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p19^{ARF} Is Dispensable for Oncogenic Stress-Induced p53-Mediated Apoptosis and Tumor Suppression In Vivo

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Recent studies have shown the p19^{ARF} tumor suppressor to be involved in the response to oncogenic stress by regulating the activity of p53. This response is mediated by antagonizing the function of Mdm2, a negative regulator of p53, indicating a pathway for tumor suppression that involves numerous genes altered in human tumors. We previously described a transgenic mouse brain tumor model in which oncogenic stress, provided by cell-specific inactivation of the pRb pathway, triggers a p53-dependent apoptotic response. This response suppresses the growth of developing tumors and thus represents a bona fide in vivo tumor suppressor activity. We further showed that E2F1, a transcription factor known to induce p19^{ARF} expression, was required for the response. Here, we use a genetic approach to test whether p19^{ARF} functions to transduce the signal from E2F1 to p53 in this tumor suppression pathway. Contrary to the currently accepted hypothesis, we show that a deficiency in p19^{ARF} has no impact on p53-mediated apoptosis or tumor suppression in this system. All measures of p53 function, including the level of apoptosis induced by pRb inactivation, the expression of p21 (a p53-responsive gene), and the rate of tumor growth, were comparable in mice with and without a functional *p19^{ARF}* gene. Thus, although p19^{ARF} is required in some cell types to transmit an oncogenic response signal to p53, it is dispensable for this function in an in vivo epithelial system. These results underscore the complexity of p53 tumor suppression and further indicate the existence of distinct cell-specific pathways that respond to similar stimuli.

p19^{ARF} was first identified as the product of an alternative transcript within the *p16^{INK4a}* gene (20). Deletions of the *INK4a/ARF* locus have been found in a variety of human cancers (21), and mice nullizygous at the *INK4a/ARF* locus develop tumors with a high frequency, usually lymphoma and sarcoma (24). Importantly, mice that specifically lack p19^{ARF} but retain p16^{INK4a} develop a similar spectrum of tumors (11), establishing the tumor suppressor function of p19^{ARF}. Recent studies in fibroblasts in vitro (2, 3, 8, 16, 35) and in B cells in vivo (4, 23) suggest a role for p19^{ARF} in p53 responses to oncogenic stress. For example, overexpression of c-Myc, E2F1, activated Ras, or E1A in primary mouse embryonic fibroblasts (MEF) induces p53-dependent growth arrest or apoptosis, and these effects are attenuated in *p19^{ARF}* null cells. Furthermore, p53-deficient cells are resistant to p19^{ARF}-induced cell cycle arrest (11), indicating that p19^{ARF} acts upstream of p53. Biochemical analyses show that p19^{ARF} can bind to Mdm2 and block Mdm2-induced p53 degradation, thus providing a molecular mechanism by which p19^{ARF} can activate p53 (10, 19, 26, 32, 33).

These studies suggest a model in which p19^{ARF} acts as a tumor suppressor by responding to oncogenic signals, possibly via direct transcriptional activation by E2F transcription factors (2, 35) and by transmitting the signal to p53 via Mdm2 regulation. Indeed, the development of B-cell lymphoma in Eμ-*myc* transgenic mice is significantly accelerated in *Ink4a-ARF$^{+/-}$* (8, 23) and *ARF$^{+/-}$* (4) backgrounds. Since a similar effect occurs in *p53$^{+/-}$* mice (7, 23), these observations support the hypothesis that p53-mediated tumor suppression in this model is dependent on p19^{ARF}. However, it is not known whether the same mechanism is broadly operative in diverse cell types susceptible to p53 tumor suppression.

Here, we address the role of p19^{ARF} in p53-mediated epithelial cell apoptosis and tumor suppression in vivo. Previously, we developed a transgenic mouse model (*TgT_{121}*) in which epithelial brain tumors are initiated by cell-specific expression of T_{121}, an oncoprotein derived from simian virus 40 (SV40) T antigen that specifically inactivates pRb and related proteins p107 and p130 (22, 27). Inactivation of these proteins in choroid plexus (CP) epithelial cells induces aberrant proliferation and p53-dependent apoptosis, resulting in the development of slow-growing tumors (Fig. 1A). In a *p53* null background, the T_{121}-induced apoptosis is significantly reduced, and tumor growth is accelerated sevenfold (Fig. 1A) (27). Thus, the development of tumors in this model serves as a paradigm for oncogenic stress-induced p53-mediated apoptosis and tumor suppression in epithelial cells in vivo.

Using a genetic approach, we further showed that the p53-mediated apoptosis in CP is dependent on E2F1 (Fig. 1B) (18). Hence, based on the observations described above, p19^{ARF} was a likely candidate for transmitting the signal from E2F1 to p53 (Fig. 1B). Here, we test this hypothesis by assessing the effect of p19^{ARF} deficiency on T_{121}-induced p53-dependent apoptosis and tumor suppression in vivo.

MATERIALS AND METHODS

Generation of p19^{ARF}-deficient *TgT_{121}* mice. Characterization of *TgT_{121}* transgenic mice (C57BL6/J;DBA2) was described previously (22, 27). Mice harboring

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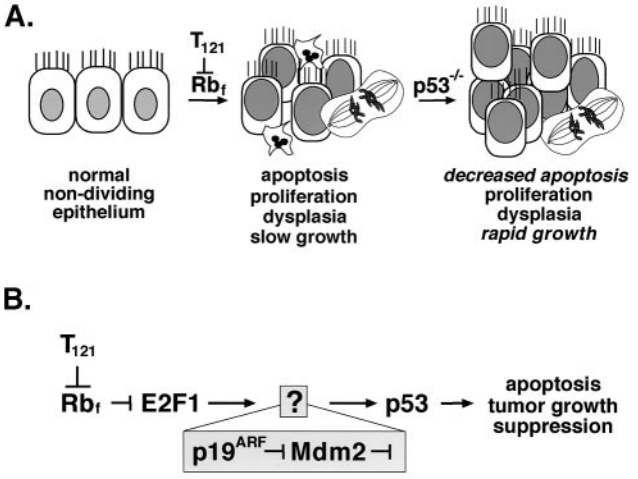

FIG. 1. p53 tumor suppression in brain epithelium. The diagram depicts previously elucidated steps in the development of choroid plexus tumors in TgT_{121} mice. (A) Cell-specific expression of the T_{121} transgene induces proliferation of normally nondividing epithelial cells by inactivating the pRb family proteins pRb, p107, and p130. p53-dependent apoptosis is then activated, as evidenced by the 85% reduction in apoptosis in TgT_{121};$p53^{-/-}$ mice. (B) Using a genetic approach, we previously showed that E2F1 acts upstream of p53 to induce apoptosis in response to T_{121}. Previously, E2F1 was shown to activate $p19^{ARF}$ transcription, and $p19^{ARF}$ was shown to induce p53 activities by regulating Mdm2 in cultured cells (see the introduction). In the current report, we tested whether this pathway is operative to induce p53-dependent apoptosis and suppression of brain epithelial tumors in vivo.

a homozygous deletion of $p19^{ARF}$ exon 1 (C57BL6/J; Sv129) (11) were kindly provided by C. J. Sherr and M. F. Roussel (St. Jude Children's Hospital). TgT_{121};$p19^{ARF+/-}$ and TgT_{121};$p19^{ARF-/-}$ mice were generated by crossing hemizygous TgT_{121} mice with $p19^{ARF-/-}$ mice through two generations. In all experiments, TgT_{121};$p19^{ARF+/-}$ and TgT_{121};$p19^{ARF-/-}$ littermates were compared to control for any variability in the genetic background. TgT_{121};$p19^{ARF+/+}$ mice were generated in a single cross between TgT_{121} mice and $p19^{ARF+/-}$ mice. TgT_{121} and $p19^{ARF}$ genotypes were identified by PCR analysis of tail DNA (11, 27).

Histology, proliferation, and apoptosis assays. Brain tissues were fixed, embedded, and sectioned as described (18). Apoptotic cells were detected in sections using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (27). Cell proliferation was determined by in situ immunodetection of bromodeoxyuridine (BrdU) incorporation as described (18). The significance of differences in apoptosis levels and cell proliferation levels between mice with different genotypes was evaluated by *t* test. $P < 0.05$ was considered significant.

In situ RNA hybridization. Sections were treated and hybridized as previously described (18). The p21 antisense probe was generated by T7 transcription of an *Eco*RI-linearized pBS-KSp21 template. The $p19^{ARF}$ antisense probe was generated by T3 transcription of a *Bam*HI-linearized pBS-KS$p19^{ARF}$ (a kind gift of Yue Xiong). Probes were labeled with [α-^{35}S]UTP (5×10^4 cpm/μl) and hybridized to slides at 50°C overnight. For the $p19^{ARF}$-specific probe, slides were exposed for 60 days; for the p21-specific probe, slides were exposed for 3 days. The sense probes did not show any signal above background in both p21 and $p19^{ARF}$ in situ hybridizations.

RESULTS

$p19^{ARF}$ expression is induced in T_{121}-expressing cells. Since oncogenic stress in cultured cells results in transcriptional induction of $p19^{ARF}$ (2, 3, 16, 35), we first determined whether $p19^{ARF}$ transcripts were induced upon T_{121} expression coincident with p53 activation in CP. In situ RNA hybridization with a mouse $p19^{ARF}$-specific probe detected a clearly positive signal in CP of TgT_{121} mice (Fig. 2C), whereas no signal above background was detected in normal nontransgenic CP (Fig. 2B). As predicted from previous studies, the $p19^{ARF}$ signal was not dependent on p53, based on the positive signal in TgT_{121};$p53^{-/-}$ CP (Fig. 2D). Although $p19^{ARF}$ transcripts were clearly induced in CP by T_{121}, levels were quite low, based on the requirement for an exposure time of 8 weeks. By comparison, p21 transcripts induced by the activation of p53 were detected in 3 days using similar procedures (18) (see Fig. 5). Nonetheless, induction of $p19^{ARF}$ transcripts specifically by T_{121} expression supported the possibility that $p19^{ARF}$ transmits a signal to p53.

$p19^{ARF}$ is not required for p53-dependent apoptosis. To test whether $p19^{ARF}$ is required for the induction of p53-dependent apoptosis by pRb protein inactivation, we generated TgT_{121} mice deficient in $p19^{ARF}$ by a series of backcrosses with $p19^{ARF-/-}$ mice (11). Apoptosis within the CP of several young (4 and 8 weeks) TgT_{121};$p19^{ARF+/+}$, TgT_{121};$p19^{ARF+/-}$, TgT_{121};$p19^{ARF-/-}$, and $p19^{ARF-/-}$ mice was measured in situ using the TUNEL assay (Fig. 3A). TgT_{121};$p53^{-/-}$ mouse brain sections were assessed as a control. Although p53 deficiency dramatically reduced the level of apoptosis, $p19^{ARF}$ deficiency had little or no effect (Fig. 3A and B). The average apoptosis level in the CP of TgT_{121};$p19^{ARF+/+}$ mice was 8.6% ± 1.9% ($n = 6$). The average relative apoptosis indices (AI) of TgT_{121};$p19^{ARF-/-}$ and TgT_{121};$p19^{ARF+/-}$ CP compared with that of TgT_{121};$p19^{ARF+/+}$ CP (100%) were 92.9% ± 19.2% and 113.0% ± 23.1%, respectively. The small difference between the TgT_{121};$p19^{ARF-/-}$ group ($n = 6$) and the TgT_{121};$p19^{ARF+/+}$ group ($n = 6$) is not statistically significant ($P = 0.601$). Moreover, as shown previously (27), the relative AI of TgT_{121};$p53^{-/-}$ CP ($n = 3$) was reduced to 12.4% ± 8.5%. Thus, contrary to the hypothesis, these data show that p53-dependent apoptosis in response to aberrant proliferation of epithelial cells in vivo does not require $p19^{ARF}$.

$p19^{ARF}$ deficiency does not interfere with tumor cell cycle. Recent studies have shown that $p19^{ARF}$ could inhibit cell growth by a p53-independent pathway (29). Thus, although $p19^{ARF}$ was clearly dispensable for the p53-dependent apoptosis of CP tumor cells, it was possible that $p19^{ARF}$ affected the tumor cell cycle by unknown p53-independent mechanisms. To determine whether this was the case, the fraction of proliferating tumor cells was determined in the presence and absence of $p19^{ARF}$ by in situ immunodetection of BrdU incorporation. TgT_{121};$p19^{ARF+/+}$, TgT_{121};$p19^{ARF+/-}$, TgT_{121};$p19^{ARF-/-}$, and $p19^{ARF-/-}$ mice of different ages (4 and 8 weeks) were examined. This analysis indicated that $p19^{ARF}$ deficiency did not affect the proliferation of CP tumor cells. The average percentage of BrdU-positive cells in the CP of TgT_{121};$p19^{ARF+/+}$ mice was 8.5% ± 2.2% ($n = 6$). The relative BrdU staining levels within the CP of TgT_{121};$p19^{ARF-/-}$ and TgT_{121};$p19^{ARF+/-}$ mice were 86.5% ± 21.2% and 107.5% ± 37.6%, respectively ($n = 6$ in both cases) (Fig. 3C). Thus, no significant effect of $p19^{ARF}$ on tumor cell proliferation was detected ($P = 0.288$).

$p19^{ARF}$ deficiency does not accelerate CP tumor growth. In previous studies of TgT_{121} mice, we showed that tumor growth was slow, becoming life-threatening only after a mean age of 26 weeks (22). Deficiency of p53 caused acceleration of tumor

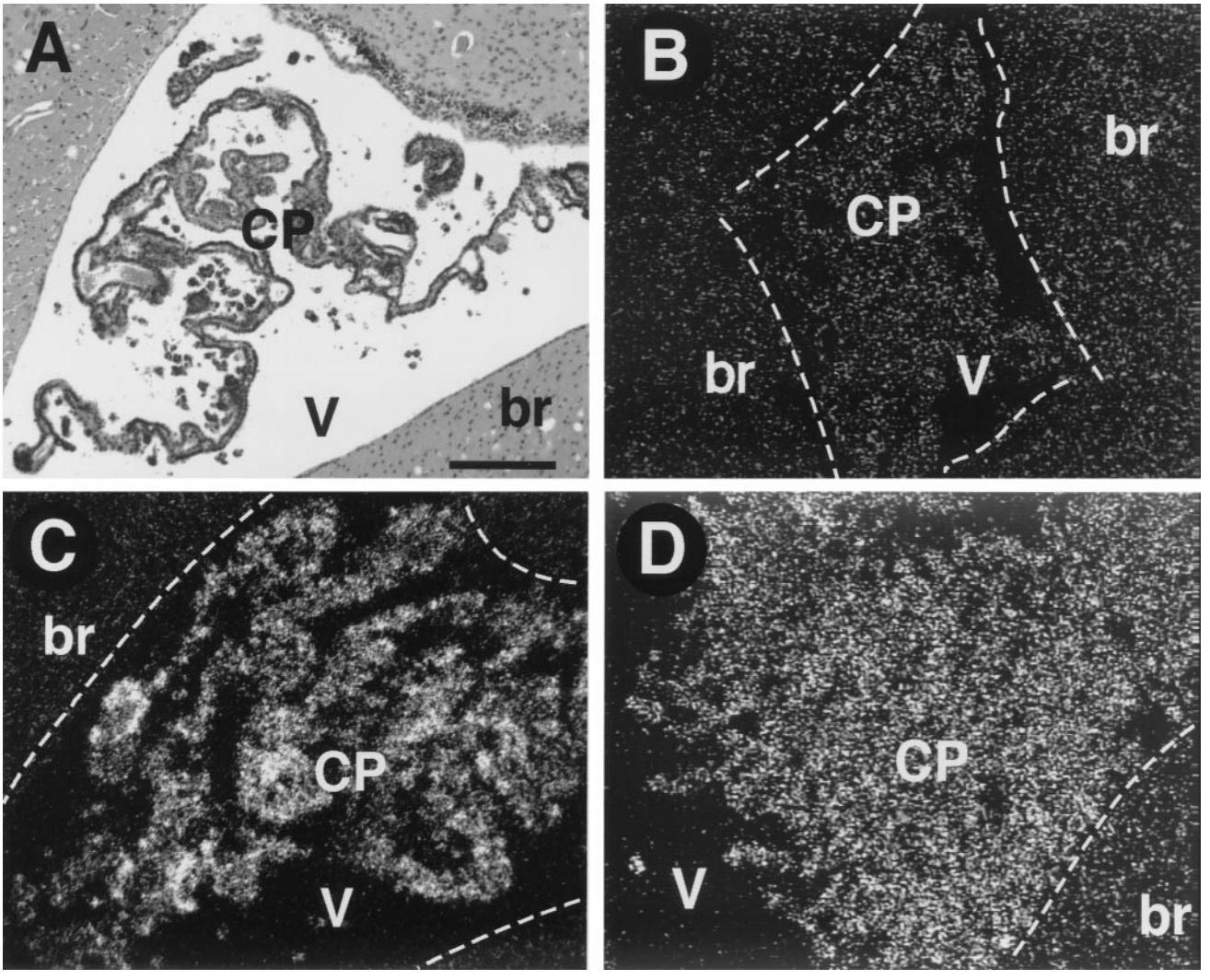

FIG. 2. $p19^{ARF}$ transcripts are induced in aberrantly proliferating CP epithelium. RNA in situ hybridization was performed with an antisense $p19^{ARF}$ RNA probe. (A) Hematoxylin and eosin (H & E)-stained section from the same brain as in panel C shows the morphology of CP in the lateral ventricle and is in an orientation similar to that of dark-field panels B to D. The borders between brain parenchyma (br) and the ventricles (V) are depicted with white dashed lines in panels B to D. No $p19^{ARF}$ expression above background is detected in the CP of nontransgenic mice (B). (Compare the signal in brain with that in CP.) $p19^{ARF}$ transcripts are induced in TgT_{121} CP (C). T_{121}-induced $p19^{ARF}$ expression is p53 independent; the level of $p19^{ARF}$ transcripts is similar in TgT_{121};$p53^{-/-}$ CP (D) and TgT_{121} CP (C). (A) Bar, 200 μm (magnification is the same in all panels). Sense probe hybridization showed no signal above background (not shown).

growth due to reduced apoptosis, and the animals died by 4 weeks of age (27). Given that neither apoptosis nor proliferation appeared to be altered by $p19^{ARF}$ deficiency, tumor growth was predicted to be unaltered as well. However, it was possible that additional unexpected parameters dependent on $p19^{ARF}$ could impact either the rate or morphological characteristics of tumor growth. Because TgT_{121};$p19^{ARF-/-}$ and TgT_{121};$p19^{ARF+/-}$ mice simultaneously develop multiple tumor types, including CP tumors, sarcoma, and lymphoid malignancies (summarized in Table 1), survival does not provide an appropriate assessment of CP tumor growth. However, unlike TgT_{121};$p53^{-/-}$ mice, all TgT_{121};$p19^{ARF-/-}$ mice survived beyond 4 weeks of age to a mean age of 17 weeks. This result confirms that $p19^{ARF}$ deficiency does not induce a phenocopy of p53 deficiency.

Further assessment of CP tumor histopathology showed no detectable differences between TgT_{121};$p19^{ARF-/-}$ and TgT_{121};$p19^{ARF+/+}$ CP tumors (for example, compare panels A and B of Fig. 4). Although survival time is clearly affected by the combination of TgT_{121} and altered $p19^{ARF}$ alleles (Table 1), evidence indicates that this effect results from exacerbation by coexisting malignancies. For example, in mice with compound genotypes, the simultaneous presence of leukemia (induced by $p19^{ARF}$ deficiency) and CP carcinoma (caused by T_{121} expression) resulted in extensive infiltration of leukemia cells into the brain (Fig. 4C), a phenotype not observed in either individual background. It is likely that such effects caused the mice to die at earlier ages (Table 1). In summary, evaluation of CP tumor growth indicates that $p19^{ARF}$ is not required for suppression of tumorigenesis in brain epithelium.

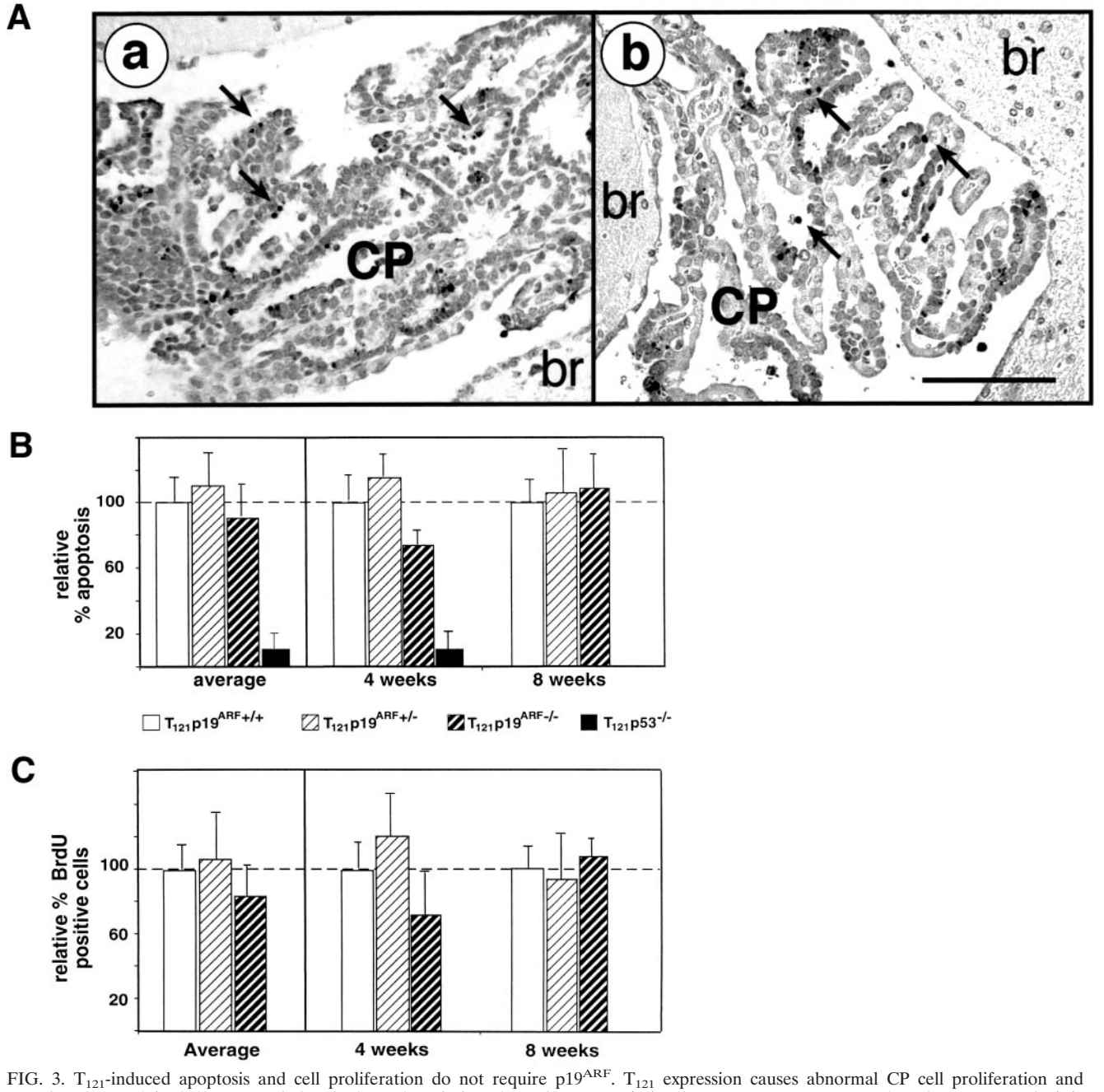

FIG. 3. T_{121}-induced apoptosis and cell proliferation do not require $p19^{ARF}$. T_{121} expression causes abnormal CP cell proliferation and apoptosis. About 85% of the apoptosis is p53-dependent (27) and requires E2F1 (18). The TUNEL assay was used to detect apoptosis (A). Apoptosis levels and morphology were indistinguishable between $TgT_{121};p19^{ARF-/-}$ (a) and $TgT_{121};p19^{ARF+/+}$ (b) CP. Apoptotic nuclei are stained and appear black; representative apoptotic cells are indicated by arrows. The bar (b) is equal to 100 μm; both panels are of the same magnification. Quantitative analysis of apoptosis (B) and proliferation (C) in the CP of three mice each at 4 and 8 weeks of age was carried out. Levels are compared to that of $TgT_{121};p19^{ARF+/+}$ tissue, which is considered to represent 100%. Average relative values for all mice of each age and genotype are shown in the left panels. For these data, error bars indicate the variation among mice. In the right panels, data for each mouse are presented, with error bars indicating the field-to-field variation in counts taken from 10 fields per brain. Different mice were used for apoptosis and proliferation assays so as to avoid any impact of BrdU incorporation on apoptosis levels. There is no significant difference in apoptotic indexes between $TgT_{121};p19^{ARF+/+}$ and $TgT_{121};p19^{ARF-/-}$ mice ($P = 0.601$). Importantly, the index of $TgT_{121};p19^{ARF-/-}$ CP was significantly higher than that of $TgT_{121};p53^{-/-}$ CP ($P < 0.05$). The level of CP cell proliferation was determined by immunostaining of BrdU incorporated in vivo (C). The data show that $p19^{ARF}$ deficiency does not significantly alter cell proliferation ($P = 0.288$).

p53 function is intact in CP of $p19^{ARF}$-deficient mice. Although $p19^{ARF}$ was not required for p53-mediated apoptosis or tumor suppression in CP epithelium, we considered whether p53 activity was at all dependent on $p19^{ARF}$ in this cell type. Furthermore, although unlikely, it was a formal possibility that the absence of $p19^{ARF}$ had triggered a switch from p53-dependent apoptosis and tumor suppression to p53-independent functions, thus masking the impact on p53. Hence, we used an

TABLE 1. Tumor frequency in TgT_{121};$p19^{ARF-/-}$ terminal-stage mice

Genotype	No. of mice	Survival (wk)[a]	% of mice with:			
			CP tumor	Sarcoma	Lymphoid malignancy[b]	Other tumors[c]
TgT_{121}; $p19^{ARF+/+}$	12	45	100	0	0	0
TgT_{121}; $p19^{ARF+/-}$	10	25	100	40	70	20
TgT_{121}; $p19^{ARF-/-}$	10	17	100	80	70	40
$p19^{ARF-/-}$	23	27	0	70	52	30

[a] Mice were sacrificed when they appeared ill. All malignancies present at the time of sacrifice were scored. Most mice with altered ARF alleles harbored multiple malignancies.
[b] Including lymphomas and leukemias.
[c] Including pheochromocytomas and endocrine tumors.
[d] Two mice of each genotype clearly contained infiltrating leukemia cells, as shown in Fig. 4C.

independent assessment of p53 function to determine whether p53 remained active in $p19^{ARF}$-deficient CP.

The p21 gene is a direct transcriptional target of p53 (5). In previous work, we demonstrated that p53-dependent p21 expression was indeed induced by T_{121} in CP (18). Furthermore, CP tumors that underwent p53 loss of heterozygosity also lost p21 expression, providing a perfect correlation between loss of p53 function and loss of p21 expression (12). If p53 activation were mediated by $p19^{ARF}$ in response to the T_{121} oncogenic signal, then p21 induction would not be observed in TgT_{121}; $p19^{ARF-/-}$ CP, as in the case of TgT_{121};$E2F1^{-/-}$ and TgT_{121}; $p53^{-/-}$ mice (18). Thus, the level of p21 expression in the CP was determined for TgT_{121};$p19^{ARF-/-}$, TgT_{121};$p19^{ARF+/-}$, and TgT_{121};$p19^{ARF+/+}$ mice ($n > 3$ in each case). In all cases, no difference in p21 induction between TgT_{121};$p19^{ARF-/-}$ and TgT_{121};$p19^{ARF+/+}$ CP was observed (Fig. 5). These results demonstrate that the p53 pathway induced by oncogenic stress in CP epithelium does not require $p19^{ARF}$ function.

DISCUSSION

We previously showed that p53 suppresses the growth of brain epithelial tumors by mediating apoptosis in response to aberrant proliferation resulting from Rb pathway inactivation

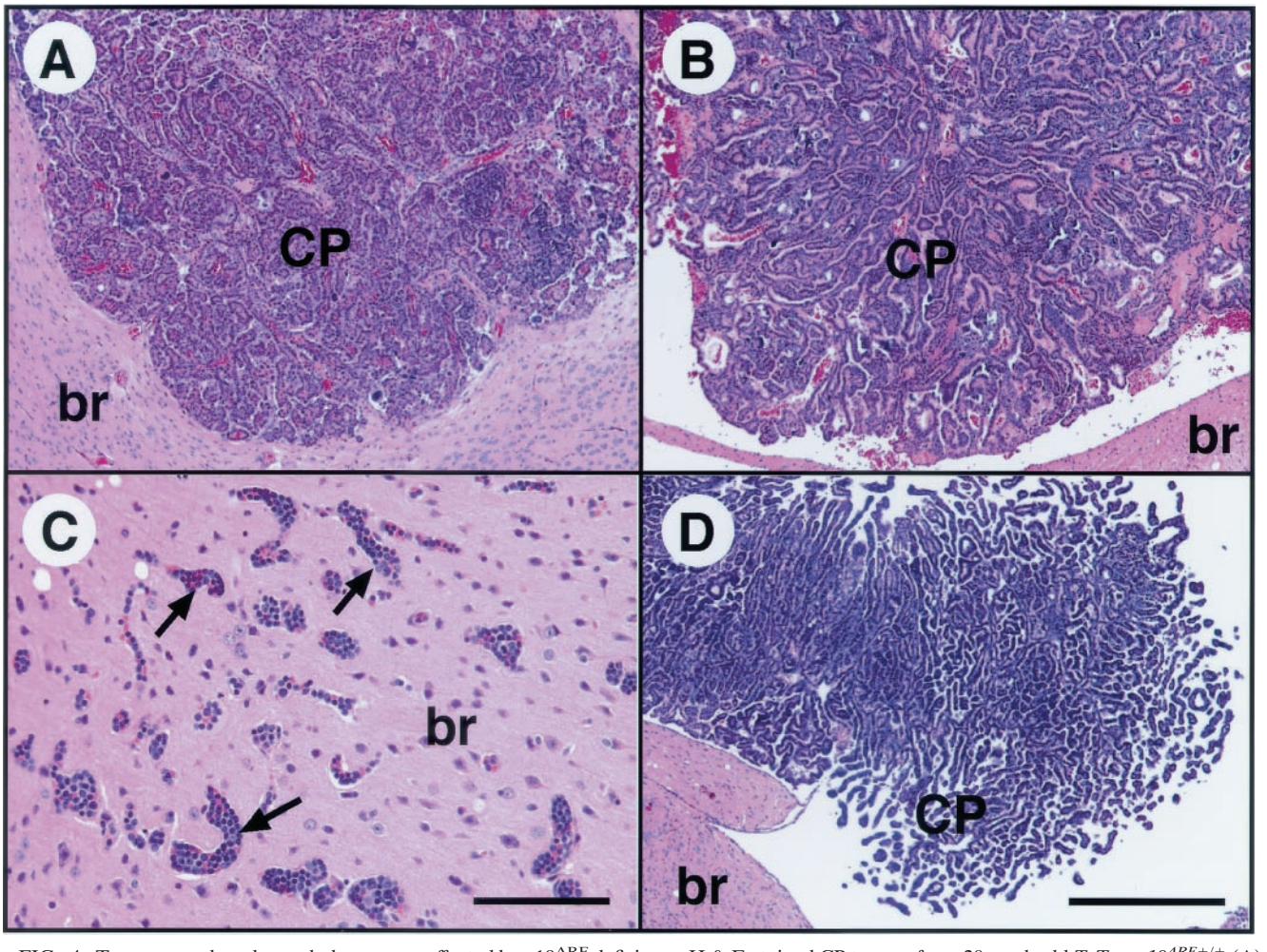

FIG. 4. Tumor growth and morphology are unaffected by $p19^{ARF}$ deficiency. H & E-stained CP tumors from 20-week-old TgT_{121};$p19^{ARF+/+}$ (A) and TgT_{121};$p19^{ARF-/-}$ (B) mice are similar in size and morphology. In contrast, tumors become life-threatening by 4 weeks of age in TgT_{121};$p53^{-/-}$ mice (D). Leukemia caused by the $p19^{ARF-/-}$ mutation is frequently observed invading the brains of TgT_{121};$p19^{ARF-/-}$ mice (C), a pathology that is present only in mice with compound genotypes and is thought to further reduce survival time (Table 1). Representative leukemia-filled vessels in the brain are indicated by arrows. The bar in panel C is equal to 100 μm. The bar in panel D is equal to 200 μm; panels A, B, and D are of the same magnification. br, brain tissue.

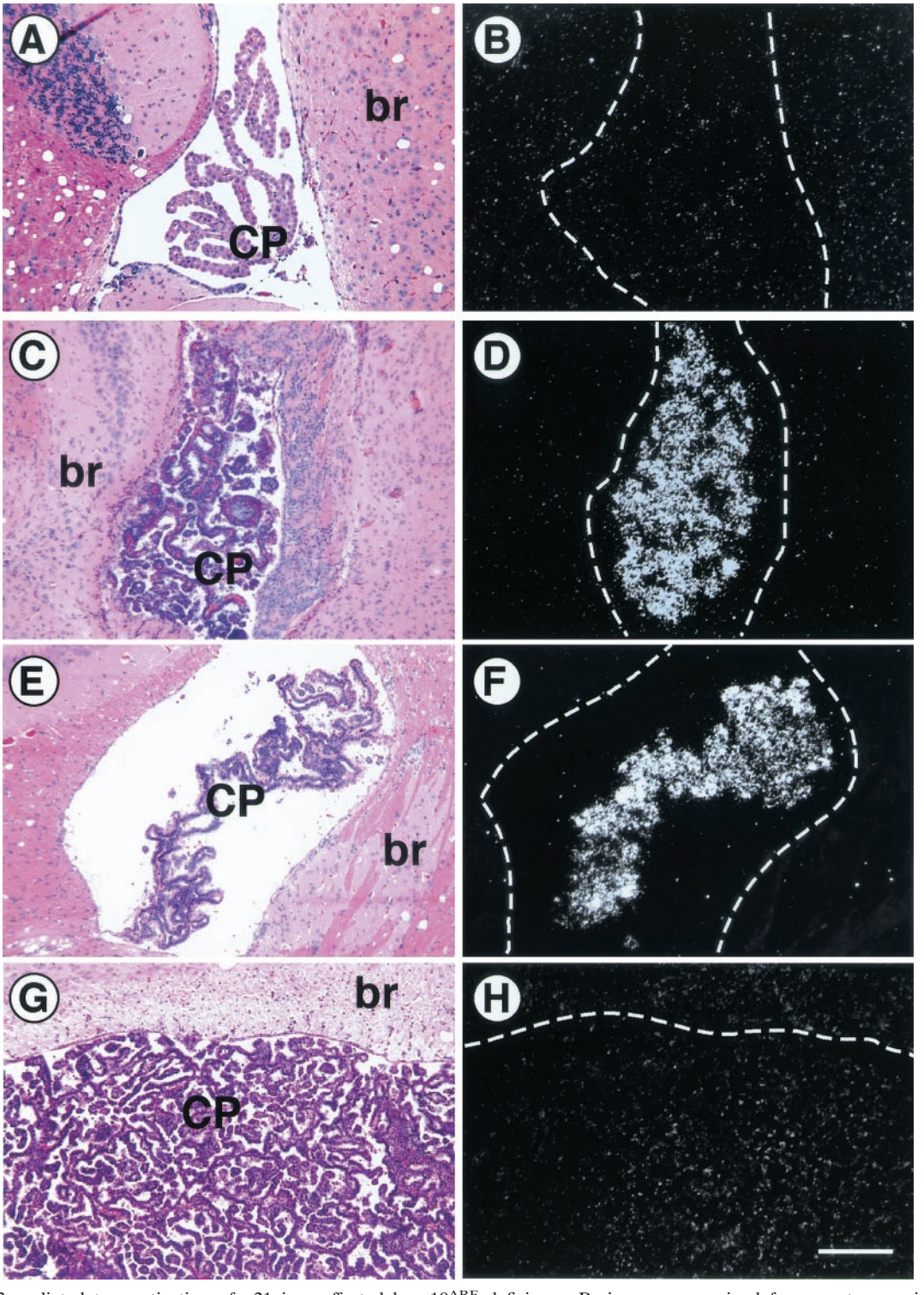

FIG. 5. p53-mediated transactivation of p21 is unaffected by p19ARF deficiency. Brains were examined from nontransgenic (A and B), TgT_{121};$p19^{ARF+/+}$ (C and D), TgT_{121};$p19^{ARF-/-}$ (E and F), and TgT_{121};$p53^{-/-}$ (G and H) mice. RNA in situ hybridization was performed on brain sections using an antisense p21 RNA probe and viewed by dark-field microscopy (B, D, F, and H). Adjacent sections were stained with H & E and viewed by bright-field microscopy to show the location and morphology of the CP (A, C, E, and G). No p21 expression is detected in normal CP (B). p21 expression is induced by expression of T_{121} (D) and remains unchanged in the absence of p19ARF (F). T_{121}-induced p21 expression in CP requires functional p53 (H) (18). The bar in panel H is equal to 200 μm; all panels are of the same magnification. br, brain tissue.

(27). Although $p19^{ARF}$ is widely believed to generally transmit oncogenic stress signals to p53 (25, 31), the studies presented here show that no defects were detected in known p53 responses in the CP of $p19^{ARF}$-deficient mice. p53-dependent apoptosis, tumor suppression, and transcriptional transactivation all remained unaffected in the absence of $p19^{ARF}$. Thus, these studies indicate that $p19^{ARF}$ does not transmit the oncogenic stress signal to p53 in this system. Furthermore, since $p19^{ARF}$ deficiency appeared to have no effect on CP tumor growth or morphology, $p19^{ARF}$ also does not appear to suppress tumors in this cell type by p53-independent mechanisms.

Previous studies by others showed that B-cell lymphoma induced in transgenic mice by c-*myc* overexpression is accelerated in *Ink4a/ARF*$^{+/-}$ and *ARF*$^{+/-}$ backgrounds, similar to the effect observed in *p53*$^{+/-}$ mice (see the introduction). Further experiments in primary B cells and in B-cell lymphomas indicate that deficiencies in p53, Ink4a/ARF, or ARF also reduce the level of c-*myc*-induced apoptosis (4, 23). Moreover, in c-*myc*-induced lymphomas, mutation of p53 or deletion of the *Ink4a/ARF* locus was frequent. These mutations were mutually exclusive (23), supporting the idea that $p19^{ARF}$ and p53 are on the same tumor suppression pathway in B cells. Thus, the fact that $p19^{ARF}$ was not required for p53-dependent apoptosis and tumor suppression in brain epithelium indicates that this pathway is not universal to all cell types and that cell-specific mechanisms exist for transmitting an oncogenic stress signal to p53 for tumor suppression. This concept is supported by a recent report that medulloblastoma induced in mice heterozygous for *patched* is accelerated by p53 deficiency but not by $p19^{ARF}$ deficiency (30). Although the mechanism for p53 tumor suppression in this model is unknown, the result clearly indicates that $p19^{ARF}$ is not required.

A recent study by K. Tsai and T. Jacks (personal communication) shows that $p19^{ARF}$ is also dispensable for p53-dependent apoptosis of embryonic central nervous system (CNS) neurons and ocular lens epithelium. pRb deficiency induces unscheduled cell proliferation in both cell types, resulting in p53-dependent apoptosis (13, 15, 17). As in the adult CP, the response in these cells requires E2F1 (28). Quantitative analysis of the apoptosis in *Rb*$^{-/-}$ *p19*$^{ARF-/-}$ embryos showed that p53-mediated apoptosis was unaffected in the CNS and was only minimally diminished in the lens. Furthermore, p53 transactivation and DNA binding remained intact in the absence of $p19^{ARF}$. Previous work in the embryonic lens had shown that a deficiency in both $p16^{Ink4a}$ and $p19^{ARF}$ inhibited p53-dependent apoptosis to a greater extent, although the relative contributions of the two factors could not be resolved (19). Interestingly, in support of cell-specific mechanisms for p53 regulation, Tsai and Jacks (personal communication) show that p53-dependent proliferation suppression in embryonic peripheral neurons (13) does appear to depend on $p19^{ARF}$.

Hence, one or more $p19^{ARF}$-independent pathways exist that trigger p53 tumor suppression in response to disrupted cell cycle regulation in vivo. What is the mechanism? One possibility is that $p19^{ARF}$ carries out this function when present, but a compensatory factor signals p53 in its absence, i.e., a redundancy exists at this step in the pathway. We consider this possibility unlikely since there are no known $p19^{ARF}$-related proteins. Thus, any compensatory pathway, such as an alternative cell-specific pathway, would be unique and hence interesting.

$p19^{ARF}$ signals to p53 by regulating Mdm2 (10, 19, 26, 32, 33), a protein that specifically binds p53 and targets it for degradation via the ubiquitin pathway (1, 6). We do not yet know whether Mdm2 is also the target for p53 regulation in CP tumor suppression. Mdm2 deficiency in the mouse causes lethality in the early embryo (9, 14), so that a cell-specific deficiency will be required to test the role of Mdm2 in this or any other in vivo tumor model. We do know that E2F1, and possibly other E2Fs (34), act upstream of p53 to facilitate the apoptotic response to pRb pathway inactivation in CP (18). Thus, as with $p19^{ARF}$, the signal(s) to p53 could be directly transcriptionally regulated by E2Fs. However, further experimentation will be required to uncover potential candidates. To this end, we are currently using an array-based approach to examine the genes whose transcription is induced in response to T_{121} in CP in an E2F1-dependent fashion.

In summary, the studies presented here indicate that, although $p19^{ARF}$ signaling to p53 is a critical tumor suppression mechanism in some cell types, it does not universally mediate p53 tumor suppression in response to disrupted cell cycle regulation. Importantly, additional mechanisms, likely dictated by the cell type, do exist.

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The first two authors contributed equally to this work.

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