotetracycline (ATc). We included *p53* in the following experiments to serve as a positive control and as a reference to evaluate the responses to Zac1 expression under the specific experimental conditions used in this study. A novel tetracycline-sensitive expression vector with lower basal activity (Hoffmann *et al.*, 1997; A.Hoffmann, M.Villalba, L.Journot and D.Spengler, submitted) was used to express Zac1 and p53 in individual LLC-PK1 and SaOs-2 cell clones. One third of the individual clones isolated from each transfection condition (L-Zac = 95, L-p53 = 92, S-Zac = 77, S-p53 = 72) revealed strong inhibition of proliferation in the absence of ATc, as deduced from light microscopic inspection. Analysis of 10 clones randomly chosen from each of these groups revealed a close correlation between growth inhibition and Zac1 or p53 expression, as shown by measurement of cell number and immunoblot analysis (data not shown) and allowed the assignment of one clone for further study. Importantly, in the presence of the repressor ATc, no major differences in growth behavior were observed between Zac1- and p53-expressing clones and the parent clones, L-tTA and S-tTA (Figure 4A and B). Therefore, the differences in cell number on day 6 were primarily due to suppression of growth in the absence of the repressor. In line with this view, results from cell counts on primary pools ($n = 3$) from each condition revealed a 4- and 7-fold difference in cell numbers on day 6 for both the LLC-PK1 and SaOs-2 cell lines (data not shown). A strong increase in protein levels of Zac1 was noted in the induced state (data not shown and Figure 7C). Similar results were also obtained for regulation of p53 in LLC-PK1 and SaOs-2 cells (data not shown and Figure 7C).

To confirm these results obtained by direct quantification of cell number, we evaluated the effects of Zac1 and p53 expression by two complementary methods. First, we studied DNA synthesis with a non-radioactive immunoassay based on incorporation of 2-bromodeoxyuridine (BrdU) into nuclear DNA on each of 6 days with or without ATc (Figure 4C). Second, we measured conversion of the tetrazolium salt Thiazolyl blue (MTT) to formazan blue, which depends on the activity of mitochondrial and cytoplasmic dehydrogenases. This activity depends on cell viability and closely correlates with cell proliferation. These experiments confirmed the measurements of cell number (Figure 4D). Similar results were obtained for L-Zac and L-p53 (data not shown).

To exclude the possibility that Zac1-induced alteration of proliferation is due to down-regulation of transduction pathways activated by mitogenic serum factors, we assessed proliferation in the presence of different serum concentrations. Cells from LLC-PK1 (data not shown) and SaOs-2 clones kept under low serum conditions (0.1% fetal calf serum) in the repressed state displayed a reduced growth rate from day 3 on, indicating serum dependence in maintaining logarithmic growth (Figure 4E). In contrast, cell number was unaffected by serum concentration with expression of Zac1 and p53 (Figure 4E). Therefore, inhibition of tumor cell growth by Zac1 and p53 proceeds through mechanisms unrelated to down-regulation of mitogenic pathways in these cellular models.

The ability of Zac1 to suppress growth could be due to a non-specific lethal effect of protein overproduction resulting in cell death. Alternatively, it could be a manifestation of a more specific effect on cell proliferation. To further investigate these two possibilities, we tested the growth pattern following re-exposure to ATc of the surviving cells. Surviving LLC-PK1 and SaOs-2 cells resumed logarithmic growth after 48 h of ATc deprivation (data not shown and Figure 4F). Therefore, Zac1- and p53-induced changes in cell growth were not permanent and, at least in part, were reversible, arguing against a non-specific effect of protein overproduction. In support of this view, high levels of expression of the inert luciferase gene from the same tetracycline-dependent expression system in LLC-PK1 or SaOs-2 cells revealed no changes in cell proliferation.

*Zac1 and p53 inhibit soft agar colony formation.* Anchorage-independent growth is often correlated with tumorigenesis and is a strong criterion for cultured cell transformation. To test the influence of Zac1 on anchorage-independent growth, we assayed LLC-PK1 and SaOs-2 cell clones for their ability to grow in soft agar. Colony formation by Zac1- or p53-expressing cells was dramatically reduced compared with the repressed state (Figure 5). Also, the few colonies formed under Zac1 or p53 expression were of smaller size. These results demonstrate that Zac1 and p53 can abate anchorage-independent growth of tumor cells, one of the hallmarks of tumorigenicity and transformed cell growth.

*Zac1 and p53 suppress tumor formation in nude mice.* The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet not all of the altered cellular growth properties commonly associated with the transformed state *in vitro* are required for neoplastic growth *in vivo* and vice versa. Therefore, loss of tumorigenicity under expression of Zac1 *in vivo* would be a critical test to substantiate the growth suppressor function of Zac1. To achieve gene regulation by Tc in nude mice, we implanted half of the animals with Tc pellets, whereas the remainder was implanted with placebo pellets. Two days later, each animal was injected s.c. on each side with S-Zac or S-p53 cells grown in the continuous presence of ATc. Due to the clonal origin of S-Zac and S-p53, we observed differences in the

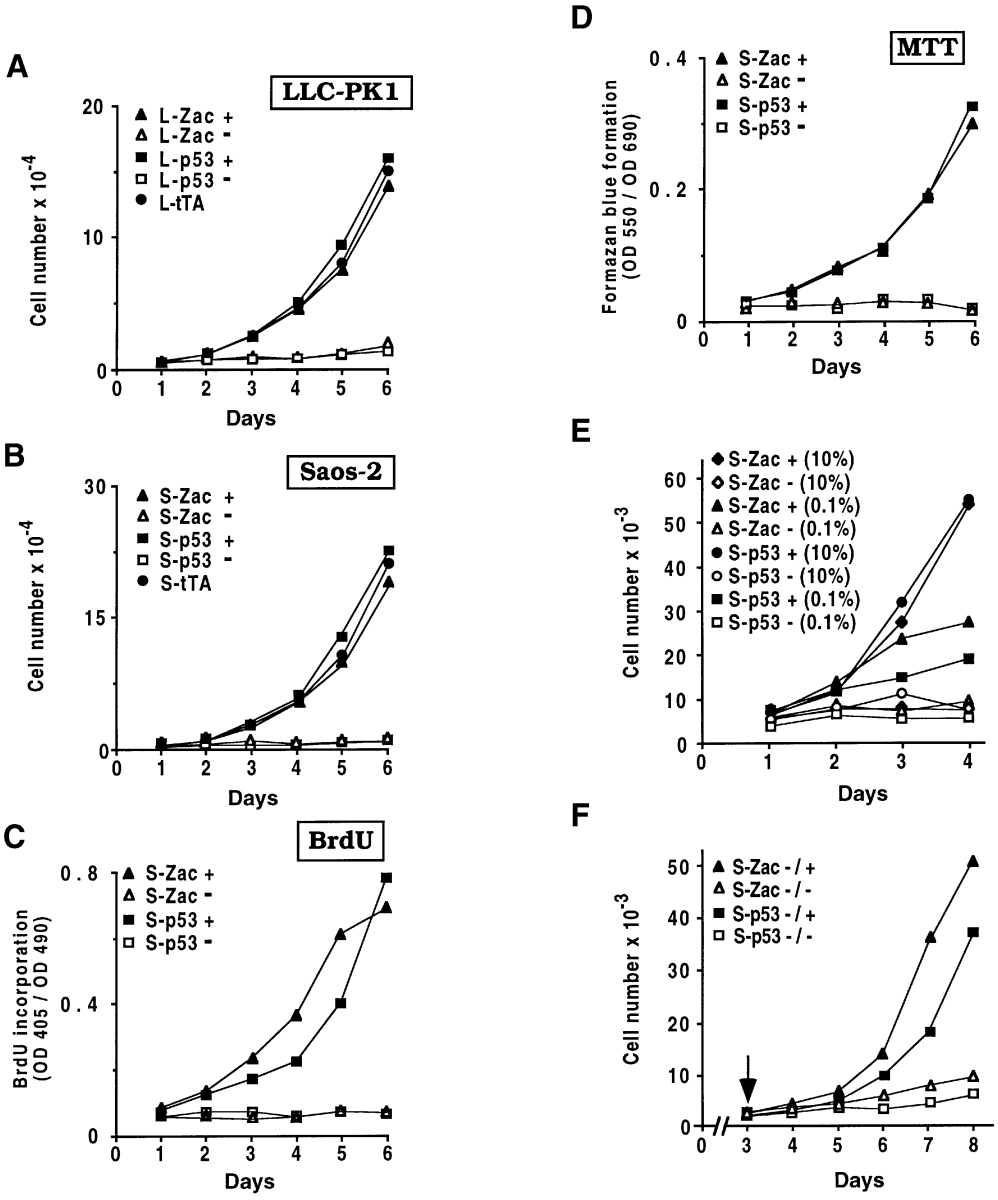



**Fig. 4.** Zac1 and p53 alter proliferation of LLC-PK1 and SaOs-2 cells. ATc-regulated expression of Zac1 and p53 was established in LLC-PK1 and SaOs-2 cells. (**A** and **B**) Cell numbers of the parent tTA clones (L-tTA and S-tTA) were compared with those obtained with Zac1- and p53-expressing LLC-PK1 (L-Zac and L-p53 respectively) and SaOs-2 (S-Zac and S-p53 respectively) clones in the presence (+) or absence (–) of ATc. (**C** and **D**) Zac1 and p53 inhibit DNA synthesis (BrdU) and cell viability (MTT). For each time point, BrdU incorporation or formazan blue formation were measured in the presence (+) or absence (–) of ATc. (**E**) Growth inhibition by Zac1 and p53 is serum independent. Cells were grown in the presence of the indicated amount of fetal bovine serum (10 or 0.1%) and in the presence (+) or absence (–) of ATc. (**F**) Growth inhibition by Zac1 and p53 is reversible. Cells were seeded in ATc-containing medium, grown in the absence of ATc for 2 days before medium was renewed (arrow) with medium containing (–/+) or lacking (–/–) ATc.

tumorigenicity of each clone, as shown by the difference in the observed lag in tumor formation, which was assessed at 11 weeks after cell injection for S-Zac and at 16 weeks for S-p53. Table I presents results from three experiments with S-Zac and one experiment with S-p53. In agreement with previous reports (Chen *et al.*, 1990), p53 expression impaired tumor formation by SaOs-2 cells *in vivo*. Interestingly, Zac1 also inhibited tumor formation, as deduced from tumor incidence (Table I) and tumor weight [193 ± 13 mg ($n$ = 14) for Tc versus 18 ± 7 mg ($n$ = 2) for placebo]. Thus, Zac1 and p53 are equivalent at inhibiting tumor formation *in vivo* in xenografted nude mice.

### *Expression of Zac1 and p53 induces apoptosis*

An increasing number of cells with signs of lost cell viability was observed from day 2 onwards following Zac1 or p53 expression. These cells, which were abundant on phase contrast microscopy, failed to convert MTT, shrank, revealed membrane blebbing and further rounded up before detaching from the plates. For Zac1 these alterations were most evident in SaOs-2 cells (S-Zac) and for p53 in LLC-PK1 cells (L-p53) (data not shown) and appeared reminiscent of apoptotic cell death. This form of cell death is often accompanied by degradation of the DNA into a ladder of regular fragments. To address this

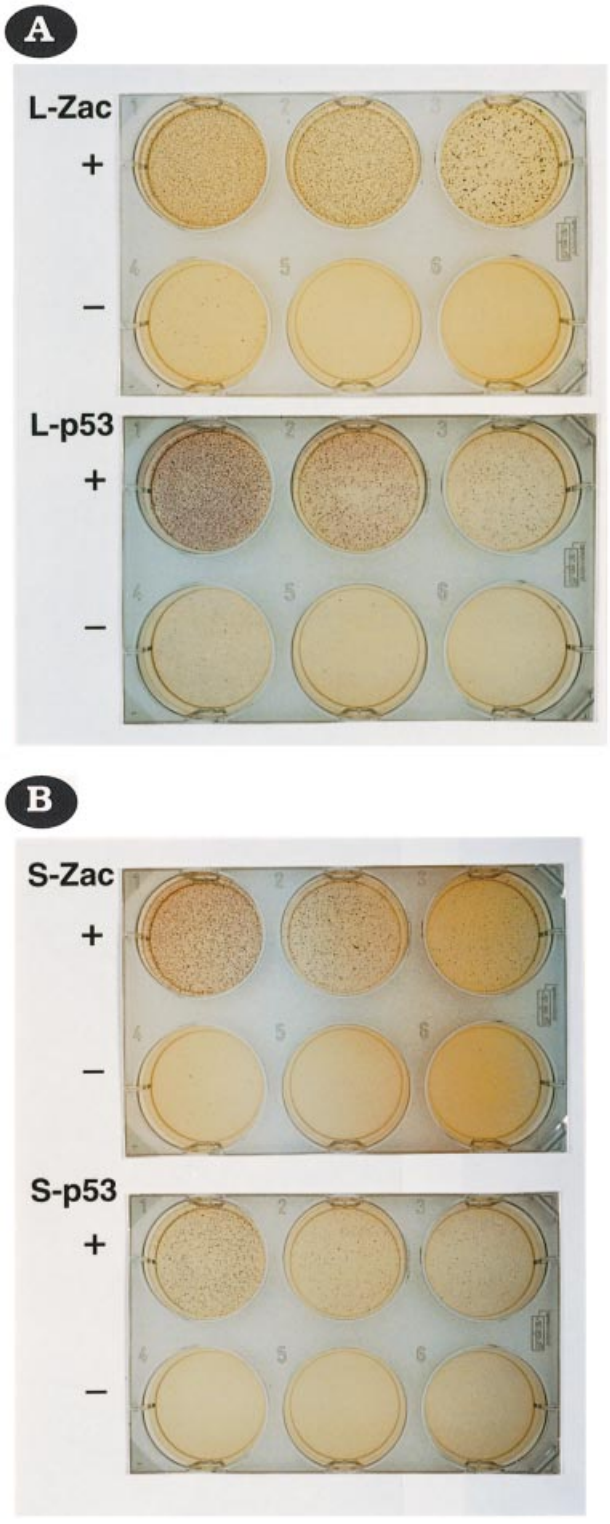



**Fig. 5.** Zac1 and p53 inhibit soft agar colony formation. Zac1 (L-Zac and S-Zac) and p53 (L-p53 and S-p53) clones were grown in the presence of ATc before plating into soft agar at densities of $1\times10^5$ (nos 1 and 4), $5\times10^4$ (nos 2 and 5) and $2.5\times10^4$ (nos 3 and 6) cells per well in 6-well plates. The repressor ATc was included in the upper rows (+) and was omitted in the lower rows (–). For photography on day 10, the soft agar was overlaid with MTT for 4 h. Pictures shown are representative of three to five independent experiments.

issue, we isolated genomic DNA from the LLC-PK1 and SaOs-2 clones kept for 3 days without ATc. When the repressor was omitted, a clearly visible degradation into

**Table I.** Zac1 and p53 inhibit tumor formation *in vivo*

| Clone | Tumor incidence (No. of tumor-bearing injection sites/No. of injection sites) | |
|---|---|---|
| | Placebo | Tc |
| S-Zac (exp. 1) | 2/12 | 14/14 |
| S-Zac (exp. 2) | 1/12 | 12/12 |
| S-Zac (exp. 3) | 1/8 | 8/8 |
| S-p53 | 1/12 | 10/12 |

Nude mice were implanted with placebo or Tc pellets s.c. Two days later, $5\times10^6$ cells from each clone were injected s.c. into each side of each animal and tumor formation was scored at 11 (Zac1) and 16 weeks (p53).

oligonucleosomal DNA fragments became evident (Figure 6A and B), which was most advanced following expression of Zac1 in SaOs-2 cells. Quantification of fragmented DNA after 48 h without ATc indicated a 17.3- and 3.9-fold increase following Zac1 expression and a 3.4- and 6.8-fold increase under p53 in SaOs-2 and LLC-PK1 cells respectively (data not shown).

The fluorescent DNA stains ethidium bromide and acridine orange were employed to examine nuclear changes under ATc-deprived conditions. Under expression of p53 a residual attached population of cells exhibited nuclear signs of apoptosis, whereas Zac1-expressing cells shrank, dislodged quickly and appeared less represented in these experiments (data not shown). Since we observed a dramatic increase in floating cells under Zac1 and p53 expression from day 2 on, we investigated these cells for structural signs of apoptosis. Decay of the nucleus involved nuclear shrinkage, condensation of the chromatin and collapse into patches, then into crescents in tight apposition to the nuclear envelope and, finally, into one or several dense spheres (Figure 6C).

To investigate the extent of DNA damage, we performed terminal deoxynucleotidyl transferase-mediated dUTP–digoxigenin nick end-labeling (TUNEL) followed by flow cytometry. Incubation of each clone in the absence of ATc induced a large increase in the number of cells with high fluorescence, indicative of increased free DNA ends and nuclear fragmentation (Figure 6D). These results confirm the data obtained with ethidium bromide stained gels and indicate that the proportion of cells displaying nuclear damage was as high as 60–70% following expression of either Zac1 or p53. Conclusively, Zac1 and p53 expression resulted in cell death with classical signs of apoptosis.

### *Expression of Zac1 and p53 induces changes in cell cycle distribution*

To test for cell cycle regulation by Zac1, propidium iodide stained SaOs-2 clones were analyzed by flow cytometry. Expression of Zac1 increased the number of cells in $G_0/G_1$ from 41 to 63% and decreased inversely the number of cells in S and $G_2$/M phase from 40 and 19% to 25 and 12% respectively (Figure 7A). In contrast, p53 expression reduced the number of cells in $G_0/G_1$ and S phase from 38 and 43% to 32 and 33% respectively, whereas the number of cells in $G_2$/M phase increased from 19 to 35% (Figure 7B), as has been previously reported in SaOs-2 cells (Roemer and Friedmann, 1993; Yamato *et al.*, 1995; Chen *et al.*, 1996; Yonish-Rouach *et al.*, 1996). Similar

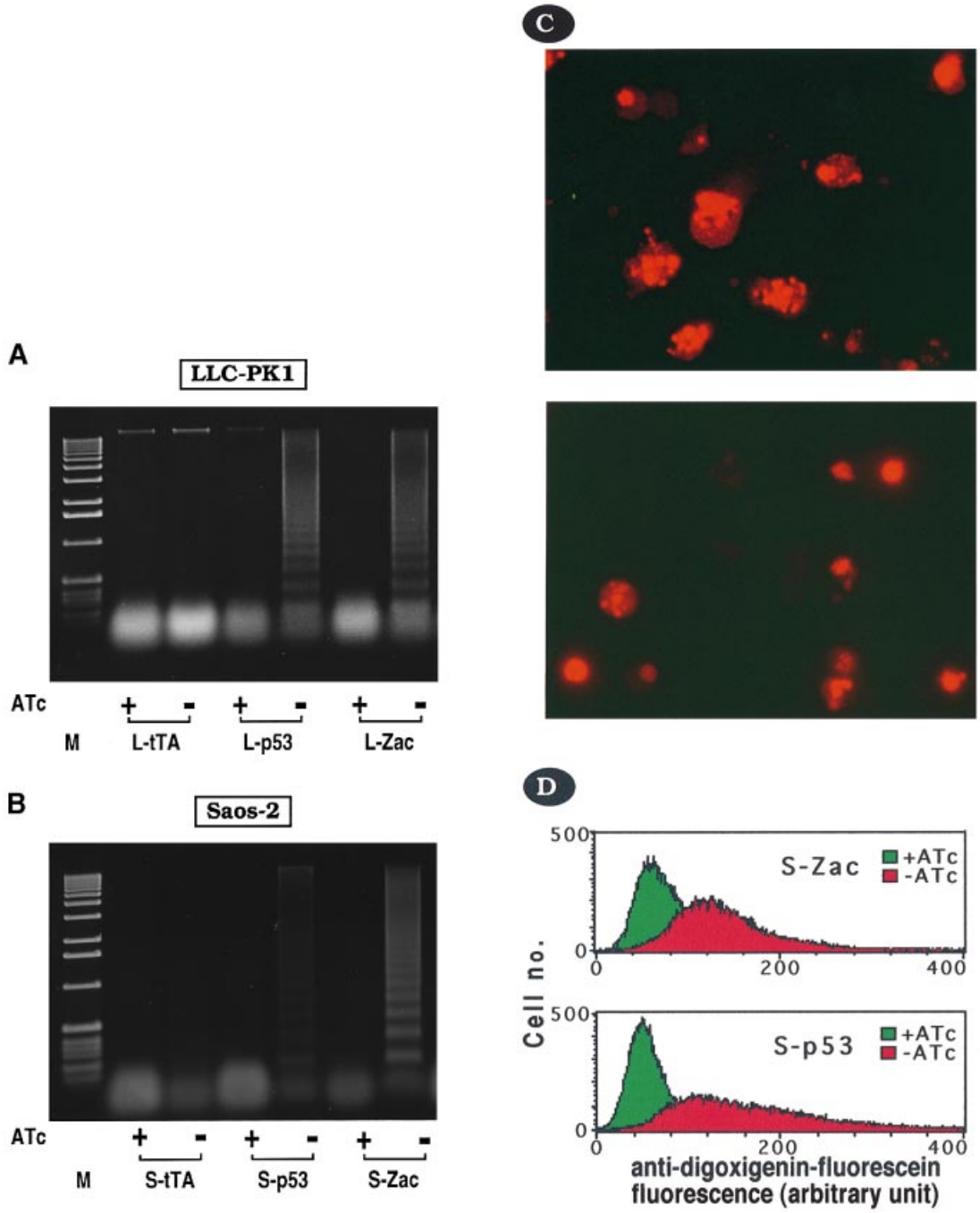
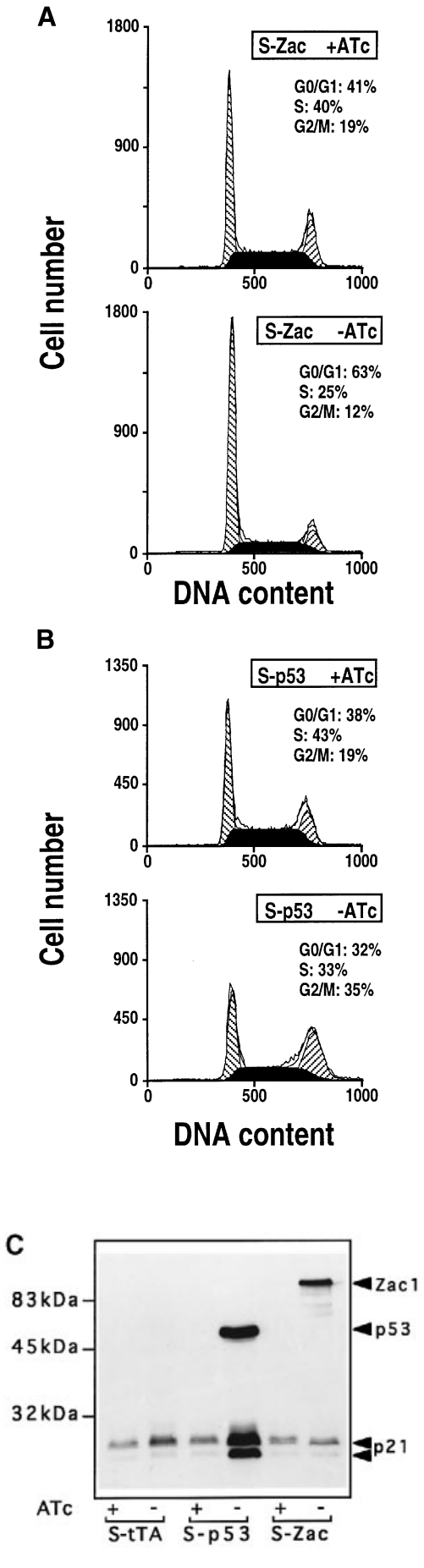



**Fig. 6.** Zac1 and p53 induce apoptotic cell death. (**A** and **B**) DNA laddering. Genomic DNA was isolated from Zac1- (L-Zac and S-Zac) and p53-expressing (L-p53 and S-p53) clones grown in the presence (+) or absence (–) of ATc for 3 days, centrifuged and soluble DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide. (**C**) Fluorescence microscopy of L-Zac1 and L-p53 displaying nuclear signs of apoptosis. Cells were grown in the absence of ATc for 3 days. Floating cells (L-Zac, upper panel; L-p53, lower panel) were collected, incubated with ethidium bromide and examined by fluorescence microscopy (510–550 nm, ×1000). (**D**) DNA end-labeling. S-Zac (Zac1) and S-p53 (p53) cells were grown for 3 days in the presence (green) or absence (red) of ATc. Permeabilized cells were subjected to terminal transferase nick end-labeling in the presence of digoxigenin-labeled dUTP (TUNEL). Cells were then incubated with fluorescein-conjugated anti-digoxigenin antiserum and subjected to flow cytometry.

results for Zac1 and p53 expression in the LLC-PK1 clones underscore that $G_1$ arrest due to Zac1 is a general property of this protein (data not shown). In support of this finding, from day 2 following induction of p53 expression, SaOs-2 cells flattened and greatly enlarged (3- to 8-fold) in average diameter, which was most evident when grown in small clusters. Similar changes, though less prominent (2- to 4-fold increase in average diameter), were also observed for L-p53 (data not shown). In contrast, Zac1-expressing LLC-PK1 or SaOs-2 clones appeared indistinguishable from the parent cell lines, supporting a functional difference between Zac1 and p53 in cell cycle regulation.

p53 induces cell cycle arrest through transactivation of




the gene encoding the cyclin-dependent kinase inhibitor p21 (also designated Cip1, WAF1, Sdi1 and Cap20) (El-Deiry *et al.*, 1993; Harper *et al.*, 1993). Increased levels of p21 inhibit the kinase activity of cdk2 and maintain retinoblastoma protein (Rb) in its underphosphorylated state in tight association with members of the E2F family. As a result, transactivation of genes driving the cell cycle is inhibited (Goodrich *et al.*, 1991; Weinberg, 1993). We tested whether Zac1-induced $G_1$ arrest utilizes the same molecular pathway as p53. Expression of p53 in SaOs-2 cells resulted in a strong induction of p21 protein, proving an intact and efficient transactivation of the endogenous gene by exogenous p53 protein (Figure 7C). Yet, no regulation of the *p21* gene in SaOs-2 cells was evident following expression of Zac1 (Figure 7C). We obtained the same results in the LLC-PK1 clones, with a strong induction of p21 solely by p53 (data not shown). In addition, Zac1 did not induce $p27^{Kip1}$, $p57^{Kip2}$ or $p16^{INK4a}$ expression (data not shown). Since SaOs-2 cells are devoid of the retinoblastoma gene (*Rb*), the $G_1$ block induced by Zac1 is independent of Rb.

Moreover, we showed that Zac1-induced growth arrest was even more pronounced, with 83% of the cell population in $G_0/G_1$, when TUNEL-negative cells were analyzed separately from TUNEL-positive ones (Figure 8). Importantly, the distribution of apoptotic SaOs-2 cells following Zac1 expression in the different phases of the cell cycle was indistinguishable from that observed in the repressed state (Figure 8). We concluded that cell cycle arrest is not a prerequisite for apoptosis and that Zac1 regulates these two pathways separately.

## Discussion

The *p53* gene has a unique role in regulating cell proliferation, since it is the only gene identified so far which concurrently controls genetic programs inducing apoptosis and cell cycle arrest *in vitro* and *in vivo* (for reviews see Bates and Vousden, 1996; Ko and Prives, 1996). In contrast, other proteins able to control cell cycle arrest, i.e. Rb or the cyclin-dependent kinase inhibitors, are devoid of the ability to induce apoptosis. On the other hand, regulators of programmed cell death such as members of the ICE/Ced-3 or bcl-2/bax families lack the ability to control cell cycle progression (Korsmeyer, 1995). Zac1 and p53 were isolated in our expression system by their common ability to induce expression of the $PACAP_1$-R gene. In addition, we unexpectedly observed that Zac1 shares with p53 the ability to inhibit growth of tumor cells by controlling apoptosis and cell cycle progression. Thus, the identification of *Zac1* provides the first example, besides *p53*, of a gene which combines concomitant induction of programmed cell death and cell cycle arrest

**Fig. 7.** Zac1 and p53 regulate cell cycle progression. (**A**) Induction of $G_1$ arrest by Zac1. S-Zac cells were grown in the presence (upper panel) or absence (lower panel) of ATc for 3 days. Propidium iodide stained cells were analyzed by flow cytometry to determine DNA content. (**B**) Induction of $G_2$/M arrest by p53. S-p53 cells were grown and analyzed as in (A). (**C**) $G_1$ arrest by Zac1 is independent of $p21^{WAF1/Cip1}$ expression. S-tTA, S-p53 and S-Zac cells were grown in the presence (+) or absence (–) of ATc for 3 days. Western blots of total cell lysates were simultaneously performed with anti-p21, anti-p53 and anti-GST–ZacΔZF antisera.

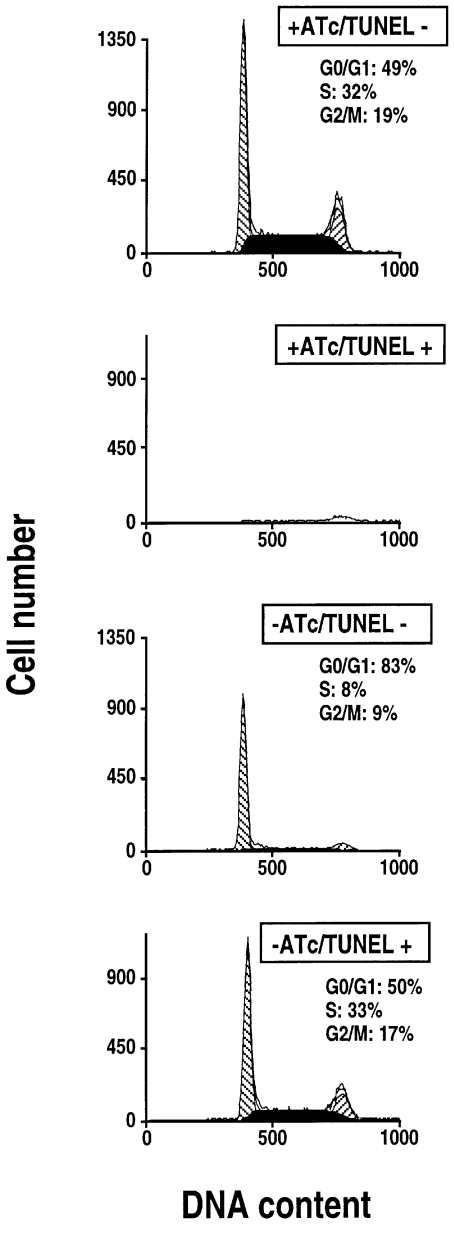



**Fig. 8.** Zac1-induced apoptosis is unrelated to cell cycle. S-Zac cells grown with or without ATc were simultaneously stained with propidium iodide and by the TUNEL technique. Cell cycle analysis was performed separately on cells unlabeled (TUNEL–) or labeled (TUNEL+) by the TUNEL procedure.

*in vitro*. We demonstrated that Zac1 recruits these programs through mechanisms at least in part unrelated to those activated by p53.

Proliferation assessment revealed that Zac1 and p53 decreased colony formation and cell number under constitutive and regulated expression in both LLC-PK1 and SaOs-2 cells. In the colony formation assay, transfection of *Zac1* and *p53* leads to a series of random integrations into the host genome, presumably resulting in large clone to clone variations in expression levels of each protein. In view of this, the comparable reductions in the number of colonies observed under Zac1 or p53 expression indicate equivalent growth inhibiting activity for both proteins. Accordingly, pools of clones transfected with Tc-sensitive expression vectors encoding Zac1 or p53 revealed similar differences in proliferation rate. Conclusively, Zac1 was as efficient as p53 at inhibiting tumor growth *in vitro*. Importantly again, this property of Zac1 was preserved in anchorage-independent growth, a hallmark of cell transformation, and for suppression of tumor formation in nude mice. Finally, the activities of Zac1 and p53 were independent of serum factors, indicating that down-regulation of mitogenic pathways is not the underlying mechanism of growth inhibition in the investigated cellular models. In contrast, inhibition of cellular proliferation and induction of apoptosis by expression of the Wilm's tumor suppressor gene *in vitro* have been associated with suppression of the insulin-like growth factor I receptor gene and of the epidermal growth factor receptor (Englert *et al.*, 1995; Werner *et al.*, 1995).

We were unable to establish Zac1- or p53-expressing clones from the AtT-20 cell line under control of the Tc-dependent expression system in five independent experiments with both cDNAs. Apart from the low transfection efficiency of this cell line (<1%), low amounts of exogenous Zac1 or p53 protein and selection for recombinants with puromycin interfered with colony formation. Since Zac1 and p53 were isolated in their wild-type form from this cell line, any additional amount of Zac1 or p53 appeared to impair cell viability. Conversely, the effectiveness of Zac1 at reducing growth of tumor cells devoid of endogenous Zac1, such as LLC-PK1 and SaOs-2 cells, not only supports a role in growth control but suggests that Zac1 employs mechanisms ubiquitously expressed and of general importance in growth control. In view of this, we identified induction of apoptosis and $G_1$ arrest as programs recruited by Zac1. The extent of cell death following p53 expression in SaOs-2 cells is in agreement with previous studies, which noted few apoptotic cells in this cell line (Diller *et al.*, 1990; Roemer and Friedmann, 1993; Yamato *et al.*, 1995; Yonish-Rouach *et al.*, 1996). In contrast, the number of cells displaying nuclear damage was comparably high (60–70%) for both S-Zac and S-p53 cell populations, as demonstrated by DNA end-labeling experiments. However, this parameter reflects only in part commitment to apoptosis, supporting the idea that additional parameters influence the decision to irreversibly enter this pathway.

In agreement with recent reports (Roemer and Friedmann, 1993; Yamato *et al.*, 1995; Chen *et al.*, 1996; Yonish-Rouach *et al.*, 1996), we observed a $G_2$/M block in SaOs-2 and notably also in LLC-PK1 cells following expression of p53. Whereas the status of Rb in LLC-PK1 cells is unknown, SaOs-2 cells lack functional Rb, which could explain the failure of p53 to arrest cells in $G_1$ despite efficient transactivation of p21. In contrast, the $G_1$ block induced by Zac1 occurred independently of a functional Rb protein and involved no induction of $p21^{WAF1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ or $p16^{Ink4}$. Thus, Zac1 might induce the expression of a yet unknown cyclin-dependent kinase inhibitor or otherwise modulate the activity or the expression of cyclins, cyclin-dependent kinases or their inhibitors. Irrespective of the actual mechanisms underlying cell cycle control by Zac1, the $G_1$ arrest was even more pronounced when only viable cells were analyzed. In line with this finding, apoptotic cells arose evenly from each phase of the cell cycle under Zac1 expression in SaOs-2 cells, emphasizing that cell cycle arrest in a

particular phase did not increase susceptibility to or protection against apoptotic cell death. Therefore, regulation of cell cycle progression and apoptotic cell death appear to be separate functions of Zac1.

At present the function of Zac1 *in vivo* can be only tentatively assigned. Proteins controlling cell cycle arrest and apoptosis have emerged during the last years as major players that coordinate cell cycle regulation during central nervous system formation and neuronal differentiation (Heintz, 1993; Raff *et al.*, 1993; Ross, 1996). The low expression of Zac1 in restricted regions of the brain of adult mice under resting conditions reflects expression in a terminally differentiated tissue with presumably low demands for growth control. Yet, due to its dual role, Zac1 could retain a critical function in controlling cell viability and in elimination of damaged cells, as has recently been reported for p53 (Sakhi *et al.*, 1994; Eizenberg *et al.*, 1995, 1996; Wood and Youle, 1995; Morrison *et al.*, 1996). In the light of the neurotrophic functions of PACAP (Pincus *et al.*, 1990; Deutsch and Sun, 1992; DiCicco-Bloom and Deutsch, 1992; Villalba *et al.*, 1997), transactivation of the $PACAP_1$-R gene by Zac1 and p53 is intriguing and points to a subtle balance between death promoting and protective mechanisms.

Concurrent expression of p53 and Zac1 *in vivo* might provide superior protection against neoplastic transformation and tumor progression. Further studies on loss of Zac1 function in tumors derived from the pituitary gland, the brain or other tissues are currently under way to substantiate the role of Zac1 in growth control and as a candidate tumor suppressor gene. In addition, a $Zac1^{-/-}$ mouse will be a valuable tool to elucidate the nature of an eventual alliance between Zac1 and p53 in protection against tumor formation. In this context, the pituitary gland, which expresses high levels of Zac1 mRNA, is an attractive candidate since, despite the high incidence of pituitary adenomas (Kovacs and Horvath, 1987; Saeger, 1995), these tumors are mostly benign (Melmed, 1993). Finally, the identification of Zac1, able to concomitantly regulate cell cycle arrest and apoptosis, appears of great potential interest for the treatment of neoplasia by gene transfer experiments to improve responsiveness (Roth *et al.*, 1996) using Zac1 on its own or in tissues refractory to p53.

## Materials and methods

### *Expression cloning and isolation of wild-type Zac1*

The expression cloning technique used to isolate p2195 and p1270 has been previously described (Spengler *et al.*, 1993). To isolate a *Zac1* wild-type cDNA, poly(A)$^+$ RNA was isolated from whole pituitary tissue obtained from 80 male BALB/c mice (BALB/cAnNCrlBR) and processed as described (Spengler *et al.*, 1993), except that sizing was carried out on a Chromaspin 1000 column (Clontech). Filters containing ~$5\times10^4$ colonies were screened with a random-primed probe derived from p2195 and one full-length clone B-16 was isolated by successive rounds of subdivision. Sequencing was carried out by subcloning restriction fragments into pBluescript using T3, T7 and internal primers. Coding regions of cDNA clones p2195, B-16 and p7150 (rat *p53*) were sequenced on both strands, p1270 and mouse *p53* on one strand. Exon–intron junctions in clones p1270 and B-16 were verified on both strands.

### *Cell culture, transfections and constructs*

The porcine epithelial cell line LLC-PK1 (ATCC CL 101) and the human osteosarcoma cell line SaOs-2 (ATCC HTB 85) were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal calf serum (FCS; Life Technologies).

*pCMVPUR sense/antisense constructs.* pCMVPUR sense/antisense constructs (1.0 μg) and pGEM4 filling DNA (3.0 μg) were transfected into $2\times10^6$ cells. pGEM4 replaced pCMVPUR in mock-transfected cells. Three electroporations for each construct were pooled and aliquots were plated in 15 cm culture dishes. Selection with puromycin (5.0 μg/ml) was started 24 h after transfection.

*tTA-regulated expression system.* Details of the modified tTA-regulated expression vector with lower basal activity are described elsewhere (Hoffmann *et al.*, 1997). Selection of tTA cell clones started 24 h after transfection using 700 and 500 μg/ml hygromycin (Merck) for LLC-PK1 and SaOs-2 cells respectively. Details on the regulatory properties of the parent L-tTA and S-tTA cell clones will be described elsewhere (A.Hoffmann, M.Villalba, L.Journot and D.Spengler, submitted). Selection of clones expressing Zac1 or p53 from the modified expression vector was carried out at 5.0 μg/ml puromycin. The following numbers of clones were screened: L-tTA Zac1, 95; L-tTA p53, 92; S-tTA Zac1, 77; S-tTA p53, 72. All clones revealed impaired cell growth to varying degrees under the activated state (–ATc), which was microscopically scored twice over 7 days. Primary pools were obtained from trypsinization of plates harboring ~100 individual colonies after 1 week of selection and measurement of cell counts was carried out as described in the following section.

### *Measurement of cell number, BrdU incorporation and MTT conversion*

Equal numbers of cells (5000) were seeded in 24-well plates in DMEM, 10% FCS supplemented with ATc ($10^{-11}$ mg/ml). After recovery for 36 h, medium was renewed and the repressor omitted for half of the samples. For samples lacking the repressor, the medium was changed again 3 h later to remove residual amounts of ATc. Growth medium was changed routinely on day 3. For measurement of DNA synthesis, cells (1000) were seeded in 48-well plates and cultured as outlined above. On each of 6 days, 10 mM BrdU was added for 8 h and subsequent steps were carried out according to the manufacturer's instructions (Boehringer Mannheim). For measurement of cell viability, 1000 cells were seeded in 24-well plates and cultured as described above. The average of OD measurements for DNA synthesis and cell viability was obtained from three independent experiments performed in triplicate. To test serum independence, cells were kept in normal medium for 36 h before serum was washed out once with DMEM and replaced by DMEM, 0.1% FCS ± ATc.

### *Soft agar assay*

Each well (35 mm) of a 6-well culture dish was coated with 4 ml bottom agar mixture (DMEM, 10% FCS, 0.6% agar ± ATc). After the bottom layer had solidified, 2 ml top agar mixture (DMEM, 10% FCS, 0.3% agar ± ATc) containing the cells was added. ATc was used at a final concentration of $3\times10^{-11}$ mg/ml. After 7 days, another 1.5 ml top agar mixture (± ATc) was added. On day 10, the wells were overlaid with 2 ml MTT (1 mg/ml) and incubated for an additional 4 h, washed once with phosphate-buffered saline (PBS) and then photographed.

### *Nude mice experiment*

Thirty six nude mice were randomly distributed into three groups of twelve animals. In each group, half of the animals were s.c. implanted with Tc pellets (63 mg, 0.7 mg/day tetracycline hydrochloride; Innovative Research of America) and the remaining half were implanted with placebo pellets (Innovative Research of America). Cells were grown in the presence of Tc, trypsinized and resuspended in PBS at a density of $5\times10^7$ cells/ml. Aliquots of 100 μl of this cell suspension were injected s.c. into each side of each animal. Two groups were injected with S-Zac cells from two independent trypsinizations, whereas one experiment was performed with S-p53 cells. Tumor formation was monitored three times a week for up to 17 weeks. S-Zac- and S-p53-injected animals were sacrificed at 11 and 16 weeks respectively, dissected and the tumors were weighed.

### *Measurement of apoptosis*

*DNA laddering.* Cells were seeded with (4000 cells/cm$^2$) or without (8000 cells/cm$^2$) ATc for 3 days and soluble DNA was prepared as described (Hockenbery *et al.*, 1990). Aliquots of DNA were fractionated on a 1.2% agarose gel.

*Fluorescence microscopy.* Cells ($5\times10^4$) were seeded in the absence of ATc in 12-well clusters and grown for 3 days. Floating cells were

collected, washed twice 01th PBS and stained with 50 µg/ml ethidium bromide and 10 mg/ml acridine orange for 10–20 min. Photography was carried out using UV filters of 510–550 nm and 1000-fold magnification. *TUNEL*. Terminal transferase end-labeling was performed with the ApopTag kit (Oncor), according to the manufacturer's instructions.

### *Flow cytometry*

Cells were seeded with (1800 cells/cm$^2$) or without (3600 cells/cm$^2$) ATc for 3 days. For propidium iodide staining, cells were pelleted, kept on ice for at least 10 min and resuspended in 900 µl propidium iodide staining solution (PISS, 50 µg/ml propidium iodide, 0.1% trisodium citrate dihydrate, 0.1 mg/ml RNase A, 0.1% Triton X-100). Following an overnight incubation, cell cycle phase distribution was determined with a FACScan flow cytometer (Becton Dickinson). A total of 20 000 events were analyzed using Modfit software (Verity Software House Inc.).

### *Immunological techniques*

The plasmid pRK8-p2195 was partially digested with *Bst*XI, blunted with T4 DNA polymerase and digested with *Not*I. The resulting 1.67 kb fragment was subcloned into pGEX-5X-3 (Pharmacia) previously digested with *Sma*I and *Not*I. The resulting plasmid encoded a GST–ZacΔZF fusion protein lacking the zinc finger domain of Zac1. The fusion protein was purified by affinity chromatography using glutathione–Sepharose beads followed by SDS–PAGE and electroelution. Rabbits were immunized with 40 µg fusion protein and antiserum was collected on a weekly basis. Purified IgGs were used for the Western blots and immunocytochemistry experiments. Western blots were performed on total cell lysates (50 µg) with the above mentioned purified IgG or with commercially available antibodies to p53 (Pharmingen, San Diego, CA; No. 14091A), p21$^{Waf1}$ (Transduction Laboratories, Lexington, KY; No. C24420), p27$^{Kip1}$ (Transduction Laboratories; no. K25020) and p16$^{Ink4}$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA; No. sc-759).

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