Mutations in the INK4a/ARF Melanoma Susceptibility Locus Functionally Impair p14ARF* 

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The INK4a/ARF locus encodes two cell cycle regulatory proteins, the cyclin-dependent kinase inhibitor, p16INK4a, and the p53 activator, p14ARF. Germinal mutations in this locus are associated with melanoma susceptibility in 20–40% of multiple case melanoma families. Many of these mutations specifically impair p16INK4a, whereas mutations uniquely targeting p14ARF are rare. Nevertheless, the importance of p14ARF has not been excluded because more than 40% of INK4a/ARF alterations affect p16INK4a and p14ARF. We now report that p14ARF is functionally impaired in melanoma kindreds carrying INK4a/ARF mutations. Of the seven INK4a/ARF mutations tested, three altered the subcellular distribution of p14ARF and diminished the ability of p14ARF to activate the p53 pathway. This work establishes the importance of p14ARF in melanoma predisposition.

The INK4a/ARF locus on the short arm of chromosome 9 is one of the most frequently altered sequences in human cancer. It is mutated, deleted, or hypermethylated in many tumors with a frequency ranging from 30% in esophageal tumors to ~60% in gliomas and mesotheliomas (1). Inherited mutations involving this locus are also associated with melanoma predisposition in 20–40% of multiple case melanoma families (2). The dual coding capacity of the INK4a/ARF locus may account for its high disruption rate in human tumors. This locus encodes two distinct proteins, which are translated in different reading frames from alternatively spliced transcripts (3). The α transcript (exons 1α, 2, and 3) encodes the p16INK4a cyclin-dependent kinase (CDK) inhibitor, whereas the β transcript (exons 1β, 2, and 3) specifies the alternative product, p14ARF (3–5).

Both INK4a/ARF-encoded proteins maintain cell cycle control. p16INK4a regulates G1-phase exit by interacting with CDK4 and CDK6 to inhibit the phosphorylation of the retinoblastoma protein (pRb) (6), whereas p14ARF interacts with hdm2 to prevent the degradation of p53 (7–10). The interaction of p14ARF with hdm2 inhibits the ubiquitin-protein isopeptide ligase activity of hdm2 (11, 12) and prevents the nuclear export sequence of p14ARF disrupted the localization of ARF and diminished the export of p53 (11, 12). The interaction of p14ARF with hdm2 stabilizes the key understood function of ARF, recent evidence confirms that p14ARF also functions in the pRb pathway. The growth inhibitory activity of ARF remains active when the p53 pathway is inactivated but can be suppressed by inactivation of both the pRb and p53 pathways (15). The various functions of p14ARF presumably reflect its multiple binding partners. In addition to hdm2, p14ARF interacts with the pRb-associated transcription factors E2F 1, -2, and -3, the protein phosphatase subunit, spinophilin, and the DNA enzyme topoisomerase I (16–19). Although all the functional details have not been determined, p14ARF is activated in response to abnormal oncogenic stimuli, including E1A, MYC, and oncogenic Ras, and therefore must play an important role in sensitizing transformed cells to either growth arrest or apoptosis (20–24).

Although the INK4a/ARF locus encodes two potent cell cycle regulators, current data indicate that tumor-associated alterations affecting this locus predominantly target p16INK4a. Many INK4a/ARF mutations have been shown to inactivate the CDK4 inhibitory activity of p16INK4a (25–28). Furthermore, whereas mutations in exon 1β are rare, constitutional and somatic inactivating p16INK4a mutations in exon 1α are common in cancer (29–32). Nevertheless, a significant proportion of mutations occurring in the shared second INK4a/ARF exon alter the amino acid sequence of both p16INK4a and p14ARF. Approximately 50% of the somatic INK4a/ARF mutations identified in human cancer (32), and 40% of those identified in multiple case melanoma families affect the amino acid sequence of both p16INK4a and p14ARF (29–31, 33–37).

Tumor-associated mutations affecting the second INK4a/ARF exon alter the C-terminal half of p14ARF. This region encodes a nucleolar localization domain (™PRRQGQLRPRPSHTPRARRCP™) that is required for full activity of the human ARF protein (14, 38). Two frameshift mutations identified in non-small cell lung and esophageal squamous cell cancers and occurring in the C-terminal nucleolar localization sequence of p14ARF disrupted the localization of ARF and diminished its ability to stabilize p53 (14). This work contradicts an earlier study, which concluded that tumor mutations dually affecting p16INK4a and p14ARF did not impair the function of the murine homologue, p19ARF (39). In this earlier study, however, missense INK4a/ARF mutations, identified in human tumors, were introduced into mouse p19ARF (39). Murine ARF does not possess a nucleolar localization domain in the exon 2-encoded C-terminal region (40, 41), and it is therefore difficult to make conclusions regarding p14ARF based solely on experiments with the mouse homologue.

Recently two families have been described in which exon 1β-specific genetic alterations are linked to melanoma susceptibility, the first in a kindred of melanoma and nervous system tumors (42), and the second in a patient with multiple primary melanoma (43). Proof of principle is lacking, however, as in the

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1 The abbreviations used are: CDK, cyclin-dependent kinase; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein.

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first case the deletion involved may affect p16INK4a expression, and in the second case the nuclear nature of the affected kindred provides only preliminary evidence of disease association.

Clear demonstration of a functional involvement of p14ARF in melanoma susceptibility is important for two reasons. First, it will implicate downstream targets of p14ARF in the investigation of other candidate molecules in melanoma susceptibility and development. Second, it will carry important implications for determination of individuals at high risk (2).

To evaluate the functional effect of INK4a/ARF exon 2 alterations we introduced a series of melanoma-associated mutations into the human p16INK4a and p14ARF genes. Seven point mutations located in the shared INK4a/ARF exon 2 and encoding non-conservative amino acid substitutions in both p16INK4a and/or p14ARF were evaluated for protein interactions, cellular distribution, cell cycle inhibitory function, and p53 pathway activation.

**EXPERIMENTAL PROCEDURES**

**Selection of INK4a/ARF Variants**—Five missense INK4a/ARF exon 2 mutations engineered in this project were identified in the germline of familial melanoma kindreds. The G67S variant was identified in a single Australian family with three affected individuals (30). The A68L and N71K variants segregated with melanoma in a single and two French melanoma-prone kindreds, respectively (29). The N71I missense mutation was found to segregate with melanoma in a single Italian family with four affected individuals (37). The R112G mutation has been found in three case Australian family (30) and in two skin metastases from a single patient (44). The missense mutation A68T was detected in the DNA derived from a primary melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45). The silent p16INK4a mutation (Ala-73) was identified in a primary sporadic melanoma; the presence of this mutation in the germline was not determined (45).

**Plasmid Constructs—p14ARF** cDNA was synthesized using reverse transcription-polymerase chain reaction and p14ARF-specific primers from total RNA extracted from a lymphoblastoid cell line, as described previously (46). p16INK4a and p14ARF point mutations were engineered by polymerase chain reaction-mediated mutagenesis. All mutants were ligated to the N terminus of the FLAG epitope encoded by the pFLAG-CMV-5b vector (Sigma) and completely sequenced.

**Indirect Immunofluorescence**—Approximately 40 h after transfection, cells were washed in PBS and fixed in 3.7% formaldehyde. Cells were immunostained for 50 min with either monoclonal mouse α-FLAG M2 antibody (Sigma) or rabbit α-p21WAF1 (Santa Cruz) or rabbit α-hdm2 (Santa Cruz) followed by a 50-min exposure to either a fluorescein isothiocyanate- or Texas red-conjugated α-mouse secondary IgG (Roche Molecular Biochemicals) or α-rabbit secondary IgG (Jackson Immunoresearch). Nuclei were visualized by Hoechst 33258 staining (2 μg/ml). Subcellular distribution was determined from a total of at least 1100 fluorescent cells, from three independent transfection experiments, unless otherwise indicated. The standard deviation obtained within a single celltransfection experiments was always less than ±6%.    

**RESULTS**

**Target Protein Interactions of p16INK4a and p14ARF Variants in Vivo**—Seven melanoma-associated INK4a/ARF mutations were selected for study; two identified in sporadic melanomas, and five identified as inherited mutations in the germline of multiple affected members of melanoma families (Table I). These mutations were located in the shared p16INK4a and p14ARF exon 2 (Fig. 1) and caused changes to the amino acid sequence of both p16INK4a and p14ARF (six mutants) or to p14ARF only (one mutant) (Table I). Six of the engineered p14ARF mutations were located within the exon 2-encoded nucleolar localization domain of this protein (Fig. 1), and all showed in vivo binding activity of these variants was assessed using a mammalian two-hybrid assay. Each p16INK4a and p14ARF mutant construct was cloned in frame with the GAL4 nuclear localization sequence and transiently co-transfected with either a CDK4 or an hdm2 expression plasmid into Saos 2 cells. 

![Image](http://www.jbc.org/)

**TABLE I** 

| Codon change | Amino acid change | Codon change | Amino acid change | Melanoma type | Reference |
|--------------|------------------|--------------|------------------|---------------|-----------|
| GCC → AGC   | G67S             | CGG → CAG    | R81Q             | Familial      | (30)      |
| GCC → TGT   | A68L             | GCC → CTT    | R82L             | Familial      | (29)      |
| GCC → AGC   | A68T             | GCC → CAC    | R82H             | Sporadic primary | (45) |
| AAC → ATC   | N71I             | CAA → CAT    | Q55H             | Familial      | (37)      |
| AAC → AAA   | N71K             | CTG → ATG    | L86M             | Familial      | (29)      |
| GCC → CTT   | AT5A             | CGA → TGA    | Rs8rter          | Sporadic primary | (45) |
| CGT → GGT   | R112G            | CCG → CCG    | P126R            | Familial, metastases | (30, 44) |

*To permit translation of the C-terminal FLAG epitope, the premature termination codon was not included in this cDNA clone. The ARF sequences encoding residues 1–57 were fused directly to the FLAG coding sequence.*
As expected, the p16INK4a variant carrying the silent mutation, Ala-73 (A73A), bound CDK4 as effectively as the wild type protein (Fig. 2A). All other tested p16INK4a mutants had reduced binding to CDK4; the G67S familial melanoma mutant gave intermediate CDK4 binding results (30 ± 6% of wild type) and the remaining p16INK4a mutants (A68L, A68T, N71I, N71K, R112G) showed minimal residual CDK4 binding (Fig. 2A).

The binding affinity of the p14ARF variants and hdm2 was also determined in Saos 2 cells, using the mammalian two-hybrid assay. Four p14ARF variants, R82L, R82H, Q85H and L86M, were indistinguishable from the wild type protein in their capacity to interact with hdm2. The R81Q and P126R familial melanoma mutants showed considerably reduced binding to hdm2. The R81Q and Arg-82 (R82L) showed hdm2 binding activity 60% higher than the wild type ARF protein (Fig. 2B).

Mutations Associated with Melanoma Disrupt the Subcellular Distribution of p16INK4a and p14ARF—Each of the p16INK4a and p14ARF mutants was cloned in frame with the FLAG epitope in the pFLAG-CMV-5b vector. The subcellular distribution of each construct was evaluated in transiently transfected NM39 melanoma cells. These cells lack endogenous p16INK4a and p14ARF but express p53 and pRb (47). As expected, p16INK4a-FLAG localized evenly throughout NM39 cells (Fig. 3). All other p16INK4a-wild type localization and was distributed evenly throughout NM39 cells (Fig. 3A) and p14ARF-FLAG localized predominantly in the nucleolus of NM39 cells (Fig. 4). The remaining p16INK4a and p14ARF mutants revealed that only the truncated R88ter mutant was more soluble than wild type ARF (Fig. 6B).

Cell Cycle Inhibitory Activity of p16INK4a and p14ARF Mutants—The effect of p16INK4a or p14ARF proteins on cell cycle progression was investigated by transiently transfecting FLAG-tagged constructs into NM39 melanoma cells. Forty hours after transfection, DNA content was assayed by flow cytometry to determine cell cycle distribution. At this time point, the expression of wild type p16INK4a or p14ARF in NM39 cells induced potent G1 cell cycle arrest. Similarly, the p16INK4a missense mutants, G67S, A68T, and A73A, remained fully active in mediating cell cycle arrest (Fig. 5A). In contrast, the remaining p16INK4a variants (A68L, N71I, N71K, R112G) reproducibly induced only partial cell cycle inhibition, compared with wild type p16INK4a, in NM39 cells (Fig. 5A).

To ensure that the diminished cell cycle inhibitory activity associated with the p16INK4a missense mutants was not due to low expression levels, the accumulation of all p16INK4a constructs was analyzed in transiently transfected NM39 cells, 40 h after transfection. Immunostaining revealed nearly equivalent expression of the wild type p16INK4a-FLAG and mutant p16INK4a-FLAG proteins, except the R112G mutant, which did not accumulate as well as the wild type protein in NM39 cells (Fig. 5B). Nevertheless, the reduced cell cycle inhibitory activity of the A68L, N71I, and N71K mutants was not due to variation in p16INK4a protein concentration. There was, however, a significant difference in the solubility of the p16INK4a proteins. Whereas the wild type p16INK4a and A73A proteins were rapidly solubilized using a standard SDS-based extraction buffer, the mutant p16INK4a proteins required the addition of 6 M urea for extraction (Fig. 5B).

Analysis of the cell cycle inhibitory activity of the p14ARF mutants revealed that only the truncated R88ter mutant was impaired, consistently inducing weaker G1 arrest in NM39 cells (Fig. 6A). Immunostaining revealed nearly equivalent expression of wild type p14ARF and R88ter proteins in transfected NM39 cells (Fig. 6B). Unlike the wild type protein, however, this truncated ARF variant extracted from NM39 cells predominately in the soluble (minus urea) protein fraction (Fig. 6B). Similarly, the other ARF mutants with reduced nuclear targeting (R81Q, R82L, and possibly L86M; see Fig. 4B) appeared more soluble than wild type ARF (Fig. 6B).

Function of p14ARF Mutants Displaying Altered Subcellular Localization—An inherent problem with flow cytometric cell cycle assays is that all transfected cells, irrespective of the ARF
subcellular distribution, are included in the final analysis. For instance, the cell cycle profile of NM39 cells transfected with the p14ARF82L-FLAG consists of 52% cells with predominantly nucleolar ARF and only 48% cells accumulating diffuse ARF (Fig. 4). In order to differentiate between the function of predominantly nucleolar p14ARF and ARF that was distributed throughout the cell, we used immunocytochemistry to assay the function of p14ARF. As a measure of p14 ARF function, we stained for the p53 transactivation targets, p21waf1 and hdm2, 24 h after transfecting NM39 cells with various p14 ARF-FLAG constructs. The endogenous levels of p21waf1 and hdm2 were almost undetectable in NM39 cells, and increased markedly in cells ectopically expressing wild type ARF. In contrast, the introduction of p16INK4a-FLAG into NM39 cells did not induce p21waf1 or hdm2 accumulation (data not shown).

As shown in Fig. 7, almost 90% of NM39 cells expressing nucleolar wild-type p14ARF accumulated high levels of hdm2, 24 h after transfection. Similarly, ~90% of NM39 cells expressing nucleolar R81Q, R82L, R82H, Q85H, L86M, or P126R expressed hdm2 above background. In contrast, of the cells expressing R81Q throughout the cell, i.e. in the nucleolus, nucleoplasm, and cytoplasm, only 20 ± 6% expressed hdm2 above background (Fig. 7). Likewise, only 22 ± 6% of NM39 cells with diffuse R82L protein accumulated high levels of hdm2 (Fig. 7). In contrast, a much higher proportion of cells (73 ± 3%) expressing diffuse R88ter ARF protein had increased
DISCUSSION

The INK4a/ARF locus encodes two cell cycle regulatory proteins, the CDK inhibitor, p16INK4a, and the p53 activator, p14ARF. Alterations affecting this locus occur in familial melanoma kindreds and cause functional defects in p16INK4a. We now show that the function of p14ARF is also impaired by INK4a/ARF mutations identified in multiple case melanoma-prone kindreds. Approximately 40% of the germline INK4a/ARF mutations identified in melanoma-prone kindreds alter both p14ARF and p16INK4a. Furthermore, p14ARF is altered along with p16INK4a in ~40% of primary (sporadic) melanoma tumors, and is specifically targeted in another 11% (32, 45, 48, 49). It follows that the pathways regulated by p14ARF deserve scrutiny, along with p16INK4a, for their potential role in the genesis of melanoma.

Tumors carrying inactive p16INK4a and p14ARF would be expected to display minimal selective pressure in inactivating other components of these pathways. Certainly, the disruption of both pRb and p16INK4a in the same tumor occurs infrequently (50–52), as does the inactivation of both p14ARF and p53 in a single tumor (53). Furthermore, in melanomas, which have a high frequency of INK4a/ARF alterations, the rate of

amounts of hdm2. The results obtained with p21waf1 expression corresponded with the hdm2 data (data not shown).

FIG. 3. Cellular distribution of FLAG epitope-tagged p16INK4a mutation constructs in NM39 melanoma cells. A, each p16INK4a-FLAG-tagged construct was transiently introduced into the NM39 melanoma cells. Approximately 40 h after transfection, cells were fixed, permeabilized, and immunostained with a monoclonal mouse α-FLAG M2 antibody followed by a 50-min exposure to a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody. Nuclei were visualized by Hoechst staining. LM, light microscopy. B, according to the relative intensity of fluorescence in the cytoplasm and nucleus of each individual cell, subcellular distributions of the FLAG fusion proteins were divided into three groups, as indicated. The subcellular distribution of the mutant p16INK4a proteins was no longer homogeneous but showed areas of intense fluorescence, which were classified as either speckled (small discrete areas of intense fluorescence) or punctate (larger aggregates of intense fluorescence). The percentage of each localization was determined from at least three separate transfection experiments and from a total of at least 1100 fluorescent cells, unless otherwise indicated. a, localization was determined from two independent transfection experiments from a total of 1018 fluorescent cells.
p53 and pRb inactivation is low compared with other tumor types (54–59).

The role of p16INK4a and p14ARF in tumorigenesis was evaluated by introducing melanoma-associated INK4a/ARF mutations into cDNAs encoding human p16INK4a and p14ARF. Five missense mutants identified in the germline of familial melanoma kindreds and two INK4a/ARF variants derived from sporadic melanomas were engineered. At present, the only functional p14ARF domain encoded by the second INK4a/ARF exon is a nucleolar localization sequence. Consequently, six of the ARF mutations tested (R81Q, R82L, R82H, Q85H, L86M, R88ter) were chosen because they affected this functional motif. This region is not conserved in the murine and Monodelphis ARF homologues, and mouse ARF does not require the exon 2-encoded domain for nucleolar targeting (40, 41). The corresponding p16INK4a residues (G67S, A68L, A68T, N71I, N71K, A73A) lie within the flexible loop 2, adjacent to the helix-turn-helix motif generated by the second ankyrin repeat. These residues are highly conserved in all human and murine INK4 inhibitors (60). The remaining p14ARF mutant, P126R, is located at the C terminus of the protein, and this proline residue is conserved in the murine ARF protein. The corresponding

![Cellular distribution of melanoma-associated p14ARF variants](image-url)

**Fig. 4.** Cellular distribution of melanoma-associated p14ARF variants. A, the subcellular distribution of p14ARF-FLAG-tagged proteins was examined in transiently transfected NM39 cells as detailed in Fig. 3 legend. LM, light microscopy. B, localization of various FLAG epitope-tagged p14ARF mutation constructs in NM39 melanoma cells. According to the relative intensity of fluorescence in the cytoplasm, nucleoplasm, and nucleolus of each individual cell, subcellular distributions of the FLAG fusion proteins were divided into four groups, as indicated. The percentage of each localization was determined from separate transfection experiments and from a total of at least 1100 fluorescent cells, unless otherwise indicated. *, localization was determined from two independent transfection experiments from a total of 899 fluorescent cells.

|              | predominantly nucleolar | throughout cell | nucleoplasm | cytoplasm | only |
|--------------|-------------------------|-----------------|-------------|-----------|------|
| p14ARF       | 76                      | 17              | 7           | 0         |      |
| R81Q         | 45                      | 43              | 11          | 1         |      |
| R82L         | 52                      | 41              | 7           | 0         |      |
| R82H         | 73                      | 24              | 4           | 0         |      |
| Q85H         | 87                      | 9               | 3           | 0         |      |
| L86M         | 69                      | 20              | 10          | 0         |      |
| R88ter       | 0                       | 56              | 0           | 44        |      |
| P126R        | 83                      | 12              | 5           | 0         |      |
residue in p16INK4a, Arg-112, is less conserved across all INK4 inhibitors, but is invariant in human and mouse p16INK4a and p15INK4b. This residue is located at the end of loop 3 and near the helix motif generated by the fourth p16INK4a ankyrin repeat (60).

More than 60 mutant p16INK4a alleles have been identified in human tumors and in individuals predisposed to melanoma. Of the variants examined, most have been shown to be functionally impaired in vitro and/or in vivo. The only familial melanoma-associated p16INK4a mutation that has not been reported to affect p16INK4a activity is the 24-base pair duplication (48), which resides at the flexible N-terminal end of the protein and segregates with melanoma in three kindreds (31, 35). As expected, therefore, all missense p16INK4a mutations examined in this work (G67S, A68L, A68T, N71I, N71K, A73A, R112G) had reduced CDK4 binding activity and altered subcellular distribution patterns (Table II). These p16INK4a mutant proteins did not display altered subcellular distribution. The localization of these tumor-associated p16INK4a mutants is reminiscent of protein aggregation. It is known that p16INK4a oligomerization occurs in vivo (62), and it is possible that altering essential residues within this protein increases the aggregation of mutant molecules. It has also been established that mutant p16INK4a aggregates are resistant to solubilization (62), and the missense p16INK4a mutants tested in this work required 6 M urea to achieve solubility.

Analysis of seven melanoma-associated p14ARF mutants revealed that four (R81Q, R82L, R88ter, and P126R) were impaired, in at least one functional assay (Table II). The R81Q, R82L, and R88ter mutants were no longer restricted to the nucleolus of the NM39 melanoma cells (Fig. 4). Proteins targeted to the nucleolus often require highly basic residues for nuclear import. The ARF mutants with diminished nucleolar retention (R81Q and R82L) had changes to basic arginine residues within the nucleolar localization domain (80PRRGAQLRRPRHSPTRARCCP101), and the R88ter mutant carried a truncated nucleolar localization sequence. Interestingly, the Q85H mutant was restricted to the nucleolus in a substantially higher proportion of transfected NM39 cells, relative to the wild type protein (Fig. 4B). The increased positive charge in the

Fig. 5. Cell cycle inhibitory activity of melanoma-associated p16INK4a variants. A, NM39 melanoma cells were transfected with the indicated p16INK4a, FLAG plasmid and pCMVEGFP-spectrin. The cell cycle distribution of green fluorescent cells was determined, 40 h after transfection, using propidium iodide staining. The G1-phase arrest induced by wild type p16INK4a was set at 100%, and the arrest induced by the p16INK4a mutants was expressed relative to the wild type protein. Each result is derived from at least two independent transfection experiments. B, the expression of p16INK4a, FLAG constructs was determined 40 h after co-transfecting NM39 cells with the indicated FLAG plasmid and the pEGFP-FPN1 vector. Lysates were initially prepared using a detergent-based extraction buffer (−urea), and the resulting insoluble protein pellet was re-extracted in a buffer containing 6 M urea (+urea). Protein expression was analyzed by sequential immunostaining with α-FLAG and α-EGFP antibodies. Re-extraction with urea did not solubilize additional EGFP (data not shown).
The hdm2 binding affinity of wild type and mutant p14 ARF was determined using a mammalian two-hybrid assay. Only two ARF mutants (R81Q and P126R) showed reduced hdm2 binding in vivo (Fig. 2B and Table II). The altered localization of the ARF variants should not influence binding to hdm2 in this assay, because the proteins are fused to the vector encoded GAL4-nuclear localization sequence. Consequently, although both R81Q and R82L mutants displayed altered subcellular distribution patterns in NM39 cells, when forced into the nucleus, only R81Q showed diminished hdm2 binding. This suggests that specific mutations may distort the tertiary structure and function of ARF, since the exon 1β-encoded ARF domain is sufficient for hdm2 binding (41, 63). Certainly the removal of the exon 2-encoded ARF domain substantially increases the binding affinity of ARF-hdm2 (38), and likewise the premature truncation at residue Arg-88 (R88ter) improves hdm2 binding.

The functional activity of the mutant p14ARF proteins was further assessed in a cell-by-cell assay, where the function of nucleolar ARF was compared with the function of diffuse ARF, i.e. p14ARF distributed throughout the nucleus and cytoplasm. In this assay the transactivating function of p53 was evaluated by determining any increase in the level of endogenous hdm2 or p21waf1. Not surprisingly, whereas nucleolar wild-type and nucleolar mutant ARF were very effective at inducing p53 activity, as seen by the marked increase in hdm2 and p21waf1 levels, diffuse ARF was a poor inducer of p53 function (Fig. 7). These data confirm that the predominantly nuclear localization of p14ARF is critical for complete function and any disruption in nuclear localization significantly reduces the p53 activating function of p14ARF. Interestingly, diffuse R88ter protein was less effective at inducing p53 when compared with the wild type protein but much more effective than the diffuse R81Q...
and R82L mutants (Fig. 7). Although the R88ter mutant has severely altered localization, it binds hdm2 with greater affinity than the wild type ARF, and this may compensate, in part, for its diffuse localization. We are currently investigating whether the disrupted localization of p14ARF interferes with the other known functions of this protein.

The specific contribution of p14ARF alterations to phenotype in those familial melanoma kindreds displaying relevant mutations is unknown. Genotype-phenotype analyses are currently under investigation by the International Melanoma Genetics Consortium, but are confounded by the small numbers of cases affected by specific mutations. So far there do not appear to be obvious distinguishing clinical features associated with the known INK4a/ARF melanoma-associated mutations. Five of the 13 INK4a/ARF mutation-positive familial melanoma kindreds in the largest series of described kindreds (30) carry mutations that alter both p16INK4a and p14ARF. These families show no obvious differences in age of onset or the number of individuals with multiple primary melanomas when compared with families carrying germline mutations specific to p16INK4a only. There is, however, a suggestion of an increased risk of cancers, other than cutaneous melanoma, in p16INK4a and p14ARF mutant gene carriers. Three of the five families with p16INK4a and p14ARF mutations have additional cancers including breast and cervical cancer, whereas only one out of eight families with p16INK4a-specific mutations have other cancers (30). A formal investigation of genotype-phenotype is an ongoing project of the International Melanoma Genetics Consortium.

Perhaps the most informative comparison can be made with
the N71I and N71K p16INK4a variants also mutate p14ARF and the pRb and p53 cell cycle pathways. The stimulation of late DNA lesions in tumor progression. 

factors. These factors also trigger p14ARF expression, which fort the suggestion, from the current functional analyses, that the late DNA lesions in tumor progression.

more likely to survive following DNA damage and to accumulate DNA lesions in tumor progression.

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table II

Effects of melanoma-associated mutations on the function of p16\(^{INK4a}\) and p14\(^{ARF}\)

| Amino acid change | CDK4 binding | Localization | G1 arrest |
|-------------------|--------------|--------------|----------|
| Wild type         | −            | Uniform      | +        |
| G67S              | −            | Uniform      | +        |
| A68L              | −            | Not uniform  | +/−      |
| A68T              | −            | Not uniform  | +/−      |
| N71I              | −            | Not uniform  | +/−      |
| N71K              | −            | Not uniform  | +/−      |
| A73A              | +            | Uniform      | +/−      |
| R112G             | −            | Not uniform  | +/−      |

Binding of p16\(^{INK4a}\) and p14\(^{ARF}\) to CDK4 and hdm2, respectively, was determined using the mammalian two-hybrid assay.

The localization of FLAG-tagged proteins was determined by immunofluorescent staining of transfected NM39 melanoma cells.

The reduced function of the R112G mutant may reflect, in part, its reduced expression level, compared to the wild type protein.
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