Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization

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Establishment of primary mouse embryo fibroblasts (MEFs) as continuously growing cell lines is normally accompanied by loss of the p53 or p19^{ARF} tumor suppressors, which act in a common biochemical pathway. *myc* rapidly activates *ARF* and *p53* gene expression in primary MEFs and triggers replicative crisis by inducing apoptosis. MEFs that survive *myc* overexpression sustain *p53* mutation or *ARF* loss during the process of establishment and become immortal. MEFs lacking *ARF* or *p53* exhibit an attenuated apoptotic response to *myc* ab initio and rapidly give rise to cell lines that proliferate in chemically defined medium lacking serum. Therefore, *ARF* regulates a p53-dependent checkpoint that safeguards cells against hyperproliferative, oncogenic signals.

[*Key Words:* Myc signaling; ARF tumor suppressor; p53; apoptosis; immortalization]

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The *INK4a–ARF* locus is a common target of deletion and mutation in human cancers, possibly second in frequency only to *p53*. The product of the *INK4a* gene, p16^{INK4a}, acts as an inhibitor of cyclin D-dependent kinases, preventing them from phosphorylating the retinoblastoma (pRb) protein and thus inhibiting S-phase entry during the cell division cycle (Serrano et al. 1993). A second product of this locus, p19^{ARF}, encoded in part by an alternative reading frame of *INK4a* exon 2, is completely unrelated in its primary structure to p16^{INK4a} and induces both G_1- and G_2-phase arrest in rodent fibroblasts (Quelle et al. 1995b) in a p53-dependent manner (Kamijo et al. 1997). Thus, both p16^{INK4a} and p19^{ARF} act as potent tumor suppressors by targeting pRb and p53 function, respectively.

Establishment of mouse embryo fibroblasts (MEFs) as continuously growing cell lines is usually accompanied by either ARF or p53 loss of function, implying that the two proteins act epistatically in a single pathway (Kamijo et al. 1997; Zindy et al. 1997). The p53 protein is a transcription factor (Kern et al. 1992) that induces several known target genes, including the cyclin-dependent kinase inhibitor *p21/Cip1/Waf1* (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993) and *mdm2* (Barak et al. 1993; Wu et al. 1993). In turn, Mdm2 acts in a feedback loop to catalyze p53 ubiquitination and degradation, limiting the p53 response (Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997). The ARF protein can physically interact in binary or ternary complexes with p53 and Mdm2, and its overexpression induces p53 stabilization and activates p53-dependent transcription (Kamijo et al. 1998; Pomerantz et al. 1998; Zhang et al. 1998). Although the levels of p19^{ARF} expressed in normal MEFs are relatively low, an unexplained feature is that p19^{ARF} expression is significantly elevated in *p53*-null fibroblasts (Quelle et al. 1995b). Conversely, reintroduction of *p53* into *p53*-null cells returns the level of p19^{ARF} to normal levels (Kamijo et al. 1998). Together, these data suggest that a feedback loop also acts to limit p19^{ARF} expression once p53 is activated, and the ability of Mdm2 to bind both p53 and p19^{ARF} raises the possibility that Mdm2 might be responsible for their joint down-regulation.

The physiologic signals that induce *ARF* remain unknown. *ARF* is dispensable for p53 activation in response to ionizing or UV radiation (Kamijo et al. 1997), suggesting that it does not function in a DNA damage-signaling pathway. Observations that *ARF*-null MEFs are immortal and can be transformed by oncogenic *ras* alleles without a requirement for collaborating oncogenes such as *myc* and adenovirus *E1A* (Kamijo et al. 1997) led us to consider the possibility that *myc* and *E1A* might regulate p19^{ARF} function. Either of these oncogenes are capable of immortalizing primary rodent fibroblasts (Land et al. 1983; Ruley 1983). Whether induced by enforced *myc* or

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E1A expression, chemical carcinogens, or by loss of *p53* or *ARF* function, establishment and immortalization enable MEFs to be transformed into tumor cells by oncogenic *ras* genes alone (Land et al. 1983; Newbold and Overell 1983; Ruley 1983, 1990; Hicks et al. 1991; Lin et al. 1995; Serrano et al. 1996; Kamijo et al. 1997). *myc* and *E1A* seem to inactivate cellular responses that are normally required for *ras*-mediated inhibition of cell proliferation, thereby converting *ras* into a growth-promoting gene (Franza et al. 1986; Hicks et al. 1991; Hirakawa and Ruley 1991).

Given their apparent immortalizing functions, it seems paradoxical that *myc* and *E1A* are also potent inducers of apoptosis (Askew et al. 1991; White et al. 1991; Evan et al. 1992; Rao et al. 1992). The sensitivity of rodent fibroblasts to *myc*- or *E1A*-induced apoptosis correlates directly with the levels of oncoprotein expression and is greatly potentiated by depriving cells of extracellular survival factors (Evan et al. 1992; Lowe and Ruley 1993). Both Myc and E1A can induce p53 stabilization and trigger p53-dependent transcription (Lowe and Ruley 1993; Hermeking and Eick 1994; Wagner et al. 1994). Several lines of evidence indicate that p53 mediates apoptosis by *myc* and *E1A* in primary fibroblasts, with *p53* loss rendering cells highly resistant to their deleterious effects (Debbas and White 1993; Lowe and Ruley 1993; Hermeking and Eick 1994; Wagner et al. 1994). For cells overexpressing *myc* to grow, programmed cell death must be actively suppressed (Askew et al. 1991; Evan et al. 1992; Hermeking and Eick 1994; Wagner et al. 1994). Therefore, *myc* overexpression should provide a strong selective pressure for events that dismantle apoptotic signaling pathways. Here, we show that *ARF* is a target of *myc* activation and that loss of *ARF*, like loss of p53, can attenuate *myc*-induced cell death. We suggest that ARF's normal role is to respond to hyperproliferative signals, thereby facilitating p53 activation through a signaling pathway that differs from those induced by DNA damage.

Results

ARF is induced by explanting MEFs into culture

ARF is not detectably expressed during mouse embryogenesis, and disruption of the gene has no effect on development (Kamijo et al. 1997; Zindy et al. 1997). However, when MEFs were explanted into culture and cells were serially transferred on a 3-day schedule (3T9 protocol), $p19^{ARF}$ was induced at early passages and increased steadily thereafter (Fig. 1, wild-type MEFs). Its accumulation inversely correlates with the rate of MEF cell proliferation, which gradually slows and eventually ceases as cells reach replicative ''crisis'' (passages 17–20 on this protocol) (Kamijo et al. 1997). Expression of $p19^{ARF}$ in *p53*-null MEFs was elevated and temporally advanced as compared to that in wild-type cells (Fig. 1), consistent with the ability of ARF and p53 to regulate each other's levels and activities (see introductory section). In contrast, the loss of $p21^{Cip1}$, a p53-responsive gene product that negatively regulates progression through the cell cycle (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993), did not affect ARF levels (Fig. 1).

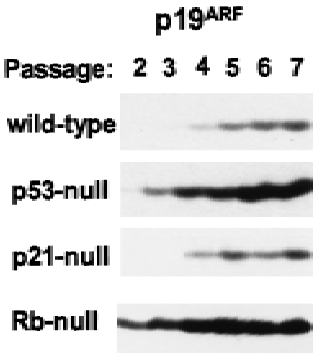

Figure 1. Expression of $p19^{ARF}$ in early-passage, primary MEF strains. MEFs of the indicated genotypes (left) propagated on a 3T9 protocol were harvested at passage numbers given at the *top,* lysed, and immunoblotted for $p19^{ARF}$ protein expression. Equal quantities of protein (200 μg) were loaded per lane.

Although expression of $p19^{ARF}$ in MEFs could connote a role in replicative senescence, the basis for its accumulation was puzzling. One clue was provided by observations that *Rb*-null MEFs greatly overexpressed $p19^{ARF}$ (Fig. 1), possibly reflecting a propensity of Rb-regulated E2F transcription factors to influence *ARF* gene expression. A previous survey of *INK4a* responses to various E2F family members noted that the level of *ARF* mRNA rose in response to infection of REF52 cells by adenoviruses encoding *E2F-1* and, to a lesser extent, *E2F-2,* but not those specifying *E2F3*, *E2F4*, or *E2F5* (DeGregori et al. 1997). Second, the ability of *ARF*-null MEFs to grow continuously and to be transformed by *ras* alone mimics the effects of *myc* and *E1A* on normal MEFs (Land et al. 1983; Ruley 1983). This led us to the idea that some immortalizing function of *myc* might be dispensable in *ARF*-null cells. The underlying hypothesis is that ARF normally functions to safeguard cells against sustained and potentially oncogenic hyperproliferative signals (as opposed to DNA damage), thereby explaining why its loss strongly predisposes to tumor development. (For supporting data involving E1A, see de Stanchina et al. 1998).

Induction of ARF by myc

We examined the effects of ectopic *myc,* Ha-*ras* (Val-12), and *E2F-1* on *ARF* gene expression by infecting early passage (p5) MEFs with retrovirus vectors expressing these genes, or with a control vector expressing the T cell coreceptor *CD8.* Wild-type, *ARF*-null, and *p53*-null cells were infected three times at 4 hr intervals with high titer replication-defective viruses. By 48 hr after infection, >95% of MEFs infected with the control virus expressed cell-surface CD8, as determined by fluorescence-activated flow cytometry (FACS) using a cognate antibody (data not shown), indicating that virtually all cells were productively infected.

Patterns of various RNAs and proteins expressed 48 hr after infection are illustrated in Figure 2, A and B, respec-

tively. When wild-type MEFs were infected with *myc* virus, we observed induction of *ARF* mRNA without significant changes in the levels of *INK4a* transcripts (Fig. 2A, lanes 1,2). This correlated with increased expression of $p19^{ARF}$ protein without an observable change in $p16^{INK4a}$ (Fig. 2B, lane 2 vs. lane 1). Thus, *myc* selectively induced *ARF* expression within the first 2 days after infection. Ectopic *myc* expression led to 2-fold increases in *p53* mRNA levels (Fig. 2A, lanes 1,2) (Hermeking and Eick 1994; Roy et al. 1994) and to 8- to 10-fold increases in p53 protein (Fig. 2B, lanes 1,2), resulting from p53 stabilization (data not shown). This was accompanied by accumulation of the p53-responsive gene products, Mdm2 (Fig. 2B, lane 2), and $p21^{Cip1}$ (see Fig. 3, below). In contrast, we did not observe effects of *myc* overexpression on the levels of the Bcl-2 or Bax proteins (data not shown). Infection of wild-type MEFs with a retrovirus vector encoding oncogenic Ha-*ras* did not affect expression of $p19^{ARF}$, p53, or Mdm2, although we did observe a slight increase in $p16^{INK4a}$ levels by 48 hr postinfection (Fig. 2B, lane 3). Virtually all wild-type MEFs infected with *E2F-1* virus died by apoptosis within 48 hr after infection (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994), preventing us from assaying $p19^{ARF}$ protein levels under these conditions.

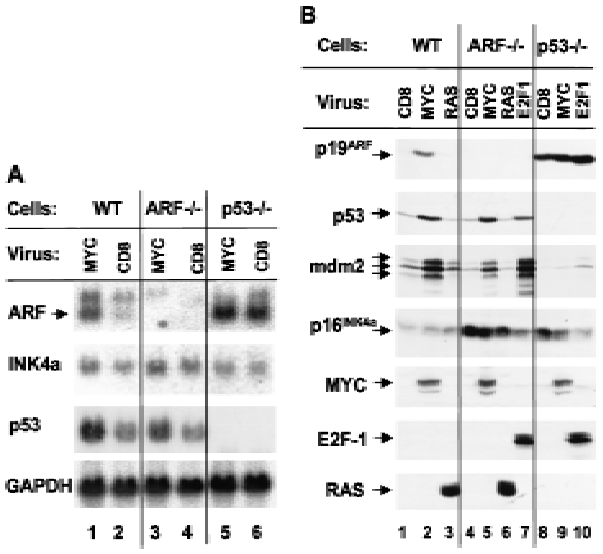

Figure 2. Expression of ARF, p53, and p53 targets in virus-infected MEFs. (*A*) Wild-type (WT), *ARF*-null, or *p53*-null MEFs (*top*) were infected with either a control (*CD8*) or *myc*-expressing retrovirus. At 48 hr postinfection, total RNA was isolated from infected cells, electrophoretically separated, blotted to filters, and hybridized sequentially with ^{32}P-labeled probes specific for *ARF* (exon 1β), *INK4a* (exon 1α), *p53*, and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). (*B*) Replicate cultures infected with *CD8* or *myc* viruses, or infected with *ras* or *E2F-1* vectors (*top*) were lysed 48 hr postinfection and immunoblotted using antibodies directed to the proteins indicated at *left*. Wild-type cells infected with the *E2F-1* virus died and could not be analyzed (see text). Because a smaller fraction of cells from other Myc-infected and E2F-1-infected cultures underwent apoptosis, equal quantities of protein were loaded per lane to provide valid comparisons.

To assess whether the effects of *myc* on p53 were *ARF*-dependent, we infected *ARF*-null MEFs with *myc* retrovirus. Here, the introduction of *myc* also increased expression of *p53* mRNA (Fig. 2A, lanes 3,4) and both p53 and Mdm2 proteins (Fig. 2B, lanes 4,5), indicating that the ability of *myc* to induce p53 was, at least in part, ARF-independent. This activity was observed in response to very high levels of Myc achieved 48 hr post infection, but was less pronounced at later times when Myc levels were reduced (see Fig. 6A, below). Again, oncogenic *ras* had no such effects (Fig 2B, lane 6). As reported previously, $p16^{INK4a}$ levels are elevated in *ARF*-null cells (Kamijo et al. 1997), and in this setting, neither Ha-*ras* nor *myc* appeared to regulate the protein (Fig. 2B, lanes 5,6).

Although *p53*-null cells express relatively high basal levels of $p19^{ARF}$, their infection by *myc* retrovirus further augmented the levels of *ARF* mRNA (Fig. 2A, lane 5 vs. lane 6) and protein (Fig. 2B, lane 9 vs. lane 8). In four such experiments using *p53*-null MEFs, enforced *myc* expression reproducibly elevated $p19^{ARF}$ levels 1.5- to 3-fold, implying that *myc* can induce *ARF* via a p53-independent pathway. In contrast, Mdm2 (Fig. 2B, lane 9) and $p21^{Cip1}$ (shown in Fig. 3, below) were not induced in *p53*-null cells, indicating that their up-regulation by *myc* was strictly p53-dependent. Interestingly, *ARF*-null cells, like *p53*-null cells (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994), were partially resistant to killing by *E2F-1*, and we were therefore able to document E2F-1 overexpression on a per protein basis (Fig. 2B, lanes 7,10). Like *myc*, *E2F-1* induced both p53 and Mdm2 (lane 7), and Mdm2 induction was p53-dependent (lane 10). Importantly, *E2F-1* induced $p19^{ARF}$ in *p53*-null cells to a level somewhat higher than that seen in *myc*-infected cells (lanes 10 vs. lane 9), whereas the amounts of $p16^{INK4a}$ were diminished (lanes 7,10). Thus, *E2F-1*, like *myc*, induced both $p19^{ARF}$ and p53, and triggered Mdm2 expression in a p53-dependent manner.

To determine the kinetics of the Myc response, we infected MEFs with a retrovirus vector encoding *myc* fused to the 4-hydroxytamoxifen (4-HT)-responsive domain of the estrogen receptor (ER; Eilers et al. 1991; Littlewood et al. 1995), together with a linked gene encoding resistance to puromycin. Following selection of infected cells for 2 days with puromycin under conditions in which all uninfected MEFs are killed, 4-HT was added to the medium and cells were assayed for $p19^{ARF}$ and p53 protein expression as Myc activity was induced. Figure 3 shows a representative experiment comparing wild-type, *ARF*-null, and *p53*-null cells. Ectopic Myc–ER protein levels were equivalent in the three cell lines (data not shown). In wild-type MEFs, 1.8-fold induction of $p19^{ARF}$ was observed within 3 hr of 4-HT treatment, rising to 8.5-fold above the basal level by 24-hr (Fig. 3, lanes 1–5). Induction of p53 was more protracted with a significant elevation (1.8-fold) occurring 6 hr after addition of 4-HT and reaching a maximum (3-fold above basal levels) by 12 hr of treatment. Both Mdm2 and $p21^{Cip1}$ were induced with kinetics similar to that of p53 (lanes 1–5) but, as expected, were not induced in *p53*-null

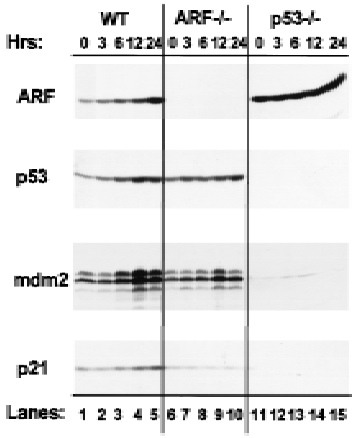

Figure 3. Induction of ARF, p53, mdm2, and $p21^{Cip1}$ by Myc–ER. MEFs of the indicated genotypes (*top*) infected with a *myc*–ER virus were treated with 4-HT for the indicated intervals (hr), and cell lysates were immunoblotted with antibodies directed to the proteins indicated at *left*. Levels of Myc–ER expressed in the three cell types were comparable (data not shown).

cells (lanes 11–15). In these experiments, the constitutively high levels of $p19^{ARF}$ expressed in *p53*-null cells were not increased further upon 4-HT treatment (lanes 11–15).

In *ARF*-null cells expressing Myc–ER, p53 levels rose only twofold during the same induction period, in agreement with the concept that p53 induction is partially ARF-dependent (Fig. 3, lanes 6–10). In accord with these findings, induction of the p53-responsive Mdm2 protein was attenuated (lanes 6–10). These results are not consistent with the idea that $p19^{ARF}$ stabilizes p53 by accelerating Mdm2 turnover (Zhang et al. 1998). Basal levels of $p21^{Cip1}$ are significantly reduced in *ARF*-null cells (lane 6; see Kamijo et al. 1997), and, surprisingly, no induction of $p21^{Cip1}$ was seen in response to 4-HT treatment (lanes 6–10). These differences in p53 response between wild-type and *ARF*-null MEFs were observed in independent experiments using two different *Myc*–ER-containing vectors (see Materials and methods). Therefore, Myc rapidly induced $p19^{ARF}$, but in its absence, p53, Mdm2, and $p21^{Cip1}$ induction were all significantly impaired. Taken together, the above data indicate that (1) *myc* induces *ARF* via p53- and Mdm2-independent pathways; (2) *myc* likely up-regulates p53 through both *ARF*-dependent and independent pathways; and (3) *myc* induction of Mdm2 and $p21^{Cip1}$ is strictly dependent on p53.

ARF loss attenuates myc-*induced apoptosis*

Apoptosis induced by *myc* in fibroblasts deprived of serum survival factors (Evan et al. 1992) depends on p53 (Hermeking and Eick 1994; Wagner et al. 1994). Because *myc* increased the levels of both $p19^{ARF}$ and p53, *myc*'s ability to trigger apoptosis might also be ARF-dependent. Cells in which the biochemical consequences of *myc* overexpression had been documented 2 days postinfection (Fig. 2) were expanded in culture for 2 additional days and then shifted into chemically defined medium containing insulin, transferrin, and BSA as the only exogenously added proteins. Under serum-free conditions, *myc*-infected wild-type MEFs rapidly underwent apoptosis as defined by propidium iodide staining for subdiploid DNA content, visualization of nuclear condensation and blebbing in Hoescht 33342-stained cells, determination of membrane integrity by vital dye exclusion, and TUNEL FACS analysis (Fig. 4A). Similarly, a majority of wild-type MEFs that had been infected with *myc*–ER virus and induced with 4-HT for 24 hr (Fig. 3) died by apoptosis within a day after transfer to serum-free medium (Fig. 4B, solid symbols). Previously uninduced cells that were shifted into serum-free medium containing 4-HT for 24 hr also died (Fig. 4B, open symbols). In each case, *p53*-null cells were highly resistant to *myc*-induced apoptosis, whereas the apoptotic response of *ARF*-null cells was less compromised (Fig. 4A,B).

To explore longer-term effects, cells infected for 4 days with *myc*- or control *CD8* vectors were propagated in either serum-containing or serum-free medium, and their growth rates were determined (Fig. 5). Early passage (p5) wild-type MEFs infected with control *CD8* virus pro-

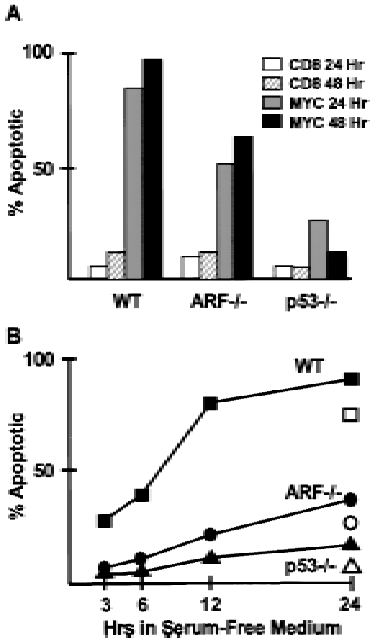

Figure 4. Myc-induced apoptosis. (*A*) MEFs of the indicated genotypes infected with *myc* or *CD8* virus for 48 hr (the same populations as in Fig. 2) were cultured for 2 more days and then transferred into serum-free medium for an additional 48 hr. Apoptosis was scored using a propidium iodide-based FACS assay to quantitate cells with subdiploid DNA content 24 and 48 hr after serum starvation. Viruses and times of infection are indicated (*top right*). All standard deviations were within 10% of the means shown. (*B*) Cells of the indicated genotypes infected with *myc*–ER virus and pretreated with 4-HT for 24 hr (the same populations as in Fig. 3) were shifted into serum-free medium (solid symbols), and apoptosis was scored by propidium iodide FACs assay at the indicated times (abscissa). Untreated, viable cells were also shifted into serum-free medium containing 4-HT and scored 24 hr later (open symbols).

liferated in medium containing serum (Fig. 5A, ●) yet underwent only one population doubling in 7 days when serum was removed (Fig. 5A, ○). More than 85% of serum-deprived cells remained viable and arrested in the G_1 phase of the cell cycle. However, as first reported by others (Evan et al. 1992), *myc*-infected MEFs harvested 4 days after infection proliferated less well in the presence of serum (Fig. 5A, ■) and had a considerably higher apoptotic index (10%–15% TUNEL-positive), so that their rate of growth was in part counterbalanced by cell death. When they were shifted to serum-free medium, cells ectopically expressing *myc* underwent apoptosis rapidly (Fig. 5A, □). After only 24 hr, the majority were already dead (Figs. 4A and 5), and by 4 days, no viable cells remained.

Passage 5 *ARF*-null and *p53*-null MEFs grew somewhat more rapidly than their wild-type counterparts in the presence of serum (Fig. 5, B and C, ●), but still exited the cell cycle when deprived of serum (Fig. 5, B and C, ○). Although *myc*-infected *ARF*-null cells transferred to serum-free medium initially underwent apoptosis, a significant fraction survived and continued to proliferate (Fig. 5B, □). By 14 days after infection, these cells were completely resistant to *myc*-induced apoptosis (data not shown) and grew as rapidly in serum-free medium as did uninfected cells propagated in the presence of serum (Fig. 5B, △). Cells lacking *p53* were even more resistant to *myc*-induced apoptosis, undergoing less cell death than *ARF*-null cells in the first few days after infection (Fig. 5C). All resistant populations continued to express MYC protein ectopically (see Fig. 6, below), confirming that they had been infected. Hence, the effects of *myc* on apoptosis were significantly attenuated in the absence of ARF or p53 function, and after a few days of selection in serum-free medium, *myc* ultimately acted as a pure growth promoter.

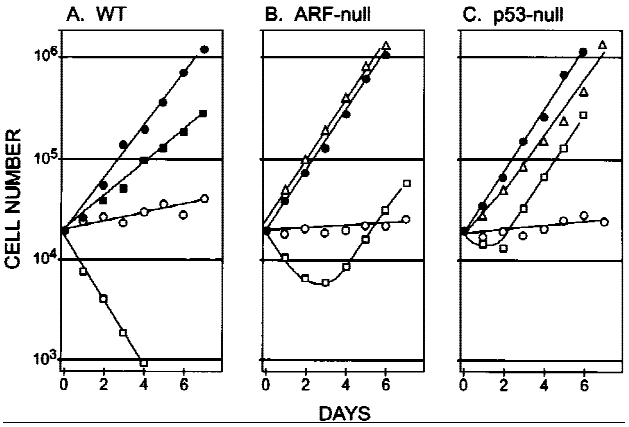

Figure 5. Rates of proliferation of virus-infected MEFs. Wild-type (*A*), *ARF*-null (*B*), and *p53*-null (*C*) MEFs infected with control *CD8* virus were transferred to serum-containing (●) or defined serum-free (○) media 4 days postinfection and counted every day thereafter. Wild-type cells infected with *myc* virus grew more slowly in serum-containing medium (*A*, ■) and died in medium lacking serum (*A*, □). A significant number of *myc*-infected *ARF*-null and *p53*-null cells survived in serum-free conditions (B, C, □). When reseeded 14 days postinfection, these *myc*-infected cells grew continuously in serum-free medium (*B,C*, △). All data points represent averages of six to eight determinations using at least three independently derived MEF strains with S.D. less than ±25% of the mean (highly significant on log scale).

Although *ARF*-null and *p53*-null cells were relatively resistant to *myc*-induced apoptosis, their response was biphasic. Significant fractions were killed in the first few days after *myc* virus infection and serum withdrawal, after which resistant cells grew out. Acute phase killing was more severe in *ARF*-null than in *p53*-null cells (Fig. 5, B and C, □), consistent with Myc's ability to target p53 through an ARF-independent pathway (see above). In addition, very high levels of Myc were achieved in the first 1–3 days after infection, but declined as infected MEFs were propagated and were reduced by almost 80% by the time that cells became completely resistant to apoptosis (about day 14) (data not shown, but see Fig. 6, below). Acutely elevated levels of Myc also killed a fraction of *p53*-null cells (Fig. 4A), accounting for their initial growth lag in serum-free medium (Fig. 5C, □). A critical issue is whether *myc* overexpression could have selected for additional genetic changes that obviated a requirement for *ARF* function. To test this possibility, we reintroduced the *ARF* gene into surviving *ARF*-null *myc* overexpressors that had acquired the ability to proliferate in serum-free medium. Reinfection of these cells with an *ARF* but not control *CD8* retrovirus resensitized them to apoptosis in serum-free medium (42% viability in *ARF*-infected cells vs. >90% in *CD8*-infected cells at 24 hr postinfection). Therefore, resistance to apoptosis was a direct consequence of *ARF* loss and was not due to mechanisms that bypass *ARF* function. Similar effects were observed using *E1A* in lieu of *myc* (de Stanchina et al. 1998).

Myc-induced apoptosis selects for cells that lose either p53 or ARF function

Because *myc* overexpression in wild-type MEFs induces apoptosis and slows their overall proliferative rate in serum-containing medium (Fig. 5A, closed symbols), we reasoned that continued passage of these cells might select for resistant, more rapidly proliferating variants that spontaneously lose ARF or p53 function. *myc*-virus-infected wild-type strains maintained in serum-containing medium and studied 7–10 days after infection initially remained sensitive to apoptosis when deprived of serum. By this time, the cells synthesized very high levels of $p19^{ARF}$ (Fig. 6A, lanes 2,3) equivalent to those seen in *p53*-null cells (lane 9). To distinguish wild-type from mutant p53, cells were metabolically labeled with [^{35}S]-methionine for 2 hr, and lysates were precipitated using conformation-specific antibodies (Yewdell et al. 1986; Gannon et al 1990). *myc* infection increased the rate of wild-type p53 synthesis (Fig. 6A, lanes 2 and 3 vs. lane 1), consistent with induction of *p53* mRNA (Fig. 2A). Because of its longer half-life, the steady-state levels of p53 in *myc*-infected versus *CD8* virus-infected cells, as

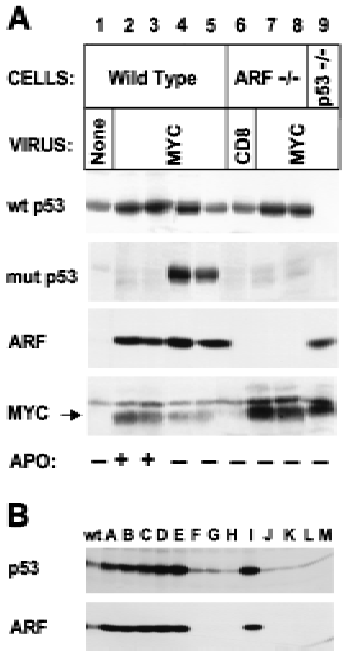

Figure 6. Myc-"immortalized" MEFs lose p53 or ARF function. (*A*) MEFs of the indicated genotype were infected with *CD8* or *myc* retroviruses at passage 5 after explantation and propagated on a 3T9 protocol. Wild-type cells tested 7–10 days after *myc* virus infection (lanes *2,3*) expressed relatively high levels of $p19^{ARF}$ and wild-type (wt) p53, and were initially sensitive to apoptosis (APO +) when transferred into serum-free medium (see text). However, by 14–21 days postinfection, rapidly growing derivatives were isolated that could grow under serum-free conditions (APO −) and expressed mutant (mut) p53 (lanes *4,5*). *ARF*-null cells infected at passage 5 and transferred 14 days after selection in serum-free medium were resistant to apoptosis but expressed only wild-type p53 (wt) (lanes *7,8*). Note that Myc protein levels were significantly higher in *ARF*-null (lanes *7,8*) and *p53*-null (lane *9*) cells than in wild-type MEFs (lanes *2–5*). Apoptosis was determined by FACS analysis of propidium iodide- and Hoescht 33342-stained cells. (*B*) Cells containing a single wild-type *ARF* allele were infected with *myc* virus for 4 days and transferred into serum-free medium for 2 days to select for variants resistant to apoptosis. Surviving cells were diluted in microtiter wells and subclones were expanded from single cells in serum-containing medium. Lysates were then blotted for $p19^{ARF}$ and p53. Results with 13 clones (designated *A–M*) are compared with those obtained with wild-type (wt) uninfected MEFs.

judged by immunoblotting, differed even more significantly (Fig. 2B). At this time after infection, no mutant forms of p53 were detected (lanes *2,3*).

By 14–21 days after infection, wild-type MEFs infected with *myc* virus and maintained in medium containing serum no longer underwent apoptosis when transferred to serum-free medium and continued to proliferate as established cell lines. Emerging variants were readily identified by their much smaller size (1.11 pL vs. 3.3 pL mean corpuscular volume), accelerated growth rate, and ability to proliferate in serum-free medium. Four such independently derived cell lines expressed mutant, dominant-negative forms of p53 in addition to the wild-type form of the protein (Fig. 6A, lanes 4 and 5 show results with two such lines). In contrast, *myc*-infected *ARF*-null cell lines growing in serum-free conditions expressed only wild-type *p53* (Fig. 6A, lanes 7,8). Therefore, *myc*-induced immortalization of wild-type cells selected for p53 loss of function, but such selection was obviated in cells lacking *ARF*.

In general, *ARF*-null cells tolerated higher levels of ectopic Myc protein than did wild-type MEFs that had acquired p53 mutations in the course of infection (cf. Myc levels in lanes 7 and 8 with those in lanes 4 and 5). Moreover, wild-type MEFs that were initially sensitive to Myc-induced apoptosis expressed higher levels of Myc than did resistant variants (cf. Myc levels in Fig. 6, lanes 2 and 3 vs. lanes 4 and 5). These results are consistent with the idea that high levels of Myc are selected against by apoptosis until resistant variants emerge.

In continuing studies of spontaneously immortalized wild-type MEFs that emerged from crisis on a 3T9 protocol, we determined that 23 of 28 individually derived cell lines had sustained p53 mutations, whereas the remainder exhibited biallelic loss of *ARF*. In principle, biallelic *ARF* loss might also occur during *myc*-induced establishment, but this should again be a less frequent event than p53 mutation, involving two-hit versus one-hit kinetics (Kamijo et al. 1997; Zindy et al. 1997). Moreover, because *myc* virus-infected populations are polyclonal, attempts to demonstrate biallelic *ARF* loss in a subset of cells would be occluded by the presence of other cells in the population containing mutant p53 and expressing high levels of $p19^{ARF}$. To determine whether *ARF* loss can also occur in response to enforced Myc expression, MEFs hemizygous for a wild-type *ARF* allele were infected with *myc* virus, propagated in serum-free medium for 2 days, and then shifted back into medium containing serum. Surviving cells were subcloned by limiting dilution, expanded, and then assayed for $p19^{ARF}$ expression and for the presence of wild-type and mutant p53. Of 26 clones, eleven exhibited p53 mutations, whereas the other 15 lacked detectable $p19^{ARF}$. Figure 6B shows results with 13 representative clones designated A–M. Mutant p53 was expressed at high levels (clones A–E and I) compared to those in uninfected wild-type MEFs. As expected, clones with mutant p53 also expressed higher levels of $p19^{ARF}$ than wild-type cells. In contrast, *ARF*-null variants (clones F–H and J–M) expressed low levels of wild-type p53. Southern blotting confirmed the loss of the wild-type *ARF* allele in the latter cases (data not shown). Therefore, immortalization of wild-type MEFs by *myc* leads to either *ARF* or p53 loss and confers resistance to Myc-induced apoptosis.

Discussion

Signaling to ARF and p53

Cells protect themselves from mutant cancer genes (i.e., mutated oncogenes or loss of tumor suppressors) through compensatory mechanisms that arrest cell growth or induce cell suicide (for review, see Sherr 1996; Weinberg

1997). Expression of activated *ras* in primary MEFs inhibits cell growth (Serrano et al. 1997), whereas overexpression of *myc* in these same cells triggers apoptosis, a process further aggravated by withdrawal of serum survival factors (Evan et al. 1992). Yet, introduction of *myc* and *ras* together into primary rodent embryo fibroblasts elicits cell transformation (Land et al. 1983). *myc* must somehow block *ras*-mediated inhibition of cell proliferation, whereas conversely, *ras* may play a role in attenuating the apoptotic function of Myc (Weinberg 1997).

One hypothesis is that cultured cells achieve replicative immortality by inactivating their *INK4a* or *p53* genes (for review, see Weinberg 1997). To some extent, this idea was based on the ability of MEFs from *INK4a/ARF*-null mice to grow continuously after explantation into culture and to be transformed by oncogenic *ras* alone (Serrano et al. 1996; 1997). Yet, MEFs from mice lacking *ARF* alone exhibit the immortalized features previously attributed to disruption of *INK4a*, implying that loss of $p19^{ARF}$ in lieu of $p16^{INK4a}$ enables oncogenic *ras* alleles to transform these cells. Given that Ras positively regulates the synthesis of D-type cyclins and their assembly with CDK4 (Cheng et al. 1998 and references therein), a role for $p16^{INK4a}$ in antagonizing these growth promoting activities of Ras would be expected (see Fig. 7 for schematic). Pomerantz et al. (1998) recently demonstrated that overexpression of *ARF* in rat embryo fibroblasts transformed by *myc* plus *ras* was significantly more potent than *INK4a* in suppressing transformation. Moreover, they found that $p19^{ARF}$, but not $p16^{INK4a}$, suppressed transformation by E1A plus Ras in a p53-dependent manner, consistent with the idea that $p19^{ARF}$ acts downstream of pRb (and E2F-1) in countering oncogenic signaling (Fig. 7). We therefore propose that either $p19^{ARF}$ or p53 inactivation provides an immortalizing function that mimics certain actions of Myc and E1A and renders primary MEFs more susceptible to *ras*-induced transformation. Clearly, this model does not preclude a requirement for other growth-promoting functions of Myc and E1A in immortalizing wild-type cells.

Overexpressed *myc* can signal through $p19^{ARF}$ and p53 to trigger apoptosis, although its effects can be overridden by serum survival factors (Fig. 7). Overexpression of *myc* induces the accumulation of $p19^{ARF}$, at least in part by increasing *ARF* gene expression. Induction of $p19^{ARF}$ synthesis by a conditionally active Myc–ER fusion protein occurred within 3 hr of 4-HT treatment and temporally preceded p53 accumulation and p53-dependent expression of Mdm2 and $p21^{Cip1}$. Although we have found that *myc* can induce p53 through an ARF-independent pathway, its induction of p53 and p53-responsive gene products is significantly compromised in *ARF*-null cells. For unexplained reasons, we observed greater attenuation of the $p21^{Cip1}$ response than that of Mdm2 in *ARF*-null cells, implying that not all p53-responsive genes are equally affected by *ARF* loss. In contrast, other signals that induce p53, such as DNA damage by radiation, are effective in the complete absence of *ARF* (Kamijo et al. 1997). Therefore, *myc* signals to p53 at least in part through an ARF-dependent pathway, which is distinct from that triggered by DNA damage.

ARF and Myc-induced apoptosis

Because enforced expression of *ARF* itself arrests wild-type MEFs but does not kill them (Quelle et al. 1995b), a function of Myc other than *ARF* induction is required to trigger apoptosis. Nonetheless, the loss of either *ARF* or *p53* confers significant resistance to *myc*-induced cell death, and these effects of ARF, like its ability to induce cell cycle arrest, are p53-dependent. In cultures of wild-type MEFs acutely infected with *myc* retrovirus, a significant proportion of the cells underwent apoptosis even when grown in the presence of serum. In the face of Myc overexpression, there was a strong selective advantage for cells that sustained p53 mutations, and once such variants emerged, these soon predominated and were able to continuously proliferate in chemically defined medium lacking serum. Results using *myc*-infected MEFs containing a single functional *ARF* allele demonstrated that *ARF* loss, rather than p53 mutation, could also lead to establishment, in agreement with previous observations made with cells that had undergone spontaneous immortalization (Kamijo et al. 1997). As in the latter cases, loss of ARF or p53 function appeared to be mutually exclusive events, indicating that *ARF* loss can relieve *myc*-induced selective pressure for p53 mutation.

A conceptual dilemma is posed by observations that *ARF*-null cells infected with *myc* virus were initially

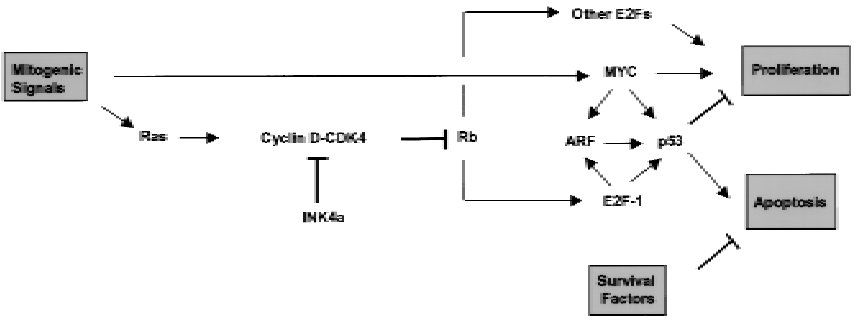

Figure 7. Model for ARF signaling. *ARF* is activated via Myc and E2F-1 and acts in turn to trigger p53-dependent cell cycle arrest or apoptosis, depending on the presence of extracellular survival factors. Ras acts through cyclin D-dependent kinases to stimulate pRB phosphorylation, resulting in release of E2F from pRb constraint and activation of *E2F*-responsive genes. Activation of *ARF* by MYC and E2F-1 need not be direct, although both transcription factors have been demonstrated to increase *ARF* mRNA levels (see text). Like Myc, different E2F isoforms are proposed to regulate both cell growth and cell death. In inhibiting cyclin D-dependent kinases, $p16^{INK4a}$ can modulate certain growth-promoting functions of Ras. Other functions of Myc and Ras are not detailed in the schematic.

sensitive to apoptosis when shifted into serum-free medium, although significantly less so than wild-type MEFs. After several days in serum-free medium, apoptosis was no longer detected, and the *myc* virus-infected, *ARF*-null cells again grew rapidly. This raised the possibility that *myc* overexpression selected for additional cryptic genetic changes that rendered the cells resistant to apoptosis. However, when cells that had resumed proliferation in serum-free medium were infected with an *ARF* virus, they promptly died, implying that attenuation of apoptosis was a direct consequence of *ARF* loss. Therefore, we favor the interpretation that the high levels of *myc* expression achieved acutely after virus infection were able to kill cells through an ARF-independent pathway, likely involving p53 directly. Myc levels fell as infected MEFs were propagated, and because both *ARF*-null and *p53*-null cells tolerate higher levels of Myc than wild-type cells, they appear to become resistant to apoptosis without further selection.

ARF function in tumor surveillance

Other immortalizing oncogenes, such as adenovirus *E1A*, can act like *myc* in triggering apoptosis in an ARF-dependent manner (see de Stanchina et al. 1998). Among its many effects, E1A releases E2F1, E2F2, and E2F3 from pRb constraint; E2F-1 can selectively induce the *ARF* gene (DeGregori et al. 1997) and protein expression, and trigger apoptosis in a p53-dependent manner (Wu and Levine 1994; Qin et al. 1994; Shan and Lee 1994; Kowalik et al. 1995). In agreement with these findings, MEFs lacking *Rb* exhibited relatively high levels of $p19^{ARF}$ expression, and E1A mutants that are unable to interact with pRb were handicapped in their ability to induce $p19^{ARF}$ (de Stanchina et al. 1998). Because *Rb*-null MEFs undergo replicative senescence in culture (F. Zindy et al., unpubl.), high $p19^{ARF}$ levels should sensitize them to apoptosis as long as p53 function is intact. Similarly, in an in vivo mouse model using the developing murine lens, *Rb* deficiency triggers apoptosis in a largely p53-dependent manner (Morgenbesser et al. 1994). Lenses from animals lacking exon 2 of the *INK4a* gene, and hence likely disrupted for both *INK4a* and *ARF* function, exhibited less apoptosis than wild-type lenses but more than that observed in a *p53*-null background (Pomerantz et al. 1998).

Unlike ARF, p53 also integrates signals emanating from DNA-damage response pathways. Cancer cells are generally considered to have conserved normal p53 function if they retain wild-type p53 and exhibit an intact p53-dependent DNA damage checkpoint response. However, if such cells lack *ARF*, they are still compromised in their p53 response, because they would fail to respond to hyperproliferative signals induced by oncogenes such as *myc*. The fact that hyperproliferative signals and DNA damage pathways can collaborate to induce p53 suggests that cells sustaining oncogenic stimulation would initially be more susceptible than their normal counterparts to chemotherapeutic drugs and to radiotherapeutic regimens that induce DNA damage (de Stanchina et al. 1998). Loss of *ARF* would disable this synergy, making tumor cells more resistant to treatment and ultimately selecting for p53 loss in the face of higher dose therapy. *ARF* function may have evolved to harness the apoptotic machinery precisely for the purpose of preventing abnormal cell growth in response to oncogenic signals. This would explain why its loss is such a common event in many different forms of cancer.

Materials and methods

Cell culture

MEFs from day 14.5 embryos (wild-type, *ARF*-null, *p53*-null, *p21*-null) or day 13.5 embryos (*Rb*-null) were explanted and maintained on a 3T9 protocol (9×10^5 cells transferred at 3-day intervals) (Kamijo et al. 1997) and propagated in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 10 μg/ml gentamycin (GIBCO). *ARF*-null cells were established in our laboratory. MEFs from *p53*-null cells were derived from mice purchased from the Jackson Laboratories. Timed *p21*-null and *Rb*-null pregnant females were generously provided by Stephen Elledge (Baylor College of Medicine, Houston, TX) and Tyler Jacks (MIT, Cambridge, MA), respectively. Where indicated, cultured MEFs at passage 5 were switched to defined, serum-free medium containing insulin, transferrin, and BSA as the only added proteins (Roussel and Sherr 1989). At the same time, cells infected for 48 hr with the indicated retroviruses were diluted and plated at 2×10^4 cells/60-mm-diam. dish in 4 ml of complete medium. The following day, fresh medium containing or lacking serum was added, and cells from replicate cultures were counted every day thereafter. Viability was determined by trypan blue exclusion, and DNA fragmentation was monitored using a terminal deoxynucleotidyl transferase (FACS TUNEL) assay (Gorczya et al. 1993) and by measurement of subdiploid (<2N) DNA content of propidium iodide-stained nuclei (Askew et al. 1991). Where indicated, cells grown for 10–14 days post-infection in complete serum-containing medium were diluted as above and their kinetics of proliferation and survival in serum-free medium were reassessed.

Cells hemizygous for a functional *ARF* allele were infected with *myc* retrovirus, and 4 days postinfection were transferred into serum-free medium for 2 days. Survivors were plated in complete medium at limiting dilution in 96-well microtiter plates, and 26 clones derived from single cells were expanded and assayed for $p19^{ARF}$ and p53 proteins as shown in Figure 6B. Cells lacking *ARF* were confirmed by Southern blotting to have segregated the residual wild-type allele. The presence of mutant and wild-type p53 was confirmed by immunoprecipitation of metabolically labeled cell lysates with conformation-specific antibodies (see below).

Virus infection

Human kidney 293T cells were from Dr. David Baltimore (California Institute of Technology, Pasadena). A helper ecotropic retrovirus plasmid defective in Ψ2 packaging sequences, and pSRα vectors containing human c-*myc* (Roussel et al. 1995) and *CD8* (Quelle et al. 1995b) were provided by Dr. Charles Sawyers (UCLA). The human *E2F1* cDNA, provided by Dr. Scott Hiebert (Vanderbilt University, Nashville, TN), or oncogenic Ha-*ras* (Val12) cDNA, from Dr. Michael M. White (Southwestern Medical Center, Dallas, TX), was cloned into the same vector in place of c-*myc*. A *myc*–ER retroviral vector containing a linked gene for puromycin-resistance was provided by Drs. Dean Felsher

and J. Michael Bishop (UCSF). The ER moiety is unable to bind estrogen yet retains its affinity for the synthetic ligand, 4-HT (Littlewood et al. 1995). The cDNA cassette encoding *myc*–ER was also expressed in the pSRα vector and MEFs infected with this retrovirus yielded similar results to those shown in Figures 3 and 4B. Viruses produced by cotransfection of 293T cells with vector and helper virus plasmids (Roussel et al. 1995) were harvested every 6 hours, 24–72 hours after transfection. Pooled, filtered supernatants (three successive additions of 2 ml at 4-hr intervals) were used to infect naive primary MEF strains (2×10^5 cells plated/100-mm-diam. dishes) in the presence of 10 μg/ml Polybrene (Sigma, St. Louis, MO). At 12 hr postinfection, 10 ml of fresh medium was added, and medium was changed 24 hr later. Cells infected with Myc–ER virus were selected in 2 μg/ml puromycin for 48 hr prior to treatment of surviving cells with 1 μM 4-HT for times indicated in Figure 3.

RNA and protein expression

Total RNA was separated electrophoretically in gels containing formaldehyde (20 μg/lane), blotted to nitrocellulose, and detected using ^{32}P-labeled probes specific for exons 1α (*INK4a*) and 1β (*ARF*) of the mouse *ARF–INK4a* locus (Quelle et al. 1995b; Zindy et al. 1997). Proteins were detected by direct immunoblotting. Frozen cell pellets (~ 2 mg protein) were lysed on ice in Tween 20 lysis buffer (50 mM HEPES at pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 1 mM PMSF, 0.4 U/ml aprotinin, 1 mM NaF, 10 mM β-glycerophosphate, and 0.1 mM Na orthovanadate), sonicated 2 × 7 sec (Virtis VirSonic 475, 12%–14% power), and left on ice for 30 min. Debris was removed by sedimentation at 4°C in a microcentrifuge (5 min at 15,000 rpm), and protein was quantitated using a BCA kit (Pierce, Rockford IL). Samples (200 μg of protein per lane) were separated by SDS–PAGE and transferred to nitrocellulose membranes (MSI, Westboro MA). Filters were washed in TBS-Tween (10 mM Tris HCl at pH 7.4, 150 mM NaCl, 0.1% Tween 20) and blocked in the same solution with 10% (wt/vol) nonfat dry milk. Filters were then exposed for 1–2 hr at room temperature to either 0.2 μg/ml affinity-purified rabbit antibody to the mouse $p19^{ARF}$ carboxyl terminus (Quelle et al. 1995b) or the $p16^{INK4a}$ carboxyl terminus (Quelle et al. 1995a); rabbit antiserum to E2F-1 (from Scott Hiebert); or monoclonal antibodies directed to p53 (Ab-7, Calbiochem La Jolla, CA), Mdm2 (2A10 provided by Gerard Zambetti, St. Jude Children's Reseach Hospital), $p21^{Cip1}$ (F-5, Santa Cruz Biochemicals, CA), human Myc (06340, Upstate Bio., Inc) or $p21^{ras}$ (rat mAb 259, Santa Cruz Biochemicals). Those filters exposed to affinity-purified rabbit antibodies to $p19^{ARF}$ were washed for 45 min in TBS–Tween and incubated 45 min with a 1/2000 dilution of donkey antibodies to rabbit IgG (Amerham) in TBS–Tween containing 5% milk. All filters were then rewashed as described above and antibody binding sites were visualized by enhanced chemiluminescence using appropriate second antibody conjugates or horseradish peroxidase-conjugated protein A (for $p16^{INK4a}$) as per the manufacturer's instructions (ECL kit, Amersham). For discrimination of mutant and wild-type forms of p53, cells were metabolically labeled with [^{35}S]methionine and lysed, and cleared lysates were precipitated with antibodies that detect either mutant or wild-type forms of the protein (Yewdell et al. 1986; Gannon et al. 1990) as described previously (Kamijo et al. 1997).

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