A *CDKN2A* Mutation in Familial Melanoma that Abrogates Binding of $p16^{INK4a}$ to CDK4 but not CDK6

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Abstract

The *CDKN2A* locus encodes two distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$, both of which are implicated in replicative senescence and tumor suppression in different contexts. Here, we describe the characterization of a novel strain of human diploid fibroblasts (designated Milan HDFs) from an individual who is homozygous for the R24P mutation in $p16^{INK4a}$. As this mutation occurs in the first exon of *INK4a* (exon 1α), it has no effect on the primary sequence of $p14^{ARF}$. Based on both *in vitro* and *in vivo* analyses, the R24P variant is specifically defective for binding to CDK4 but remains able to associate with CDK6. Nevertheless, Milan HDFs behave as if they are $p16^{INK4a}$ deficient, in terms of sensitivity to spontaneous and oncogene-induced senescence, and the R24P variant has little effect on proliferation when ectopically expressed in normal fibroblasts. It can, however, impair the proliferation of U20S cells, presumably because they express more CDK6 than primary fibroblasts. These observations suggest that CDK4 and CDK6 are not functionally redundant and underscore the importance of CDK4 in the development of melanoma. [Cancer Res 2007;67(19):9134–41]

Introduction

Germ line mutations in the human *CDKN2A* locus (commonly referred to as *INK4a/ARF*) are associated with predisposition to melanoma and other malignancies (1). The locus is also subject to mutation, homozygous deletion, and transcriptional silencing in a range of sporadic cancers (2, 3). In what seems to be a unique arrangement in the human genome, *INK4a/ARF* encodes two structurally distinct proteins, $p14^{ARF}$ and $p16^{INK4a}$, that incorporate amino acid sequences specified by different translational reading frames in a shared exon (2, 3). Both products have the capacity to block cell proliferation and are implicated in replicative senescence and tumor suppression, albeit in different contexts. Whereas $p16^{INK4a}$ acts by preventing phosphorylation of the retinoblastoma protein (pRb) by cyclin-dependent kinases (CDK), $p14^{ARF}$ acts primarily by preventing the MDM2-mediated ubiquitylation of p53 (2, 3).

More specifically, $p16^{INK4a}$ binds directly to CDK4 and CDK6, two closely related kinases that serve as catalytic partners for the D-type cyclins (4). Much of the existing literature implies or assumes that CDK4 and CDK6 are functionally interchangeable. By binding to the kinase subunits, $p16^{INK4a}$ compromises their interactions with D-type cyclins and thereby blocks the formation of active kinase complexes (5, 6). However, most of the D-type cyclins in the cell participate in high molecular weight complexes that contain members of the CIP/KIP family of CDK inhibitors, in addition to CDK4 or CDK6 (e.g., see refs. 7–9). Whether these ternary complexes are catalytically active remains open for debate; however, they nevertheless perform an important function by regulating the availability of $p21^{CIP1}$ and $p27^{KIP1}$ to inhibit other kinases. Thus, $p16^{INK4a}$ can indirectly inhibit CDK2 by sequestering CDK4 and CDK6 and causing the redistribution of $p21^{CIP1}$ and $p27^{KIP1}$ from cyclin D–dependent complexes onto cyclin E– or cyclin A–CDK complexes (7, 10, 11).

When ectopically expressed in primary human diploid fibroblasts (HDF), $p16^{INK4a}$ induces a growth arrest that phenotypically resembles the M1 stage of replicative senescence (12–14). As $p16^{INK4a}$ spontaneously accumulates when cells reach the end of their proliferative life span (15–17), it is generally assumed that it contributes to the implementation of M1. Classic experiments have established that M1 can be bypassed by agents that interfere with pRb and p53 function, such as the SV40 large T-antigen, or the human papilloma virus (HPV) E6 and E7 proteins (13). If both pRb and p53 are inactivated, the cells proliferate until they reach the M2 phase of senescence where continued cell proliferation is offset by cell death (13, 14). If only one of the tumor suppressor pathways is disabled, then HDFs senesce at a stage that is operationally between M1 and M2, referred to as M^{INT} or M1.5 (18–20). In line with these ideas, we have previously shown that $p16^{INK4a}$-deficient HDFs, isolated from individuals who have mutations in both alleles of *INK4a*, undergo senescence at this intermediate stage (20).

Germ line mutations occur throughout the coding region of the *INK4a* gene, as well as in noncoding regions, and a variety of strategies have been used to assess their effect on the function of the resultant protein (2). The most reliable indicators are whether the $p16^{INK4a}$ variants associate with CDK4 and CDK6, either *in vitro* or in a cell-based setting, and whether the variants can block cell proliferation when ectopically expressed. In general, mutants that do not bind to CDK4 and CDK6 are unable to cause cell cycle arrest. However, a substantial proportion of melanoma-associated germ line variants are able to interact with CDK4 and CDK6 under experimental conditions, yet show reduced potency in cell proliferation assays (21). The latter category of mutations may affect protein folding or stability, or cause relatively subtle changes in kinase binding affinity that are difficult to quantify using currently available assays.

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The three-dimensional structure of $p16^{INK4a}$ comprises four ankyrin-like repeats, and residues from different repeat units contribute to the interaction with CDKs (5, 6). The distribution of inactivating mutations suggests that the structural integrity of $p16^{INK4a}$ is critical for its function (2). In the vast majority of cases, disabling mutations affect binding to both CDK4 and CDK6, in line with the idea that these related kinases are likely to adopt very similar structures. The one exception we have noted thus far is the R24P variant, which, in a simple *in vitro* test, appeared to be specifically defective for binding to CDK4 (22), although opinions differ about this interpretation (23–25). Identification of an individual who is homozygous for the R24P mutation prompted us to reexamine its properties by isolating and characterizing primary fibroblasts from this patient. In a variety of contexts, we confirm that the R24P variant is unable to interact with CDK4 but continues to bind to CDK6. Despite this unusual property, fibroblasts homozygous for this mutation behave as if they are $p16^{INK4a}$ deficient, as judged by delayed implementation of replicative senescence and resistance to oncogene-induced senescence. Importantly, although the R24P variant seems to be incapable of halting the proliferation of human fibroblasts, it does impair the growth of U20S cells, which express higher levels of CDK6. As well as underscoring the importance of $p16^{INK4a}$ rather than $p14^{ARF}$ in tumor suppression, our findings suggest a pivotal role for CDK4 rather than CDK6 in melanoma development, consistent with the evidence that *CDK4* is also a high-risk melanoma susceptibility gene (1).

Materials and Methods

Cell culture and retroviral infection. The Hs68 and TIG3 strains of normal human fibroblasts, and the Milan fibroblasts described here, were grown as monolayers in DMEM supplemented with 10% fetal bovine serum. Cells were passaged at a 1:4 split ratio, equivalent to two population doublings or a 1:8 ratio (three population doublings) before they reached confluence. Methods for infecting human fibroblasts with ecotropic retroviruses and cell proliferation assays have been described in detail elsewhere (12, 21). Cells were pulse-labeled with BrdUrd for 1 h, and incorporation was assayed using a kit from Roche according to the manufacturer's protocol.

The EH1 strain of U20S cells, expressing wild-type $p16^{INKa}$ from an isopropyl-L-thio-β-D-galactopyranoside (IPTG)–inducible promoter, has been described elsewhere (7). A similar strategy was used to generate cells expressing the R24P variant.

Immunoprecipitation and immunoblotting. For direct immunoblotting, cells were washed twice with PBS, resuspended in lysis buffer containing 62.5 mmol/L Tris-HCl (pH6.8) and 2% (w/v) SDS, and boiled for 10 min. Protein concentrations were determined using the Pierce BCA system according to the manufacturer's protocol. For immunoprecipitation, the cells were suspended in NP40 buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1% (v/v) NP40, 20 μmol/L AEBSF, 10 μmol/L EDTA, 1.3 μmol/L bestatin, 0.14 μmol/L E-64, 0.01 μmol/L leupeptin, and 0.003 μmol/L aprotinin; snap frozen on dry ice; and clarified by centrifugation at 18,000 × *g* for 15 min at 4°C. Samples of total protein (200–500 μg) were incubated with 2 to 20 μL of antibody and 20 μL of protein A or G beads, overnight at 4°C. The beads were washed four times in cold NP40 buffer, and the protein complexes were removed from the beads by the addition of 25 μL 2× sample buffer followed by boiling for 10 min.

Proteins were fractionated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and immunoblotted with appropriate primary antibodies in PBS containing Tween 20 (0.2% v/v) and dried milk powder (5% w/v). Immune complexes were detected using horseradish peroxidase–linked secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Where possible, we used the monoclonal antibody DCS50 to detect $p16^{INK4a}$ because the other commonly used antibody, JC8, does not recognize the R24P variant or versions of $p16^{INK4a}$ with mutations in adjacent residues.[3] In some experiments, $p16^{INK4a}$ was visualized with sc468 (Santa Cruz Biotechnology) because of loss of sensitivity in batches of DCS50. The $p14^{ARF}$ protein was immunoprecipitated with the JR14 polyclonal antibody and immunoblotted using the 4c6 monoclonal antibody (26). Proteins carrying the HA epitope were detected with the 12CA5 antibody. Other antibodies were as follows: CDK4 (sc601), CDK6 (sc177), $p21^{CIP1}$ (sc397), $p16^{INK4a}$ (sc468 and sc759), and p53 (DO-1) were from Santa Cruz Biotechnology. The mitogen-activated protein/extracellular signal-regulated kinase 1/2 (MEK1/2) antibody was obtained from Cell Signalling Technology, and antibodies against Ras (pan-Ras, AB-4) and MDM2 (4B11) were obtained from Oncogene Science.

***In vitro* binding assays and kinase assays.** *In vitro* binding assays were carried out as previously described (26) using components synthesized *in vitro* by coupled transcription and translation. The protein complexes were immunoprecipitated using the DPAR12 polyclonal antibody, separated by SDS-PAGE, and visualized by autoradiography.

Results

Functional evaluation of the R24P variant of $p16^{INK4a}$. In our original assessment of the R24P variant of $p16^{INK4a}$, based on an *in vitro* association assay, we provided evidence that it was specifically defective for binding to CDK4 (22). In its simplest form, the assay measures the coprecipitation of small amounts of labeled proteins produced by coupled *in vitro* transcription and translation (27). Whereas wild-type $p16^{INK4a}$ is capable of binding to CDK4 and CDK6 in this system, mutants with complete loss of function, such as A20P, show no significant association with either kinase (Fig. 1*A*). Under similar conditions, the R24P variant showed relatively robust binding to CDK6 but not to CDK4 (Fig. 1*A*).

To extend these findings, we used recombinant retroviruses to express 2×HA-tagged versions in normal HDFs (20). The resulting drug-resistant cell pools were analyzed ~6 days postinfection. Expression of the exogenous 2×HA-tagged proteins was confirmed by immunoblotting with an anti-HA antibody (Fig. 1*B, top*). Cell lysates were then immunoprecipitated with antibodies against CDK4 and CDK6 and the amounts of coprecipitated $p16^{INK4a}$ were determined by immunoblotting with the monoclonal antibody, DCS50. This antibody detects both the exogenous tagged versions and the endogenous $p16^{INK4a}$, which serves as an internal control. As expected, 2×HA-tagged wild-type $p16^{INK4a}$ coprecipitated with both CDK4 and CDK6, whereas the A20P variant did not. In line with the *in vitro* binding data, the R24P variant coprecipitated with CDK6 but not with CDK4.

We also monitored the proliferative potential of the infected cell pools by comparing the relative numbers of viable cells over time (Fig. 1*C*). As anticipated, expression of wild-type $p16^{INK4a}$ caused a substantial reduction in cell proliferation, whereas the cells expressing the nonfunctional A20P variant continued to proliferate as well as the control cells infected with empty vector. Surprisingly, the R24P variant had only a marginal effect on cell proliferation and similar results were obtained in several independent comparisons with other $p16^{INK4a}$ variants (additional data not shown). By these criteria, the R24P variant would register as a complete loss of function mutant (21) despite its clear ability to interact with CDK6.

[3] F. Gregory, J. Rowe, and S. Brookes, unpublished observations.

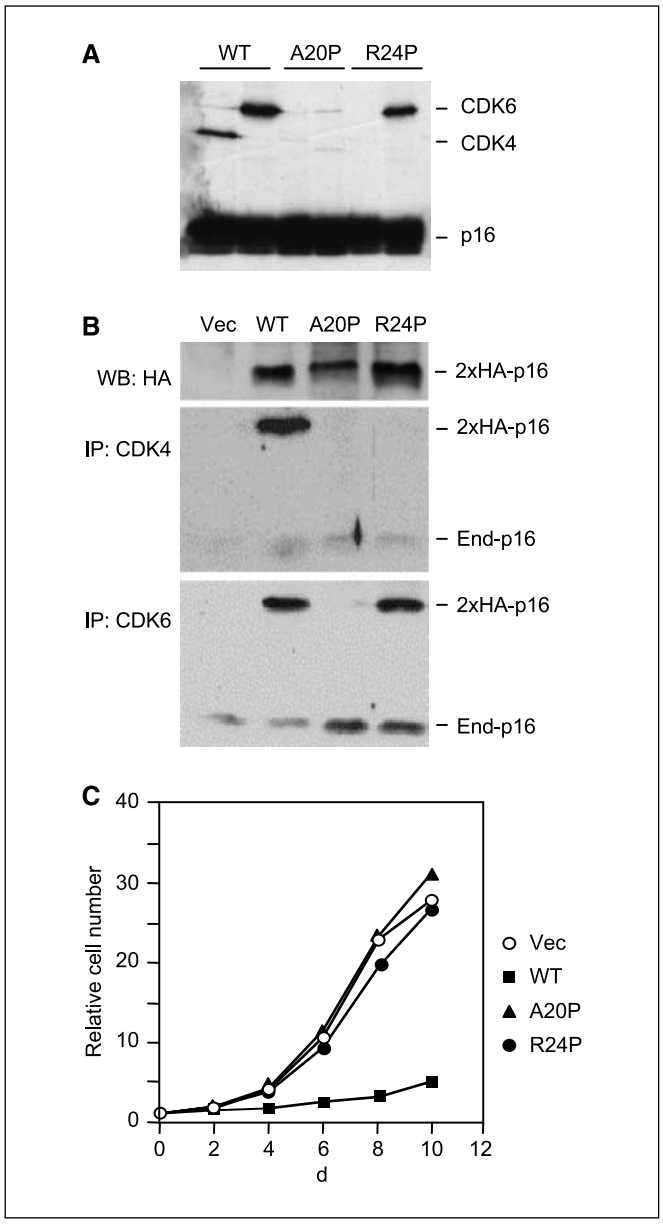

Figure 1. Functional evaluation of the R24P variant of $p16^{INK4a}$. *A,* the wild-type (*WT*), A20P, and R24P variants of $p16^{INK4a}$ were labeled with [^{35}S]methionine by coupled transcription and translation *in vitro*. CDK4 and CDK6 were synthesized in the same way, mixed with equivalent amounts of the $p16^{INK4a}$ products at 30°C, and then immunoprecipitated with a polyclonal antibody against $p16^{INK4a}$. The precipitated proteins were analyzed by SDS-PAGE in a 12% gel and visualized by autoradiography. *B,* HDFs were infected with recombinant retroviruses encoding 2×HA-tagged $p16^{INK4a}$ variants, or the empty vector (*Vec*), and pools of cells were recovered by selection in the appropriate antibiotic. Samples of total protein (30 μg) were separated by SDS-PAGE and immunoblotted directly with an HA antibody (*top*). Equivalent amounts of cell lysate (300 μg) were immunoprecipitated (*IP*) with antibodies against CDK4 or CDK6, fractionated by SDS-PAGE in a 12% gel, and immunoblotted (*WB*) with an antibody against $p16^{INK4a}$. The positions of the 2×HA-tagged and endogenous $p16^{INK4a}$ are indicated. *C,* proliferation assays conducted on the cells transduced with the $p16^{INK4a}$ variants. Cells were stained with crystal violet and relative cell numbers were determined by measurement of the absorbance at 590 nm, normalized to the value on day 0.

Isolation and initial characterization of the Milan strain of HDFs. Analysis of the segregation of the R24P allele within an extended Italian family identified an individual who is homozygous for the mutation (Fig. 2). In view of the rarity of this situation, and the particular attributes of the R24P variant, we developed a culture of primary skin fibroblasts from a biopsy conducted on the homozygous patient. We have designated this strain as "Milan HDFs."

Access to Milan HDFs made it possible to confirm that the endogenous $p16^{INK4a}$ in these cells showed a capacity to discriminate between CDK4 and CDK6 *in vivo*. To enhance the expression of endogenous $p16^{INK4a}$, we introduced the receptor for mouse ecotropic retroviruses and then infected the cells with a retrovirus encoding SV40 large T-antigen. Normal fibroblasts were analyzed in parallel. Following selection of infected cell pools, the cell lysates were immunoprecipitated with polyclonal antibodies against $p16^{INK4a}$, CDK4, and CDK6, and the complexes were analyzed by immunoblotting for the same proteins. In the normal HDFs, both CDK4 and CDK6 were present in the $p16^{INK4a}$ immunoprecipitates and endogenous $p16^{INK4a}$ was coprecipitated with CDK4 and CDK6 (Fig. 2*B*). In contrast, the 16^{INK4a} immune complexes from Milan cells contained CDK6 but not CDK4, and $p16^{INK4a}$ was not detectable in the CDK4 immunoprecipitate (Fig. 2*B*).

Another important feature of the R24P mutation is that it occurs within exon 1α and therefore does not affect the coding sequence of $p14^{ARF}$. We nevertheless sought to verify that the ARF protein functions normally in the Milan cells. To enhance the expression of $p14^{ARF}$, which is generally below detectable levels in primary HDFs, we infected the cells with a recombinant retrovirus encoding an ER-E2F1 fusion protein (26). Upon addition of 4-hydroxy tamoxifen to activate E2F1, 14^{ARF} became detectable by immunoblotting. No signal was observed in the control cells containing the empty vector or in the absence of inducer (Fig. 2*C*). Induction of ARF was accompanied by a corresponding up-regulation of MDM2 and p53, and under these conditions it was possible to show an association between endogenous ARF and MDM2 (Fig. 2*C, bottom*). Although this experiment does not provide a quantitative measure of $p14^{ARF}$ activity, it suggests that the major pathway downstream of ARF is operating in the Milan cells.

Replicative senescence of Milan HDFs. When propagated continuously in standard conditions of tissue culture, the Milan cells had a replicative life span of ~40 population doublings (Fig. 3). This was considerably less than the maximum population doublings achieved by two other strains of $p16^{INK4a}$-deficient fibroblasts that we characterized previously, which reached ~60 and 80 population doublings, respectively (20). In the Leiden and Q34 strains, the mutant $p16^{INK4a}$ is incapable of binding to either CDK4 or CDK6. It was therefore important to establish whether Milan cells were arresting at M1 or at the stage between M1 and M2 that we have termed M1.5. To explore this issue, we used recombinant retroviruses to express the HPV E6 protein, which disables p53, or the SV40 T-antigen, which disables both pRb and p53. Normal fibroblasts transduced with E6 senesce at the M1.5 stage, whereas cells expressing T-antigen proceed to M2 (Fig. 3*A*). With Milan fibroblasts, both agents allowed the cells to proliferate for a further 18 to 22 population doublings, at which point they had features characteristic of M2 (Fig. 3*B*). This implied that although the R24P variant retains the ability to bind CDK6, the Milan fibroblasts were senescing at the M1.5 stage, as if the p16/pRb pathway was completely disabled. Consistent with this, the Polycomb group protein Bmi1, which suppresses the expression of $p16^{INK4a}$, had no effect on the life span of the Milan HDFs (Fig. 3*B*) but was able to extend the life span of the control HDFs, as previously described (9, 20, 28).

Ability of Milan cells to evade oncogene-induced senescence. One of the reasons for our interest in the Milan strain of HDFs is that the homozygous germ line mutation does not affect the coding sequence of $p14^{ARF}$. In principle, therefore, they provided a unique opportunity to confirm the importance of $p16^{INK4a}$ in oncogene-induced senescence in human fibroblasts, without any collateral effects from $p14^{ARF}$. To this end, we infected the cells with a retrovirus encoding the V12 allele of H-Ras and compared their behavior to that of normal HDFs. Whereas the normal cells underwent growth arrest, as judged by reduced labeling with BrdUrd, the effect on Milan cells was much less dramatic (Fig. 4*A*). After a transient period of adaptation, the majority of the cells resumed proliferation at a marginally faster rate than the cells transduced with the empty vector (Fig. 4*B*). This is in sharp contrast to the control Hs68 cells, which essentially ceased proliferation and developed features of senescence within 2 weeks postinfection. In this regard, the Milan cells were again very similar to the Leiden strain of HDFs. However, the interpretation is complicated by the fact that in several independent experiments, expression of oncogenic Ras in Milan cells caused only a modest increase in the level of $p16^{INK4a}$ compared with that achieved in the control cells (Fig. 4*C*). It is therefore impossible to tell whether the behavior of the Milan cells reflects impaired $p16^{INK4a}$ function or the muted response of $p16^{INK4a}$ to Ras.

The R24P variant impairs proliferation of U20S cells. At face value, the ability of the R24P variant to bind to CDK6 seemed to have little effect on the proliferation or senescence of HDFs. A straightforward explanation for these data would be that CDK4 is the more abundant and therefore more relevant kinase in human fibroblasts and that CDK4 and CDK6 are functionally redundant.

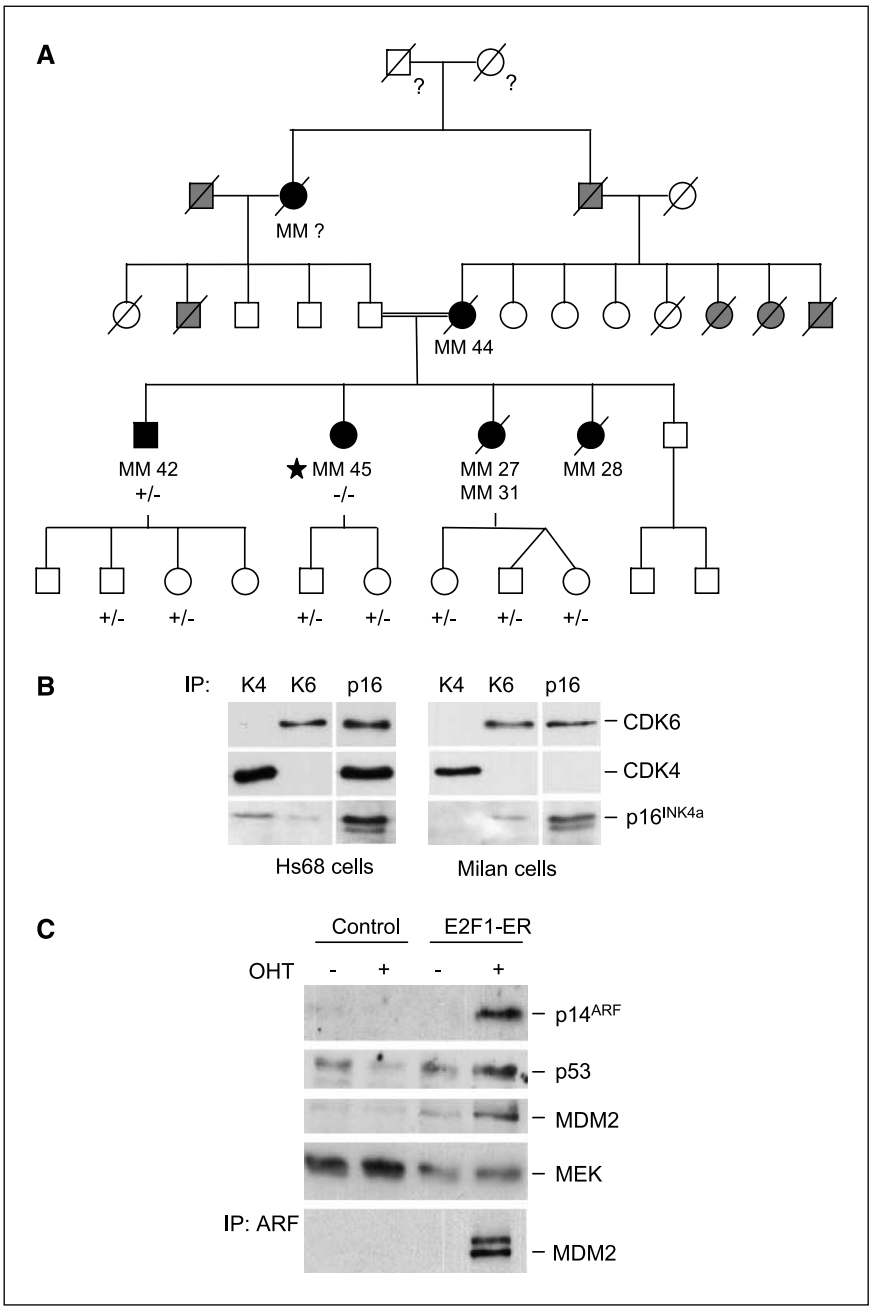

Figure 2. Functional evaluation of endogenous $p16^{INK4a}$ and $p14^{ARF}$ in Milan HDFs. *A,* pedigree of the melanoma kindred from which the Milan cells were derived, showing the incidence of malignant melanoma (*MM, black shading*) and unspecified cancer (*gray shading*) within the affected family, with numbers referring to the age of diagnosis, where known. ?, clinical records of malignant melanoma are not available or information is missing (grandparents). +/−, germ line status of *CDKN2A*: +, wild-type; −, R24P mutation. ★, the homozygous individual; note that the parents are consanguineous. *B,* control (Hs68) and the Milan strain of fibroblasts were infected with a retrovirus encoding SV40 T-antigen to enhance expression of endogenous $p16^{INK4a}$. Samples (500 μg) of total protein were immunoprecipitated with polyclonal antibodies against CDK4 (K4), CDK6 (K6), or $p16^{INK4a}$ and immunoblotted with antibodies against CDK4, CDK6, and $p16^{INK4a}$ as indicated. *C,* Milan HDFs infected with a retrovirus encoding the E2F1-ER fusion protein, or an empty vector control, were treated with 4-hydroxy tamoxifen (*OHT*) for 24 h. Samples (25 μg) of total protein were fractionated by SDS-PAGE in a 15% gel, and immunoblotted with antibodies against the indicated proteins. In the lower panel, samples (1 mg) of total protein were immunoprecipitated with a rabbit polyclonal antiserum against human $p14^{ARF}$, fractionated by SDS-PAGE in a 10% gel, and immunoblotted with a monoclonal antibody against MDM2.

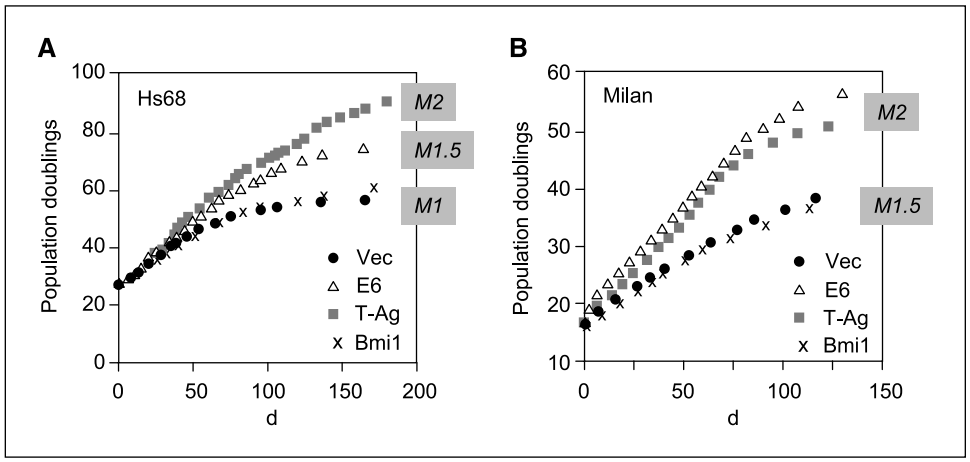

Figure 3. Replicative senescence in Milan HDFs. Hs68 (*A*) or Milan cells (*B*) were infected with retroviruses encoding HPV E6, SV40 T-antigen, Bmi1, or the empty vector control, and after drug selection, the infected cell pools were propagated continuously in culture until they failed to double in 4 to 6 wk. The curves show cumulative population doublings against time. The phenotypic properties of the senescent cells are indicated on the right as M1, M1.5, and M2.

Another possibility is that R24P is in fact functionally compromised despite retaining some capacity to interact with CDK6. This prompted us to consider whether R24P would have a demonstrable effect in a cell system in which CDK6 plays a more prominent role. Although there are human tumor cell lines that do not express detectable levels of CDK6 (29, 30), we have not found a human cell line that lacks CDK4. To try to model such a situation, we explored the possibility of reducing CDK4 expression or enhancing CDK6 expression in HDFs but both approaches have serious limitations. Ectopic expression of CDK4 or CDK6 causes a substantial up-regulation of the endogenous $p16^{INK4a}$ (9, 19), confounding attempts to show increased sensitivity to exogenous $p16^{INK4a}$. Conversely, short hairpin RNA–mediated knockdown of CDK4 significantly reduces cell proliferation, making it virtually impossible to monitor the additional antiproliferative effects of the $p16^{INK4a}$ variants in these cells. As an alternative, we turned to the U20S osteosarcoma line, which has been used to assess the effects of $p16^{INK4a}$ on proliferation in several previous studies (7, 10, 11) and expresses significantly higher levels of CDK6 than present in HDFs. U20S cells also have functional pRb and p53 and the additional advantage that the endogenous *INK4a/ARF* locus is silenced.

As an initial test, U20S cells were transiently transfected with plasmids encoding wild-type $p16^{INK4a}$, or the A20P and R24P variants. The cells were then plated at clonal density and the numbers of colonies counted after 3 weeks. As shown in Fig. 5*A*,

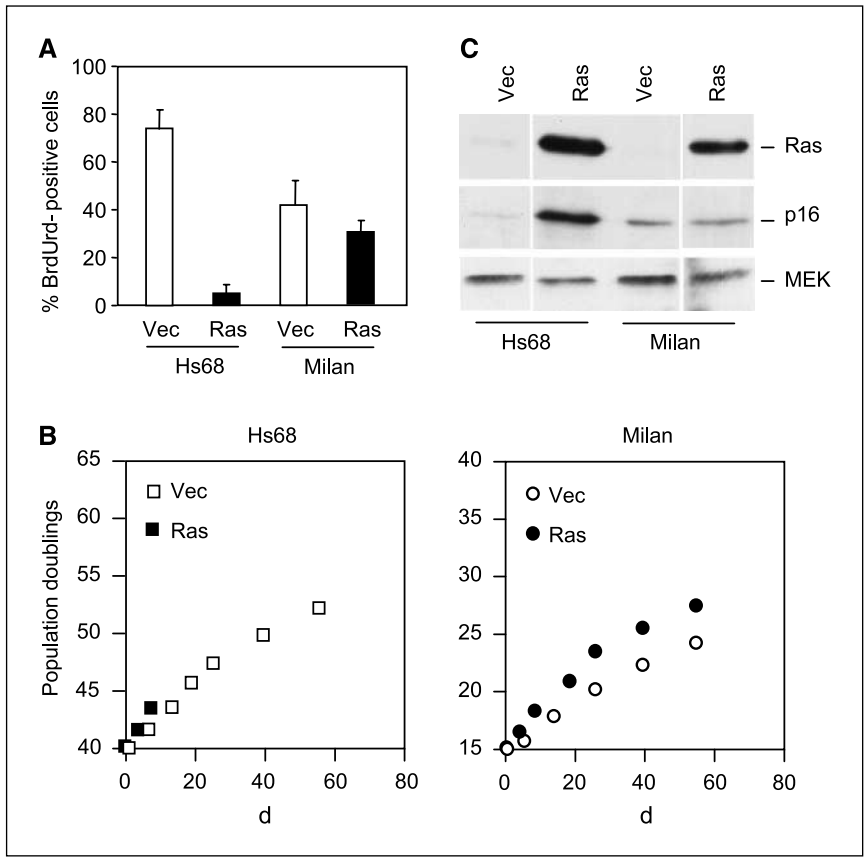

Figure 4. Milan HDFs are resistant to Ras-induced senescence. Milan and control (Hs68) HDFs were infected with a retrovirus encoding the V12 allele of H-Ras or empty vector. *A,* after drug selection, the cell pools were labeled with BrdUrd and the proportion of BrdUrd-positive cells were determined by immunohistochemistry. The proportion of stained versus unstained cells was determined by counting at least 300 cells of each type. *Columns,* average of counts; *bars,* SD. *B,* samples (30 μg) of total protein were fractionated by SDS-PAGE and immunoblotted for Ras, $p16^{INK4a}$, and MEK. *C,* proliferation of Ras-infected cells over an extended period. The plots show cumulative population doublings plotted against time.

wild-type $p16^{INK4a}$ caused an ~10-fold reduction in colony numbers, whereas the A20P variant had only a marginal effect. In this assay, the R24P variant caused a clear reduction in colony numbers, although the difference was less pronounced than with wild-type $p16^{INK4a}$. Similar results were obtained in the MCF7 cell line (data not shown).

To substantiate these findings, we generated a derivative of the U20S cell line (JR1) in which expression of the R24P variant can be regulated by IPTG. We have previously described an equivalent cell line (EH1) that expresses wild-type $p16^{INK4a}$ from an IPTG-regulated promoter (7). In both cell lines, addition of IPTG caused a substantial induction of $p16^{INK4a}$, although the JR1 line also showed some basal expression in the absence of the inducer (Fig. 5*B*). The $p16^{INK4a}$ in EH1 cells has a lower molecular weight because these cells were generated using a version of $p16^{INK4a}$ that lacked the amino-terminal eight amino acids. As expected, induction of wild-type $p16^{INK4a}$ in EH1 cells caused G_1 arrest, as judged by BrdUrd incorporation (Fig. 5*C*) and flow cytometry (data not shown). Importantly, induction of the R24P variant also caused an arrest in the JR1 cells, although the effects were less pronounced than in EH1 cells (Fig. 5*C*). The muted effects would be consistent with the idea that R24P targets only one arm of the cyclin D–dependent kinases.

We also used the inducible system to compare the half-lives of the wild-type and R24P form of $p16^{INK4a}$. Following induction with IPTG, the cells were treated with cycloheximide and the levels of $p16^{INK4a}$ were assessed by immunoblotting (Fig. 5*B*). As previously observed, $p16^{INK4a}$ is a relatively stable protein with a half-life of the order of 12 to 16 h. Importantly, the R24P variant was not measurably less stable than the wild-type protein.

In line with published studies (7), the induction of wild-type $p16^{INK4a}$ in EH1 cells caused a reduction in the amount of $p21^{CIP1}$ associated with CDK4 and CDK6 (Fig. 6). The effects applied to both CDK4 and CDK6, and there was no residual $p21^{CIP1}$ associated with CDK6. In cells expressing the R24P variant, induction of $p16^{INK4a}$ again caused redistribution of $p21^{CIP1}$ from CDK6 but with little if any effect on the $p21^{CIP1}$-CDK4 association. We presume that the displaced $p21^{CIP1}$ associates with and inhibits CDK2 but it has proved difficult to document a quantitative difference between EH1 and JR1 cells in this respect, in part because of changes in the overall levels of CDK2 in the arrested cells (ref. 6 and data not shown). Nevertheless, the results would be consistent with the idea that the ability of R24P to cause cell cycle arrest depends on the level of endogenous CDK6 in the cell and therefore the proportion of the total $p21^{CIP1}$ (and other CIP/KIP proteins) that is sequestered in cyclin D-CDK6 complexes.

Discussion

The R24P variant of *INK4a* has been identified in a number of melanoma-prone families from different geographic locations (1, 22, 24, 31–33). We became particularly interested in this variant because it has the unusual property of being specifically defective for binding to CDK4 (22). However, several other studies have drawn different conclusions regarding its properties. For example, one reported impaired binding to CDK4 in a two-hybrid screen (24), whereas another suggested that R24P binds to CDK4 but is unable to cause arrest in a melanoma cell line (23). In a separate report, R24P was unable to inhibit CDK4 kinase activity in a reconstituted system, which would concur with lack of binding, but also seemed unable to arrest U20S cells in a transient transfection assay (25). Our data go some way to reconciling these reports by demonstrating that although R24P is unable to arrest primary fibroblasts, it can partially impair the proliferation of U20S cells, both in transient transfection assays and following inducible expression in stable cell clones.

Assays based on the ectopic expression of *INK4a* variants are open to the concern that they represent artificial situations that are prone to technical variability. However, access to the Milan fibroblasts provided unambiguous evidence that the endogenous R24P associates exclusively with CDK6. These analyses were made possible by the identification of an individual who is homozygous for the mutation. Such situations are very rare, and to date we are only aware of two other examples (34, 35), and an even more remarkable case with distinct alterations in each *INK4a* allele (26). Characterization of primary dermal fibroblasts from two of these

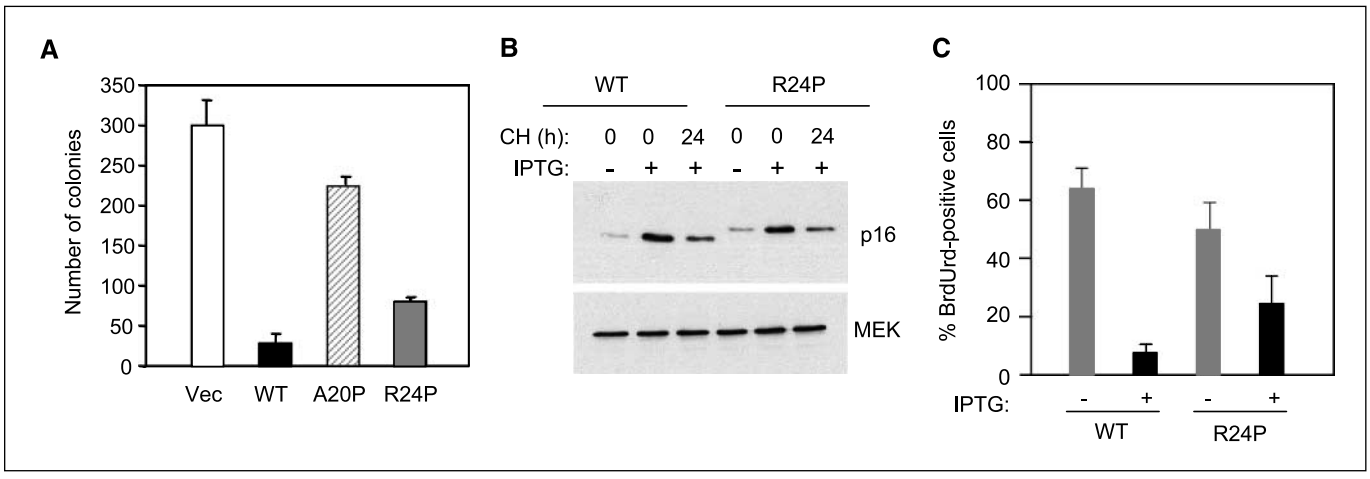

Figure 5. Ability of the R24P variant of $p16^{INK4a}$ to arrest U20S cells and inhibit CDK6 activity. *A,* U20S cells were transfected with plasmids encoding the wild-type, and A20P and R24P variants of $p16^{INK4a}$, and maintained in medium containing G418. Colonies were counted after 3 wk. The data represent an average of three independent transfections. *B,* induction of $p16^{INK4a}$ in U20S cells expressing wild-type $p16^{INK4a}$ or the R24P variant from an IPTG-inducible promoter. *Lanes 3* and *6,* cells were treated with cycloheximide (*CH*) for 24 h to block new protein synthesis. Note that the wild-type $p16^{INK4a}$ used in these experiments lacked the amino-terminal eight amino acids. MEK was used as a control for loading. *C,* cells treated with or without IPTG as in *B* were pulse-labeled with BrdUrd and the percentage of BrdUrd-positive cells was assessed by immunostaining. *Columns,* averages from six independent wells; *bars,* SD.

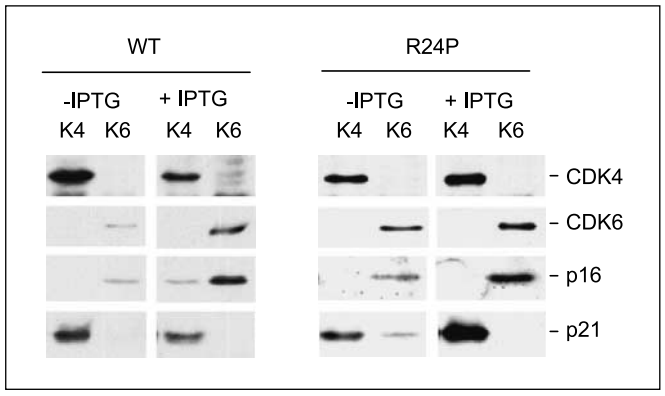

Figure 6. Subunit rearrangement in U20S cells expressing inducible $p16^{INK4a}$. The EH1 and JR1 cell lines were treated with and without IPTG to induce expression of $p16^{INK4a}$. Cell lysates were then precipitated with antibodies against CDK4 or CDK6 and immunoblotted for CDK4, CDK6, $p16^{INK4a}$, and $p21^{CIP1}$ as indicated.

cases, the so-called Leiden and Q34 cells, suggested that they are $p16^{INK4a}$ deficient but retain functional ARF, although the respective mutations occur within exon 2 and have the potential to affect both proteins (26, 36). An attraction of the Milan cells is that the R24P mutation occurs within exon 1α and therefore has no bearing on the sequence of $p14^{ARF}$. Functional evaluation confirmed that the endogenous $p14^{ARF}$ in Milan cells can bind to MDM2 and stabilize p53.

The Milan cells therefore provided a unique platform to substantiate our previous findings without concerns about the influence of $p14^{ARF}$. In the event, their behavior was remarkably similar to that of the other $p16^{INK4}$-deficient strains in terms of delayed senescence (at M1.5), insensitivity to Bmi1, and resistance to Ras-mediated arrest. Although these findings would be consistent with the inability of the R24P variant to impair the proliferation of normal HDFs, they are less easy to reconcile with the potential role of CDK6 in senescence. For example, a number of studies, including our own, have shown that the accumulation of $p16^{INK4a}$ in senescent fibroblasts causes the quantitative disruption of cyclin D-CDK6 complexes, whereas the cyclin D-CDK4 complexes are relatively unaffected (9, 15, 37). This is exactly what happens when Milan cells reach senescence, due to the association between R24P and CDK6 (data not shown), yet the cells clearly arrest at a state that is operationally distinct from M1.

We therefore have to consider the possibility that the R24P variant has a reduced affinity for both CDK4 and CDK6. Reports in the literature suggest that $p16^{INK4a}$ and other members of the INK4 family bind more avidly to CDK6 than to CDK4 (8, 38–40). A plausible explanation for the behavior of the R24P variant would be that this bias has become more accentuated due to an overall loss of affinity or stability. The counter-argument, however, is that the R24P variant shows no discernible loss of stability and is able to arrest U20S cells, albeit less efficiently than wild-type $p16^{INK4a}$. The crystal structure of the CDK6-INK4 complex does not reveal a critical role for R24 in the interaction (5, 6) and another germ line variant of the same residue, R24Q, remains able to interact with both CDK4 and CDK6 and does not seem to be functionally compromised.[4] When we changed the nearby R22 residue to P, the resultant protein was completely inactive.[5]

Despite extensive efforts to probe the mechanistic differences between R24P and wild-type $p16^{INK4a}$, by altering the relative levels of CDK4 and CDK6, the matter remains unresolved. The simplest explanation would be that the ability of R24P to cause cell cycle arrest depends on what proportion of the CIP/KIP proteins, or other members of the INK4 family, are involved in complexes with CDK6. Many cell types express all six of the possible pairings between D cyclins and CDK4/CDK6, making it difficult to accept that they are all performing the same function. Most debates about this topic revolve around the relative abundance of the components in different lineages or the specificity of their associated kinase activity, but it is also conceivable that the various cyclin D–CDK combinations have different capacities for sequestering the CIP/KIP proteins. Given the recent interest in kinase-dependent versus kinase-independent roles for the D-cyclins in cancer and development (41, 42), this is clearly an issue that warrants further investigation.

There is a wealth of published data implicating both CDK4 and CDK6 in cancer. For CDK6, this relates to the amplification and translocation of the relevant region of chromosome 7 (43–46), and there is a single report of a sporadic mutation in a neuroblastoma that renders the protein insensitive to inhibition by $p16^{INK4a}$ (47). However, we are not aware of CDK6 mutations associated with melanoma (48). In contrast, germ line mutations of CDK4 have been observed in several melanoma families (33, 49–51) and the gene maps within a frequently amplified region of chromosome 12, associated with a variety of sporadic cancers, including melanoma (52, 53). The germ line alterations change R24 to either C or H, in each case disrupting the association between CDK4 and the INK4 proteins. Mice in which the endogenous *Cdk4* locus has been replaced by the $Cdk4^{R24C}$ version of the gene develop a wide spectrum of tumors, including hemangiosarcomas and tumors of the pancreas, pituitary, brain, mammary tissue, and skin (54, 55).

In conclusion, both *CDKN2A* and *CDK4* are now considered to be high-penetrance melanoma susceptibility genes (1). Our findings with the R24P variant and Milan cells are consistent with this view but further underscore the pivotal importance of $p16^{INK4a}$ rather than $p14^{ARF}$, and CDK4 rather than CDK6, in the development of melanoma.

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[4] Kannengiesser et al., submitted for publication.
[5] S. Brookes and G. Peters, unpublished observations.

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