p16^{Ink4a} Interferes with Abelson Virus Transformation by Enhancing Apoptosis

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Pre-B-cell transformation by Abelson virus (Ab-MLV) is a multistep process in which primary transformants are stimulated to proliferate but subsequently undergo crisis, a period of erratic growth marked by high levels of apoptosis. Inactivation of the p53 tumor suppressor pathway is an important step in this process and can be accomplished by mutation of *p53* or down-modulation of p19^{Arf}, a p53 regulatory protein. Consistent with these data, pre-B cells from either *p53* or *Ink4a/Arf* null mice bypass crisis. However, the *Ink4a/Arf* locus encodes both p19^{Arf} and a second tumor suppressor, p16^{Ink4a}, that blocks cell cycle progression by inhibiting Cdk4/6. To determine if p16^{Ink4a} plays a role in Ab-MLV transformation, primary transformants derived from *Arf*$^{-/-}$ and *p16*$^{Ink4a-/-}$ mice were compared. A fraction of those derived from *Arf*$^{-/-}$ animals underwent crisis, and even though all *p16*$^{Ink4a-/-}$ primary transformants experienced crisis, these cells became established more readily than cells derived from +/+ mice. Analyses of *Ink4a/Arf*$^{-/-}$ cells infected with a virus that expresses both v-Abl and p16^{Ink4a} revealed that p16^{Ink4a} expression does not alter cell cycle profiles but does increase the level of apoptosis in primary transformants. These results indicate that both products of the *Ink4a/Arf* locus influence Ab-MLV transformation and reveal that in addition to its well-recognized effects on the cell cycle, p16^{Ink4a} can suppress transformation by inducing apoptosis.

Abelson murine leukemia virus (Ab-MLV) transforms pre-B cells in vivo and in vitro (34). Expression of the v-Abl protein tyrosine kinase, the single protein product of Ab-MLV, provides a strong growth stimulus that is countered by cellular tumor suppressor pathways. Thus, Ab-MLV-mediated transformation is a multistep process (11, 49, 54). In vitro, Ab-MLV infection induces pre-B-cell proliferation and formation of primary transformants. As these cells proliferate, they enter crisis, a period characterized by widespread apoptosis and erratic growth. Only a fraction of primary transformants survive to emerge as highly malignant, established cell lines (29, 51). Escape from crisis correlates with inactivation of the p53 pathway, either by acquiring a p53 mutation or by down-modulating the p53 regulatory protein, p19^{Arf} (15, 29, 49). Consistent with the importance of these proteins in transformation, bone marrow cells from either *p53* or *Ink4a/Arf*$^{-/-}$ mice fail to undergo crisis (29, 51).

The *Ink4a/Arf* locus encodes two tumor suppressor proteins, p19^{Arf} and p16^{Ink4a}, both of which play important roles in suppressing tumor development in humans and mice (22, 35, 40, 43, 44). p19^{Arf} affects p53 by blocking Mdm2-mediated inhibition, thereby allowing p53 to interfere with cell cycle progression and promote apoptosis; p16^{Ink4a} inhibits Rb phosphorylation through effects on cdk4 and cdk6 and causes G$_1$

arrest (46). Mutations or epigenetic changes affecting the *Ink4a/Arf* locus occur frequently in human tumors, and while some changes affect both products, others affect p16^{Ink4a} or p19^{Arf} alone (35, 44). Consistent with the importance of this locus in tumorigenesis, mice lacking these products are tumor prone (17, 22, 41, 43).

Although the way in which p19^{Arf} affects transformation has been studied extensively (46), less is known about the ways in which p16^{Ink4a} influences oncogenesis. Animals lacking only p16^{Ink4a} are tumor prone (22, 43), and the protein influences resistance to chemotherapy in a mouse lymphoma model (40). In vitro, loss of p16^{Ink4a} enhances the growth of a variety of cells, including fibroblasts, macrophages, keratinocytes, and glia (3, 10, 14, 30, 43). In addition, p16^{Ink4a} inhibits integrin-mediated cell spreading on vitronectin (1, 8) and induces apoptosis in some tumor cells (4, 36), suggesting that this protein can affect cell growth in multiple ways. Interestingly, BALB/c, a mouse strain that is highly susceptible to Ab-MLV in vivo and in vitro (32, 33), expresses a hypomorphic *p16*^{Ink4a} allele, and inheritance of this allele correlates with susceptibility to carcinogen-induced tumors (9, 13, 56), raising the possibility that p16^{Ink4a} contributes to susceptibility to Ab-MLV transformation.

Here we tested the role of p16^{Ink4a} in Ab-MLV transformation by comparing the susceptibility of bone marrow cells from *Ink4a/Arf*$^{-/-}$, *Arf*$^{-/-}$, and *p16*$^{Ink4a-/-}$ mice to all phases of transformation. These analyses revealed that a fraction of primary transformants from *Arf*$^{-/-}$ mice exhibit crisis and that primary transformants derived from *p16*$^{Ink4a-/-}$ mice became established more readily than those from +/+ mice. Coexpres-

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sion of p16^{Ink4a} and v-Abl in transforming pre-B cells increased apoptosis, suggesting that p16^{Ink4a} can influence survival of cells stimulated by an oncogenic signal. These data reveal that both p19^{Arf} and p16^{Ink4a} affect the ability of Ab-MLV to induce transformation.

MATERIALS AND METHODS

Cells, viruses, and mice. Ab-MLV-transformed pre-B cell lines were maintained as described previously (29). Ab-MLV-P120 stocks were prepared by using the pMIG vector (12, 52) and the pSV-Ψ⁻-E-MLV retroviral packaging plasmid (27) and titrated as described elsewhere (24). To prepare viruses expressing both v-Abl and p16^{Ink4a}, v-abl coding sequences were used to replace Gfp sequences 3′ of the internal ribosome entry site (IRES) in the pMIG vector (12, 52), and the coding sequences for p16^{Ink4a} from pKS-mp16 (28) were inserted into the EcoRI site upstream of the IRES. This virus and a control virus in which v-abl sequences were inserted downstream of the IRES were titrated by infecting bone marrow cells with dilutions of virus stock. DNA was prepared from the cells 24 h later, and real-time PCR was used to quantitate the number of Ab-MLV copies. Amplification of sequences within the Rag1 gene was used to standardize the number of copies of cellular sequence. Bone marrow transformation assays were carried out as described elsewhere (33). To monitor the frequency with which primary transformants became established, the cells were explanted from agar and plated in liquid medium (29, 51). Growth was monitored on a daily basis, and when the cells could be subcultured on a regular and predictable basis and levels of apoptosis were less than 10%, the cells were considered established. Ink4a/Arf$^{-/-}$ mice (41) backcrossed with C57BL/6 mice for seven generations, Arf$^{-/-}$ mice from a breeding pair on a mixed C57BL/6-129 background obtained from C. J. Sherr (St. Jude's Children's Hospital), and p16$^{Ink4a-/-}$ animals (43) on a mixed 129Sv-FvB/n background were studied. Genotypes were assessed by PCR amplification of the allele of interest.

Apoptosis and cell cycle analysis. Cells were suspended in 1.12% sodium citrate containing 2 mg of RNase A/ml and then mixed with an equal volume of 0.2% Triton X-100–0.1% sodium citrate containing 100 μg of propidium iodide/ml (6). The samples were analyzed by using a FACScan instrument (Becton Dickinson) and ModFit LT software (Verity Software). To monitor apoptosis, cells were stained with propidium iodide or merocyanin 540, a dye that specifically stains apoptotic cells (31); comparable results were obtained with each procedure.

Protein analysis. Cell lysates were prepared and quantitated as described previously (6) and fractionated on sodium dodecyl sulfate-polyacrylamide gels. Proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore) and probed with anti-p19^{Arf} (Novus), anti-p16^{Ink4a} (RDI), anti-Gag/v-Abl (39), or anti-β-actin antibody (Sigma). The bands were visualized by developing the blots with a chemiluminescence kit (Tropix) according to the manufacturer's instructions. To quantitate levels of p16^{Ink4a}, multiple exposures were evaluated by densitometry using the amount of p16^{Ink4a} in the L1-2 cell line as a standard. This level of p16^{Ink4a} was set at 1.

RESULTS

Absence of p16^{Ink4a} or p19^{Arf} does not affect pre-B-cell primary transformation frequency. The first stage of Ab-MLV transformation, called primary transformation, involves Ab-MLV-mediated stimulation of pre-B-cell growth (29, 51). To determine if loss of p16^{Ink4a} or p19^{Arf} affects this phase of transformation, bone marrow from Ink4a/Arf$^{-/-}$, Arf$^{-/-}$, and p16$^{Ink4a-/-}$ mice and littermate controls was infected and plated in soft agar. Ten days later, macroscopic colonies of primary transformants were counted. Primary transformants were recovered in all infected samples, and although the numbers varied between the different +/+ and −/− animals, no difference that correlated with genotypes was observed (Table 1). Indeed, variation in primary transformation frequencies of as much as fourfold can be observed between genetically identical animals (15, 33, 51). Similar transformation frequencies indicate that loss of Ink4a/Arf locus products does not significantly

TABLE 1. Transformation frequencies are similar in the different Ink/Arf locus knockout mice^a

Genotype	No. of primary transformants per 10⁶ nucleated cells	
	Expt 1	Expt 2
Ink4a/Arf$^{-/-}$	59 ± 3.5	72 ± 3.1
Ink4a/Arf$^{+/+}$	95 ± 4.5	28 ± 2.1
Arf$^{-/-}$	54 ± 5.0	31 ± 2.5
Arf$^{+/-}$	21 ± 1.9	23 ± 2.9
p16$^{Ink4a-/-}$	162 ± 7.0	115 ± 5.0
p16$^{Ink4a+/+}$	146 ± 4.0	159 ± 6.0

^{a} Bone marrow cells were infected with matched titers of Ab-MLV-P120 and plated in agar; macroscopic colonies of primary transformants were counted 10 days later (33). One +/+ or heterozygous animal and one −/− animal were used in each experiment. The transformation frequencies for p19$^{Arf+/-}$ mice are similar to those obtained for +/+ mice from the p19^{Arf} colony. The values given represent the average number of colonies obtained per 10⁶ nucleated bone marrow cells ± the standard error of the mean. In each experiment, at least 6 × 10⁶ cells were evaluated in 72 independently plated cultures. The same virus stock was used to compare +/+ and −/− mice of each genotype, but different virus stocks were used with different groups of mice. Uninfected cultures did not contain macroscopic colonies.

alter Ab-MLV target cell populations or the ability of Ab-MLV to stimulate pre-B-cell growth.

Some Arf$^{-/-}$ Ab-MLV-transformed pre-B cells undergo crisis. The second phase of the transformation process involves expansion of primary transformants. During this phase, cells from +/+ mice enter a period of crisis, characterized by erratic growth and high levels of apoptosis (49, 51, 54). Cells from p53$^{-/-}$ and Ink4a/Arf$^{-/-}$ mice bypass the crisis phase (29, 51). To test whether p19^{Arf} is the only Ink4a/Arf locus product involved in crisis, primary transformants derived from Ink4a/Arf$^{-/-}$, Arf$^{-/-}$, and control mice were plated in liquid medium and their growth and survival were monitored. As expected (29, 49, 51), all of the primary transformants derived from +/+ mice entered crisis within 2 to 5 days, and 10% of them became established in 46 to 67 days (Fig. 1). Consistent with previous results (29), all of the primary transformants from Ink4a/Arf$^{-/-}$ mice bypassed crisis. In contrast, even though all Arf$^{-/-}$ primary transformants became established, 39% (28 of 72) displayed a variable period of crisis lasting 20 to 50 days (P < 0.001). These data suggest that p16^{Ink4a} contributes to crisis.

Arf$^{-/-}$ Ab-MLV transformants that bypass crisis express low levels of p16^{Ink4a}. Both Ink4a/Arf locus products are expressed during crisis (29), and previous work has suggested that expression of p19^{Arf} is particularly important in modulating the p53 response (29). However, p16^{Ink4a} expression, perhaps particularly in the absence of p19^{Arf}, may also be important during crisis. To explore this question, p16^{Ink4a} levels were analyzed in Arf$^{-/-}$ transformants undergoing crisis. All seven of the transformants tested that exhibited crisis, including the representatives shown (Fig. 2A), expressed readily detectable levels of p16^{Ink4a}, and levels increased with the onset of crisis. This pattern is consistent with involvement of p16^{Ink4a} in crisis. Although some cells that did not experience crisis also expressed detectable levels of p16^{Ink4a}, most of these cell lines expressed lower levels of the protein than cells undergoing crisis (P < 0.006) (Fig. 2B). Although the precise amounts of p16^{Ink4a} that are needed for effects on crisis are not known, the strong proliferative signals provided by v-Abl may allow cells
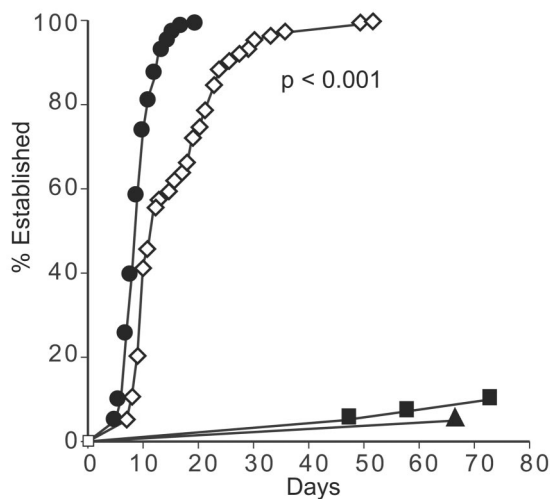
FIG. 1. $Arf^{-/-}$ primary transformants exhibit crisis. Primary transformants derived by infecting bone marrow from $Ink4a/Arf^{-/-}$ (●), $Arf^{-/-}$ (◇), $Ink4a/Arf^{+/+}$ (■), and $Arf^{+/+}$ (▲) mice with Ab-MLV were monitored for establishment. The $Arf^{+/+}$ animals are wild-type mice from our $Arf^{-/-}$ breeding colony. Cells were considered established when they displayed <10% apoptotic cells and grew regularly (15, 29). These data represent analyses of at least 70 primary transformants per genotype. The P value represents comparison of the curves obtained for cells from $Ink4a/Arf^{-/-}$ and $Arf^{-/-}$ mice using a log-rank test.
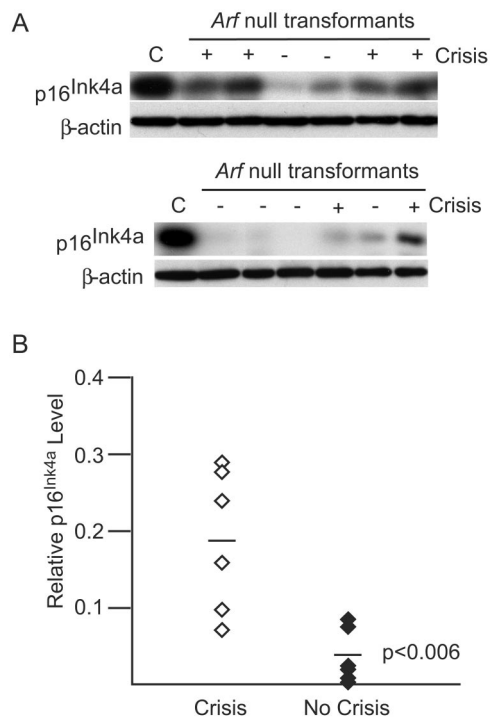

FIG. 2. p16^{Ink4a} expression correlates with crisis in $Arf^{-/-}$ transformants. (A) Primary transformants from $Arf^{-/-}$ bone marrow cells were analyzed during the outgrowth period by Western blotting using anti-p16^{Ink4a} and anti-β-actin antibodies. Representative samples are shown. The designations above the lanes indicate the presence (+) or absence (−) of crisis. C, L1-2, a fully established Ab-MLV transformant that expresses abundant p16^{Ink4a} (29). (B) Densitometry was used to compare levels of p16^{Ink4a} in primary transformants undergoing crisis to that in those that did not display crisis. Each point represents an individual cell line; the P value was obtained by using an unpaired, two-tailed t test.

expressing lower levels to circumvent these effects. Consistent with this idea, cells downregulate p16^{Ink4a} levels after becoming established (29; also our unpublished data), indicating that loss of p16^{Ink4a} expression may contribute to crisis resolution.

Lack of p16^{Ink4a} facilitates establishment. Analyses of Ab-MLV-infected cells derived from $Arf^{-/-}$ null mice indicate that p16^{Ink4a} contributes to crisis. To examine this possibility more fully, primary transformants from $p16^{Ink4a+/+}$ and $p16^{Ink4a-/-}$ mice were studied. These analyses revealed that both types of cells experienced crisis. However, 43% (91 of 213) of the $p16^{Ink4a-/-}$ cells recovered and became established, while only 29% (36 of 123) of those derived from $p16^{Ink4a+/+}$ animals survived (Fig. 3A). In addition, although the overall crisis period for both types of primary transformants was similar in duration, 9.4% (20 of 213) of those from $p16^{Ink4a}$ primary transformants became established in less than 40 days while only 1.6% (2 of 123) of those derived from +/+ littermates became established in the same rapid time frame (Fig. 3B). Thus, the absence of p16^{Ink4a} enhances the ability of primary transformants to survive crisis and can shorten the crisis period significantly. However, the loss of p16^{Ink4a} has a greater impact on some transformants than others and mirrors the results observed with the $p19^{Arf}$ null cells, where only a fraction of all primary transformants undergo crisis. These data suggest that the mechanism by which individual primary transformants recover from crisis is not identical and that several pathways influence the transformation process.

p19^{Arf} levels are similar in $p16^{Ink4a}$ null and wild-type transformants. Mice lacking $p16^{Ink4a}$ usually retain normal expression of p19^{Arf} (3, 43). However, to exclude the possibility that the enhanced survival of $p16^{Ink4a-/-}$ transformants reflects aberrant expression of p19^{Arf} in Ab-MLV-infected cells, the lev-

els of p19^{Arf} in primary transformants derived from $p16^{Ink4a-/-}$ were analyzed early in the crisis phase. Consistent with our earlier studies (29), most primary transformants analyzed expressed detectable p19^{Arf} as the crisis phase began (representatives shown in Fig. 4A). As cells recovered from crisis, 4 of 17 transformants derived from $p16^{Ink4a-/-}$ cells expressed readily detectable p19^{Arf}, while 3 of 15 derived from +/+ cells showed a similar pattern (representatives shown in Fig. 4B). Thus, the establishment advantage of the $p16^{Ink4a-/-}$ transformants is not due to differences in p19^{Arf} expression. When considered along with the results of the establishment experiment, these data suggest that p16^{Ink4a} contributes to crisis induction in a p19^{Arf}-independent fashion but is not necessary for crisis to occur.

p16^{Ink4a}-v-Abl infection induces transformation. p16^{Ink4a} expression is classically associated with effects on cell growth (3, 10, 14, 30, 43) but has also been shown to influence other cellular processes (1, 4, 8, 36). To examine the mechanism by which p16^{Ink4a} contributes to crisis, a retroviral vector expressing v-Abl and p16^{Ink4a} in *cis* (p16^{Ink4a}-v-Abl) was prepared. Matched titers of this virus and a control virus in which v-Abl was expressed in a similar fashion were used to infect $Ink4a/Arf^{-/-}$ bone marrow. To facilitate the isolation of a large number of cells, the infected cells were plated in liquid medium and monitored for expansion of pre-B cells (37). These
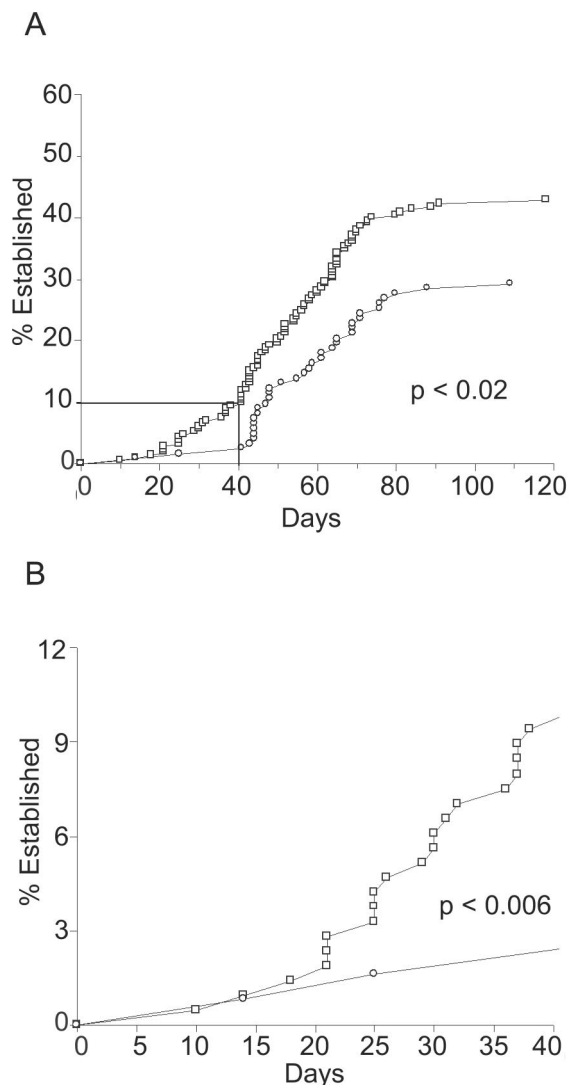
A

B

FIG. 3. $p16^{Ink4a-/-}$ colonies become established more readily than +/+ colonies. (A) Primary transformants from $p16^{Ink4a-/-}$ (□) and +/+ littermates (○) were monitored for establishment. The data shown represent analysis of 213 and 123 primary transformants from −/− and +/+ mice, respectively. The boxed area identifies transformants that became established within the first 40 days and is illustrated in an enlarged form in panel B. The P values represent comparison of each of the curves using a log-rank test.

types of cultures are scored as transformed when the density of rapidly growing pre-B cells exceeds 2×10^6 cells per ml (24), a process that requires between 10 and 15 days. All (18 of 18) of the cultures infected with the v-Abl control virus and 17 of 18 cultures infected with the p16^{Ink4a}-v-Abl virus became transformed within a similar time frame (Fig. 5A), reinforcing our earlier result that the presence of $Ink4a/Arf$ locus products does not influence the primary phase of the transformation process (Table 1). Cells did not expand in the absence of virus infection. Analyses of cultures by using Western blotting revealed that all of the transformants infected with the v-Abl-p16^{Ink4a} virus expressed both proteins (Fig. 5B). In addition, the levels of v-Abl protein expressed in cells infected with the
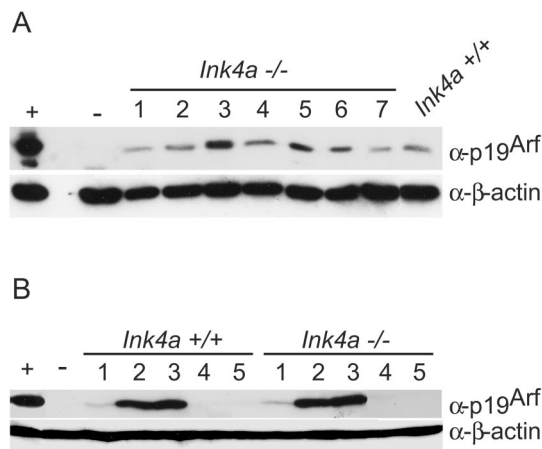
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FIG. 4. p19^{Arf} is expressed normally in $p16^{Ink4a}$ null transformants. Lysates were prepared from different cell populations and analyzed by Western blotting using anti-p19^{Arf} and anti-β-actin antibodies. (A) Analysis of lysates from $p16^{Ink4a-/-}$ and a control $p16^{Ink4a+/+}$ primary transformant prepared 8 days post-explant from agar, near the onset of crisis. (B) Analysis of lysates from $p16^{Ink4a-/-}$ and $p16^{Ink4a+/+}$ transformants prepared at the end of the crisis period. In both panels, an established cell line expressing abundant p19^{Arf} (+) and an established cell line from a $p19^{Arf}$ null animal (−) are included as controls. The experiments shown are representative of analyses of 14 null and 6 wild-type transformants at crisis onset and 17 null and 15 wild-type transformants at the end of crisis. α-p19^{Arf}, anti-p19^{Arf} antibody; α-β-actin, anti-β-actin antibody.

v-Abl-p16^{Ink4a} virus were similar to those found in cells infected with the control, v-Abl virus, indicating that the presence of $p16^{Ink4a}$ sequences did not interfere with expression of v-Abl.

Apoptosis is increased in the primary transformants expressing p16^{Ink4a}. To understand the mechanism by which p16^{Ink4a} contributes to crisis, cell cycle parameters and the level of apoptosis in $Ink4a/Arf^{-/-}$ primary transformants expressing the p16^{Ink4a}-v-Abl and v-Abl viruses were compared. Analyses of propidium iodide-stained cells by flow cytometry revealed that the percentage of cells in the G_1, S, and G_2/M phases did not differ among the cultures (Fig. 6A). In contrast, primary transformants expressing the p16^{Ink4a}-v-Abl virus had higher levels of apoptosis than those expressing v-Abl alone ($P < 0.003$) (Fig. 6B). The levels of apoptosis seen in v-Abl-infected $Ink4a/Arf^{-/-}$ cells were low and not apparent by visual inspection of the cells, in accord with a previous report of low levels of apoptosis in such cultures (29). These data indicate that cells expressing p16^{Ink4a} undergo more apoptosis than cells lacking this protein and suggest that p16^{Ink4a} contributes to the apoptotic response that characterizes crisis. Despite differences in the apoptotic response, primary transformants expressing the p16^{Ink4a}-v-Abl virus could be established with kinetics that are similar to those observed for cells expressing v-Abl alone.

To determine if the apoptotic effects of p16^{Ink4a} require p53, bone marrow from $p53^{-/-}$ mice was infected with either the p16^{Ink4a}-v-Abl or the v-Abl virus and plated in liquid medium. All of the infected cultures became transformed within 10 to 13 days. Analyses of apoptosis in these primary transformants revealed that the $p53^{-/-}$ cells transformed by p16^{Ink4a}-v-Abl
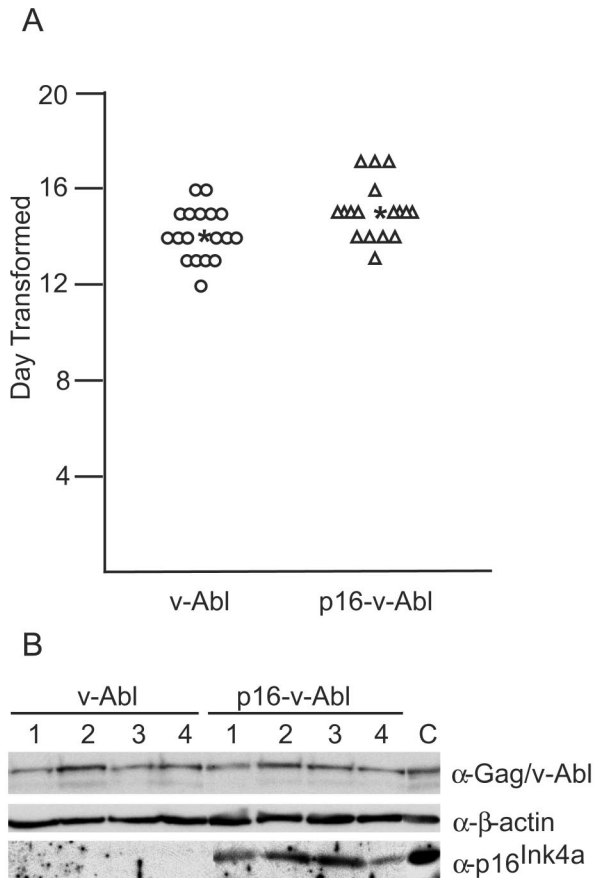
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FIG. 5. p16^{Ink4a}-v-Abl transforms cells. *Ink4a/Arf*$^{-/-}$ bone marrow was infected with a virus expressing either v-Abl alone or both p16^{Ink4a} and v-Abl and plated in liquid medium. Dishes were scored as transformed when they contained more than 2×10^6 pre-B cells per ml. (A) The day of transformation is indicated for p16^{Ink4a}-v-Abl- and v-Abl-transformed cells. The asterisk indicates the average day of transformation. All 18 cultures infected with the v-Abl virus and 17 of 18 cultures infected with the p16^{Ink4a}-v-Abl virus became transformed. (B) Lysates of primary transformants were analyzed by Western blotting with anti-p16^{Ink4a}, anti-Gag/v-Abl (39), and anti-β-actin antibodies. The experiment shown is representative of experiments in which a total of 17 v-Abl-infected transformants and 18 v-Abl-p16-infected transformants were tested.

displayed levels of apoptosis similar to those found in cells transformed with the v-Abl virus (Fig. 6B). These levels were similar to those seen in the *Ink4a/Arf*$^{-/-}$ cells infected with the v-Abl virus, indicating that the *p53*$^{-/-}$ cells infected with the p16^{Ink4a}-v-Abl virus exhibit a baseline level of apoptosis. These data indicate that p16^{Ink4a} induces apoptosis only in cells with an intact *p53* gene, suggesting that p53 may be required for this response.

DISCUSSION

Our results demonstrate that Ab-MLV-induced pre-B-cell transformation is influenced by both products of the *Ink4a/Arf* locus. Thus, oncogenic signals from v-Abl stimulate a cellular response that is orchestrated by both p19^{Arf} and p16^{Ink4a}. Consistent with this idea, recent evidence indicates that both of these proteins influence melanoma development stimulated by
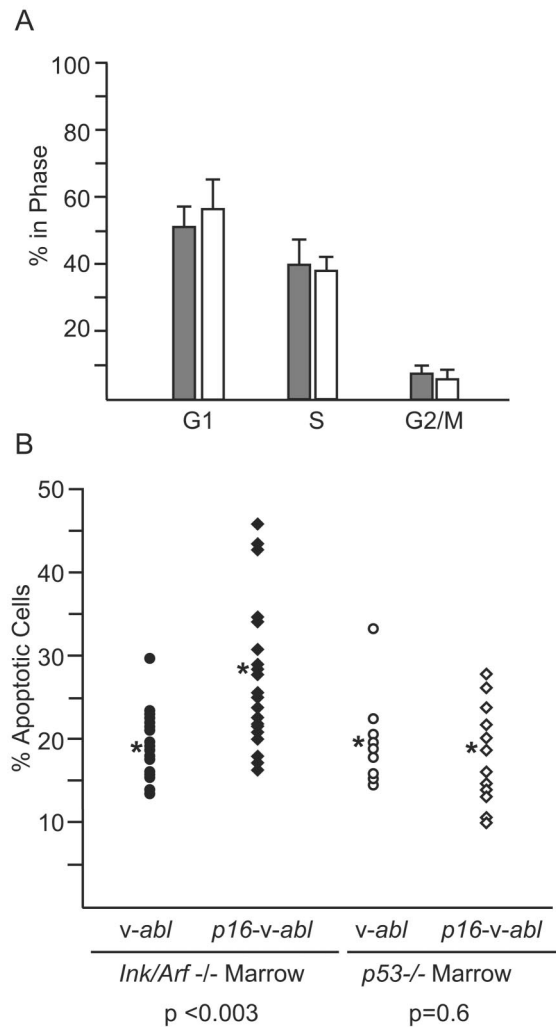
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FIG. 6. Expression of p16^{Ink4a} in primary transformants leads to apoptosis. (A) *Ink4a/Arf*$^{-/-}$ cells infected with p16^{Ink4a}-v-Abl (open bar) or v-Abl (filled bar) were harvested 24 to 72 h after transformation, stained with propidium iodide, and analyzed by flow cytometry to determine the percentage of cells in the G$_1$, G$_2$/M, and S phases of the cell cycle. The data are averages obtained from analyses of 18 independent cultures infected with v-Abl and 17 independent cultures infected with v-Abl-p16; the error bars represent standard deviations. (B) The percentage of apoptotic cells in the cultures analyzed in panel A and cultures transformed with either v-Abl or v-Abl-p16 derived from *p53*$^{-/-}$ mice were stained with propidium iodide and analyzed for the frequency of apoptotic cells by flow cytometry. Each point represents an individual clone derived from one of three independent experiments. The asterisk indicates the mean; the *P* values were obtained by using an unpaired, two-tailed *t* test.

activated Ras (45); the central role of Ras activation in Ab-MLV transformation is well documented (38, 57), and it is likely that Ras is involved in activating expression of both *Ink4a/Arf* locus products in Ab-MLV-infected cells. Once activated, both products influence the apoptosis that characterizes the crisis phase of transformation. Although p16^{Ink4a} expression is usually associated with effects on the cell cycle (44, 46, 47), p16^{Ink4a} may influence apoptosis more commonly than originally thought. p16^{Ink4a} expression is associated with apoptosis in some human carcinoma and leukemia cells (2, 4, 20,

36), and transgene-mediated expression of p16^{Ink4a} in normal T cells causes enhanced apoptosis and differentiation arrest at an immature stage (23).

Analyses of primary transformants expressing the p16^{Ink4a}-v-Abl virus derived from $p53^{-/-}$ mice indicate that a functional p53 protein is important for the apoptotic effects of p16^{Ink4a} during crisis. Consistent with these data, among human tumors evaluated for p53 status, the subset that underwent apoptosis in response to p16^{Ink4a} expression did so in a p53-dependent manner (20, 36). However, detectable changes in p53 levels are not observed during crisis even in wild-type cells (our unpublished data), probably because stabilization of p53 leads to rapid induction of apoptosis. Nonetheless, in many human tumors both p53 and p16^{Ink4a} function is lost, suggesting that these products also act independently to influence tumor development (26, 44). In addition, spontaneous tumors arising in $p16^{Ink4a-/-}$ mice often lose p53 function, and $p53/p16^{Ink4a}$ double $-/-$ mice are more tumor prone than either singly deficient animal (42). Taken together, these data indicate that the response to each of these tumor suppressors is influenced by the cell type and that the relationship between p16^{Ink4a} and p53 is complex. A likely model predicts that an intact p53 pathway is important for some p16^{Ink4a}-mediated responses, including the apoptotic response, but not for others (e.g., cell cycle arrest). Perhaps functional p53 sensitizes cells to the effects of p16^{Ink4a} in a fashion that does not require direct cross talk between the two pathways.

Although p16^{Ink4a} contributes to crisis during Ab-MLV-induced transformation, the effects appear to be more subtle than those of the second product of the $Ink4a/Arf$ locus, p19^{Arf}, in this transformation model. However, the response observed in the $p16^{Ink4a}$ null mice used here may be partly influenced by the strain background, one that is more permissive to all phases of Ab-MLV transformation than that used for the $Ink4a/Arf$ and Arf null mice. Indeed, a much higher frequency of primary transformants from normal mice on 129/FvB backgrounds become transformed than on 129/C57BL/6 backgrounds (our unpublished data). Presumably this difference reflects the effects of unknown genes that differ in these strain combinations. Thus, it is possible that effects of p16^{Ink4a} loss might be enhanced if the null allele were expressed on a C57BL/6 background.

p19^{Arf} exerts a strong influence on the establishment phase of the Ab-MLV transformation process compared to the effects documented for p16^{Ink4a}. Upregulation of p19^{Arf} leads to p53 activation via effects on Mdm2 (46) and also affects growth and survival of cells by p53-independent pathways (53, 55). The dominant role of p19^{Arf} in vitro is best revealed in primary transformants derived from $Arf^{-/-}$ mice. More than half of all the primary transformants bypass crisis, and all of those that undergo crisis recover and do so more rapidly than transformants from $+/+$ mice. In addition, even though primary transformants from $p16^{Ink4a-/-}$ animals have a survival advantage, crisis still occurs. Consistent with these effects, some fully established transformed cell lines continue to express p16^{Ink4a}, and apoptosis was not observed following overexpression of the protein in several of these cell lines (29). Such cells have probably acquired additional mutations that circumvent the effects of p16^{Ink4a} and will be a useful resource for probing the pathways involved.

p16^{Ink4a} does not affect the cell cycle profile of Ab-MLV-transformed pre-B cells even though analyses of many human malignancies have linked the tumor suppressor function of p16^{Ink4a} to its effects on Rb-mediated G$_1$ progression (44, 46). Interestingly, analyses of $Ink4a/Arf^{-/-}$ bone marrow cells have revealed that p16^{Ink4a} affects the growth of myeloid cells but not interleukin 7-dependent pre-B cells (30), the cells that are the primary target of Ab-MLV transformation (21). However, these cells were expanded under the influences of interleukin 7, a cytokine that normally influences their growth and differentiation, not under the influences of a strong oncogenic signal, and such a signal may be needed to reveal p16^{Ink4a} function. Indeed, analyses of several different tumor models highlight the cell type specificity of p16^{Ink4a}-mediated effects (3, 19, 22, 43).

Although the role of p16^{Ink4a} is subtle in some in vitro transformation systems, studies of the Eμ-Myc lymphoma model have shown that p16^{Ink4a} expression can have dramatic effects on the response of tumors to chemotherapy (40). In this situation, cells containing both functional p16^{Ink4a} and p53 become cytostatic for extended periods of time. Because these lymphoma cells express markers associated with senescence pathways, p16^{Ink4a} appears to activate this program in these tumors. These data, and our results that p16^{Ink4a} affects a late stage in transformation, emphasize that studying only the induction phase of oncogenesis does not reveal a complete picture of the effects of p16^{Ink4a}.

Our experiments demonstrate an underappreciated role for p16^{Ink4a} in blocking oncogenesis by inducing apoptosis. p16^{Ink4a} expression is strongly selected against in many types of human tumors by deletion, mutation, or epigenetic silencing (26, 35, 44), and its loss is a negative prognostic indicator for several tumors (5, 7, 16, 18, 48, 50). Loss of p16^{Ink4a} has also been associated with tumor progression in human hematological tumors, where $p16^{Ink4a}$ gene deletions have been documented in late-stage leukemias and lymphomas that retained the gene at diagnosis (7, 25). Thus, loss of p16^{Ink4a} can be an important late event in the multistage process of malignant transformation, allowing an indolent disease to become more aggressive. Escape from apoptosis may be one mechanism that is critical for acquisition of a more aggressive, malignant phenotype. Additional studies using the well-defined and simple model of Ab-MLV pre-B-cell transformation should help to uncover the mechanism by which this response is orchestrated.

REFERENCES

1. Adachi, Y., S. S. Lakka, N. Chandrasekar, N. Yanamandra, C. S. Gondi, S. Mohanam, D. H. Dinh, W. C. Olivero, M. Gujrati, T. Tamiya, T. Ohmoto, G. Kouraklis, B. Aggarwal, and J. S. Rao. 2001. Down-regulation of integrin α$_v$β$_3$ expression and integrin-mediated signaling in glioma cells by adenovirus-mediated transfer of anti-sense urokinase-type plasminogen activator receptor (*uPAR*) and sense *p16* genes. J. Biol. Chem. **276:**47171–47177.
2. Ausserlechner, M. J., P. Obexer, G. J. Wiegers, B. L. Hartmann, S. Geley, and R. Kofler. 2001. The cell cycle inhibitor p16(INK4A) sensitizes lymphoblastic leukemia cells to apoptosis by physiologic glucocorticoid levels. J. Biol. Chem. **276:**10984–10989.

3. **Bachoo, R. M., E. A. Maher, K. L. Ligon, N. E. Sharpless, S. S. Chan, M. J. You, Y. Tang, J. DeFrances, E. Stover, R. Weissleder, D. H. Rowitch, D. N. Louis, and R. A. DePinho.** 2002. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer Cell **1:**269–277.

4. **Calbo, J., M. Marotta, M. Cascallo, J. M. Roig, J. L. Gelpi, J. Fueyo, and A. Mazo.** 2001. Adenovirus-mediated transfer of wt-p16 induces cell cycle arrest or apoptosis in pancreatic cancer. Cancer Gene Ther. **8:**740–750.

5. **Calero Moreno, T. M., G. Gustagsson, S. Garwicz, D. Grander, G. K. Jonmundsson, B. M. Frost, A. Makipernaa, O. Rasool, E. R. Savolainen, K. Schmiegelow, S. Soderhall, K. Vettenranta, F. Wesenberg, S. Einhorn, and M. Heyman.** 2002. Deletion of the Ink4-locus (the p16Ink4a, p14ARF and p15Ink4b genes) predicts relapse in children with ALL treated according to the Nordic protocols NOPHO-86 and NOPHO-92. Leukemia **16:**2037–2045.

6. **Chen, Y. Y., and N. Rosenberg.** 1992. Lymphoid cells transformed by Abelson virus require the v-abl protein tyrosine kinase only during early G_1. Proc. Natl. Acad. Sci. USA **89:**6683–6687.

7. **Elenitoba-Johnson, K. S. J., R. D. Gascoyne, M. S. Lim, M. Chhanabai, E. S. Jaffe, and M. Raffeld.** 1998. Homozygous deletions at chromosome 9p21 involving p16 and p15 are associated with histologic progression in follicle center lymphoma. Blood **91:**4677–4685.

8. **Fahraeus, R., and D. P. Lane.** 1999. The p16(INK4a) tumour suppressor protein inhibits $\alpha_v\beta_3$ integrin-mediated cell spreading on vitronectin by blocking pCK-dependent localization of focal $\alpha_v\beta_3$ contacts. EMBO J. **18:**2106–2118.

9. **Festing, M. F. W., L. Lin, T. R. Devereux, F. Gao, A. Yang, C. H. Anna, C. M. White, A. M. Malkinson, and M. You.** 1998. At least four loci and gender are associated with susceptibility to the chemical induction of lung adenomas in A/J × BALB/c mice. Genomics **53:**129–136.

10. **Foster, S. A., D. J. Wong, M. T. Barrett, and D. A. Galloway.** 1998. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. Mol. Cell. Biol. **18:**1793–1801.

11. **Green, P. L., D. A. Kaehler, and R. Risser.** 1987. Clonal dominance and progression in Abelson murine leukemia virus lymphomagenesis. J. Virol. **61:**2192–2197.

12. **Hawley, R. G., F. H. Lieu, A. Z. Fong, and T. S. Hawley.** 1994. Versatile retroviral vectors for potential use in gene therapy. Gene Ther. **1:**136–138.

13. **Herzog, C. R., T. R. Devereux, B. Pittman, and M. You.** 2002. Carcinogenic induction directs the selection of allelic losses in mouse lung tumorigenesis. Cancer Res. **62:**6424–6429.

14. **Hout, T. J., J. Rowe, M. Harland, S. Drayton, S. Brookes, C. Gooptu, P. Purkis, M. Fried, V. Bataille, E. Hara, J. Newton-Bishop, and G. Peters.** 2002. Biallelic mutations in p16^{INK4a} confer resistance to Ras- and Ets-induced senescence in human diploid fibroblasts. Mol. Cell. Biol. **22:**8135–8143.

15. **Jenab-Wolcott, J., D. Rodriguez-Correa, A. Reitmair, T. Mak, and N. Rosenberg.** 2000. The absence of Msh2 alters Abelson virus pre-B cell transformation by influencing p53 mutation. Mol. Cell. Biol. **20:**8373–8381.

16. **Jin, M., S. Inoue, T. Umemura, J. Moriya, K. Arakawa, K. Nagashimia, and H. Kato.** 2001. Cyclin D1, p16 and retinoblastoma gene product expression as a predictor for prognosis in non-small cell lung cancer at stages I and II. Lung Cancer **34:**207–218.

17. **Kamijo, T., F. Zindy, M. F. Roussel, D. E. Quelle, J. R. Downing, R. A. Ashmun, G. Grosveld, and C. J. Sherr.** 1997. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell **91:**649–659.

18. **Kamiryo, T., K. Tada, S. Shiraishi, N. Shinojima, H. Nakamura, M. Kochii, J. Kuratsu, H. Saya, and Y. Ushio.** 2002. Analysis of homozygous deletion of the p16 gene and correlation with survival in patients with glioblastoma multiforme. J. Neurosurg. **96:**815–822.

19. **Kannan, K., N. E. Sharpless, X. Xu, R. C. O'Hagan, M. Rosenberg, and L. Chin.** 2003. Components of the Rb pathway are critical targets of UV mutagenesis in a murine melanoma model. Proc. Natl. Acad. Sci. USA **100:**1221–1225.

20. **Kawabe, S., J. A. Roth, D. R. Wilson, and R. E. Meyn.** 2000. Adenovirus-mediated p16INK4a gene expression radiosensitizes non-small cell lung cancer cells in a p53-dependent manner. Oncogene **19:**5359–5366.

21. **Kelliher, M. A., D. J. Weckstein, A. G. Knott, H. H. Wortis, and N. Rosenberg.** 1993. ABL oncogenes directly stimulate two distinct target cells in bone marrow from 5-fluorouracil-treated mice. Oncogene **8:**1249–1256.

22. **Krimpenfort, P., K. C. Quon, W. J. Mooi, A. Loonstra, and A. Berns.** 2001. Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. Nature (London) **413:**83–86.

23. **Lagresle, C., B. Gardie, S. Eyquem, M. Fasseu, J. C. Vieville, M. Pal, F. Sigaur, and J. C. Bories.** 2002. Transgenic expression of the p16(INK4a) cyclin dependent kinase inhibitor leads to enhanced apoptosis and differentiation arrest of CD4-CD8-negative immature thymocytes. J. Immunol. **168:**2325–2331.

24. **Mainville, C., K. Parmar, I. Unnikrishnan, G. D. Raffel, L. Gong, and N. Rosenberg.** 2001. Alteration of the FLVRES motif in the v-Abl SH2 domain

25. **Maloney, K. W., L. McGavran, L. F. Odom, and S. P. Hunger.** 1999. Acquisition of $p16^{INK4A}$ and $p15^{INK4B}$ gene abnormalities between initial diagnosis and relapse in children with acute lymphoblastic leukemia. Blood **93:**2380–2385.

26. **Malumbres, M., and M. Barbacid.** 2001. To cycle or not to cycle: a critical decision in cancer. Nat. Rev. Cancer **1:**222–231.

27. **Muller, A. J., J. C. Young, A. M. Pendergast, M. Pondel, N. R. Landau, D. R. Littman, and O. N. Witte.** 1991. BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. Mol. Cell. Biol. **11:**1785–1792.

28. **Quelle, D. E., M. Cheng, R. A. Ashmun, and C. J. Sherr.** 1997. Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. Proc. Natl. Acad. Sci. USA **94:**669–673.

29. **Radfar, A., I. Unnikrishnan, H.-W. Lee, R. A. DePinho, and N. Rosenberg.** 1998. p19^{Arf} induces p53-dependent apoptosis during Abelson virus-mediated pre-B cell transformation. Proc. Natl. Acad. Sci. USA **95:**13194–13199.

30. **Randle, D. H., F. Zindy, C. J. Sherr, and M. F. Roussel.** 2001. Differential effects of p19^{Arf} and p16^{Ink4a} loss on senescence of murine bone marrow-derived pre-B cells and macrophages. Proc. Natl. Acad. Sci. USA **96:**9654–9659.

31. **Reid, S., R. Cross, and E. Snow.** 1996. Combined Hoechst 33342 and merocyanin 540 staining to examine murine B cell cycle stage, viability and apoptosis. J. Immunol. Methods **192:**43–54.

32. **Risser, R., M. Potter, and W. P. Rowe.** 1978. Abelson virus-induced lymphomagenesis in mice. J. Exp. Med. **148:**714–726.

33. **Rosenberg, N., and D. Baltimore.** 1976. A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. J. Exp. Med. **143:**1453–1463.

34. **Rosenberg, N., and O. N. Witte.** 1988. The viral and cellular forms of the Abelson (*abl*) oncogene. Adv. Virus Res. **35:**39–81.

35. **Ruas, M., and G. Peters.** 1998. The p16Ink4a/CDKN2A tumor suppressor and its relatives. Biochim. Biophys. Acta **1378:**F115–F177.

36. **Sandig, V., K. Brand, S. Herwig, J. Lukas, J. Bartek, and M. Strauss.** 1997. Adenovirally transferred p16INK4/DCKN2 and p53 genes cooperate to induce apoptotic tumor cell death. Nat. Med. **3:**313–319.

37. **Sawyers, C. L., W. Callahan, and O. N. Witte.** 1992. Dominant negative myc blocks transformation by abl oncogenes. Cell **70:**901–910.

38. **Sawyers, C. L., J. McLaughlin, and O. N. Witte.** 1995. Genetic requirement for ras in the transformation of fibroblasts and hematopoietic cells by the bcr-abl oncogene. J. Exp. Med. **181:**307–313.

39. **Schiff-Maker, L., M. C. Burns, J. B. Konopka, S. Clark, O. N. Witte, and N. Rosenberg.** 1986. Monoclonal antibodies specific for v-abl- and c-abl-encoded molecules. J. Virol. **57:**1182–1186.

40. **Schmitt, C. A., J. S. Fridman, M. Yang, S. Lee, E. Baranov, R. M. Hoffman, and S. W. Lowe.** 2002. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell **109:**335–346.

41. **Serrano, M., H.-W. Lee, L. Chin, C. Cordon-Cardo, D. Beach, and R. A. DePinho.** 1996. Role of the *INK4a* locus in tumor suppression and cell mortality. Cell **85:**27–37.

42. **Sharpless, N. E., S. Alson, S. S. Chan, D. P. Silver, D. H. Castrillon, and R. A. DePinho.** 2002. p16(INK4a) and p53 deficiency cooperate in tumorigenesis. Cancer Res. **62:**2761–2765.

43. **Sharpless, N. E., N. Bardeesy, K. H. Lee, D. Carrasco, D. H. Castrillon, A. J. Aguirre, E. A. Wu, J. W. Horner, and R. A. DePinho.** 2001. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature (London) **413:**86–91.

44. **Sharpless, N. E., and R. A. DePinho.** 1999. The *Ink4A/Arf* locus and its two gene products. Curr. Opin. Genet. Dev. **9:**22–30.

45. **Sharpless, N. E., K. Kannan, J. Xu, M. W. Bosenberg, and L. Chin.** 2003. Both products of the mouse *Ink4a/Arf* locus suppress melanoma formation in vivo. Oncogene **22:**5055–5059.

46. **Sherr, C. J.** 2001. The *Ink4a/ARF* network in tumour suppression. Nat. Rev. Mol. Cell Biol. **2:**731–737.

47. **Sherr, C. J., and J. M. Roberts.** 1999. CDK inhibitors: positive and negative regulators of G-1 phase progression. Genes Dev. **13:**1501–1512.

48. **Straume, O., I. Sviland, and L. A. Akslen.** 2000. Loss of nuclear p16 protein expression correlates with increased tumor cell proliferation (Ki-67) and poor prognosis in patients with vertical growth phase melanoma. Clin. Cancer Res. **6:**1845–1853.

49. **Thome, K., A. Radfar, and N. Rosenberg.** 1997. Mutation of Tp53 contributes to the malignant phenotype of Abelson virus transformed lymphoid cells. J. Virol. **77:**8149–8156.

50. **Tutor, O., M. A. Diaz, M. Ramirez, P. Algara, L. Madero, and P. Martiniz.** 2002. Loss of heterozygosity of p16 correlates with minimal residual disease at the end of the induction therapy in non-high risk childhood B-cell precursor acute lymphoblastic leukemia. Leuk. Res. **26:**817–820.

51. **Unnikrishnan, I., A. Radfar, J. Jenab-Wolcott, and N. Rosenberg.** 1999. p53 mediates apoptotic crisis in primary Ab-MLV-transformed pre-B cells. Mol. Cell. Biol. **19:**4825–4831.

confers a temperature-sensitive transformation phenotype. J. Virol. **75:**1816–1823.

52. **Van Parijs, L., Y. Refaeli, J. D. Lord, B. H. Nelson, A. K. Abbas, and D. Baltimore.** 1999. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation induced cell death. Immunity **11:**281–288.

53. **Weber, J. D., J. R. Jeffers, J. E. Rehg, D. H. Randle, G. Lozano, M. F. Roussel, C. J. Sherr, and G. P. Zambetti.** 2000. p53-independent functions of the p19^{Arf} tumor suppressor. Genes Dev. **14:**2358–2365.

54. **Whitlock, C. A., S. F. Ziegler, and O. N. Witte.** 1983. Progression of the transformed phenotype in clonal lines of Abelson virus-infected lymphocytes. Mol. Cell. Biol. **3:**596–604.

55. **Yarbrough, W. G., M. Bessho, A. Zanation, J. E. Bisi, and Y. Xiong.** 2002. Human tumor suppressor ARF impedes S-phase progression independent of p53. Cancer Res. **62:**1171–1177.

56. **Zhang, S. L., W. DuBois, E. S. Ramsay, V. Bliskovski, H. C. Morse, L. Taddesse-Heath, W. C. Vass, R. A. DePinho, and B. A. Mock.** 2001. Efficiency alleles of the *Pctr1* modifier locus for plasmacytomas susceptibility. Mol. Cell. Biol. **21:**310–318.

57. **Zou, X., S. Rudchenko, K.-K. Wong, and K. Calame.** 1997. Induction of c-myc transcription by the v-Abl tyrosine kinase requires Ras, Raf1, and cyclin-dependent kinases. Genes Dev. **11:**654–662.