Loss of ARF Sensitizes Transgenic BRAF^{V600E} Mice to UV-Induced Melanoma via Suppression of XPC

Chi Luo, Jinghao Sheng, Miaofen G. Hu, Frank G. Haluska, Rutao Cui, Zhengping Xu, Philip N. Tsichlis, Guo-Fu Hu, and Philip W. Hinds

Abstract

Both genetic mutations and UV irradiation (UVR) can predispose individuals to melanoma. Although BRAF^{V600E} is the most prevalent oncogene in melanoma, the BRAF^{V600E} mutant is not sufficient to induce tumors in vivo. Mutation at the CDKN2A locus is another melanoma-predisposing event that can disrupt the function of both p16^{INK4a} and ARF. Numerous studies have focused on the role of p16^{INK4a} in melanoma, but the involvement of ARF, a well-known p53 activator, is still controversial. Using a transgenic BRAF^{V600E} mouse model previously generated in our laboratory, we report that loss of ARF is able to enhance spontaneous melanoma formation and cause profound sensitivity to neonatal UVB exposure. Mechanistically, BRAF^{V600E} and ARF deletion synergize to inhibit nucleotide excision repair by epigenetically repressing XPC and inhibiting the E2F4/DP1 complex. We suggest that the deletion of ARF promotes melanomagenesis not by abrogating p53 activation but by acting in concert with BRAF^{V600E} to increase the load of DNA damage caused by UVR. Cancer Res; 73(14): 4337–48. ©2013 AACR.

Introduction

Derived from the professional pigment-producing melanocytes, melanoma is considered the most lethal form of skin cancer (1). Mutation in the BRAF protein, especially the V600E point mutation, is the most prevalent genetic alteration in human melanoma (2). Interestingly, the BRAF^{V600E} mutation on its own is not sufficient to induce tumor formation in vitro and in vivo, consistent with the fact that expression of BRAF^{V600E} in melanocytes either in the culture dish or in animals results in senescence (3–6). Therefore, additional genetic alterations are required for the progression of BRAF^{V600E}-expressing melanocytes to melanoma, at least in part to achieve suppression of the senescence response.

In addition to BRAF mutation, the CDKN2A locus is frequently targeted in melanoma (7). Two distinct tumor suppressors, p16^{INK4a} and p14^{ARF} (p19^{ARF} in mice, hereafter referred to as ARF), are encoded by this locus via alternative reading frames (8). In contrast to p16^{INK4a}, the importance of ARF in melanomas has been debated, as germline mutations in ARF fail to associate with melanoma susceptibility (9), and CDKN2A mutations that target only p16^{INK4a} but not ARF for inactivation have been described previously (10). However, the recent discovery of mutations specific to the ARF-coding region and ARF-specific deletions (11), as well as the observation that ARF ablation facilitates oncogenic induction of murine melanoma (12–15), highlight the significance of ARF in melanomagenesis. Therefore, it is increasingly accepted that p16^{INK4a}-independent inactivation of ARF can also predispose individuals to melanoma.

The mechanistic basis of ARF’s ability to suppress melanoma may lie in ARF’s canonical role in the MDM2-p53 pathway, and indeed p53 is mostly wild-type (WT) in human melanomas (16), suggesting loss of ARF may abrogate the need for p53 mutation. However, ARF also has p53-independent functions (17, 18), which explains the fact that it can act as a melanoma suppressor in the absence of p53 (14). For example, ARF has been shown to bind to DRTF polypeptide 1 (DP1), a coactivator of E2F transcription factors, and inhibit the formation of active DP1–E2F complexes, thereby regulating the expression of E2F target genes (19–21). Therefore, the mechanism of melanoma suppression by ARF remains unclear. Understanding this mechanism may have impact on the design of future therapies for melanoma.

The ability of ARF to modulate E2F activity also extends ARF’s function to activation of the nucleotide excision repair (NER) pathway (21). NER is a DNA repair mechanism that functions to remove bulky DNA adducts such as cyclobutane pyrimidine dimers (CPD) and 6-4-photoproducts mostly caused by UV irradiation (UVR; ref. 22). Interestingly, solar UVR is the major etiologic factor for skin cancers including melanoma, although the relationship between UVR and melanoma is less evident than that of squamous and basal
cell carcinomas (23). Recent studies showed neonatal UVB irradiation (275–320 nm) can exacerbate melanoma penetrance in various genetic mouse models (12, 13, 24); however, the mechanism leading to UV sensitivity in melanocytes is not well understood. Although BRAF mutations are not over-represented in melanomas arising from chronic sun-exposed areas, it is unclear whether BRAFV600E alone or in combination with other genetic events increases the sensitivity to UVB.

In the present study, we used a transgenic BRAFV600E mouse model (3) to show that loss of ARF is able to shorten the latency of melanoma formation and causes profound sensitivity to neonatal UVB exposure. Oncogenic BRAFV600E instigates transient DNA damage in melanocytes, which triggers senescence that cannot be bypassed by ARF deletion in vivo. However, loss of ARF cooperates with BRAF mutation to suppress the senescence in various genetic mouse models (12, 13, 24); however, the mechanism leading to UV sensitivity in melanocytes is not well understood. Although BRAF mutations are not over-represented in melanomas arising from chronic sun-exposed areas, it is unclear whether BRAFV600E alone or in combination with other genetic events increases the sensitivity to UVB.

Materials and Methods

Mice

All mouse experiments were carried out with the approval of Tufts University/Tufts Medical Center (Boston, MA) Institutional Animal Care and Use Committee. The generation of melanocyte-specific BRAFV600E transgenic mice was described previously (3). Founder mice were backcrossed to C57BL/6 mice for more than 10 generations. The exon 1b-specific p19<sup>ARF</sup> knockout mouse in C57BL/6 background used in this study has been described elsewhere (25) and was a generous gift from N. Rosenberg (Tufts University School of Medicine, Boston, MA). All mice were maintained in a pathogen-free mouse facility at Tufts University School of Medicine.

Cell culture and recombinant vectors

All cells were cultured in a 5% CO<sub>2</sub> humidified incubator at 37°C. Primary mouse melanocytes were isolated from neonatal mouse skins as described previously (26) and maintained in F12 medium supplemented with 3% FBS (Gibco), 1% antibiotic-antimycotic (Invitrogen), 48 nmol/L 12-O-tetradecanoylphorbol-13-acetate (Sigma), 0.1 mmol/L isobutylmethyl xanthine (Sigma), 10 μg/mL bovine pituitary extract (Invitrogen), and 0.1 mmol/L dibutyryladenosine 3′,5′-cyclic monophosphosphate (Sigma). Contaminating fibroblasts and keratinocytes were eliminated by treatment with 100 μg/mL geneticin (Invitrogen) for 24 hours. Primary mouse melanoma cells were isolated by collagenase/hyaluronidase digestion of tumor fragments for 30 minutes and grown in RPMI-1640 (Gibco) supplemented with 10% FBS and 1% antibiotic-antimycotic (27). Human melanoma cell line CHL1 was cultured in Dulbecco's Modified Eagle Medium (Gibco) with 10% FBS and 1% penicillin-streptomycin.

The retroviral vector pBabe-puro-p19<sup>ARF</sup> was generously provided by N. Sharpless (University of North Carolina, Chapel Hill, NC), pBabe-hygro-dominant-negative-p53 was obtained from Addgene (#9058; ref. 28). The lentiviral construct FG12-HA-BRAF<sup>V600E</sup>-eGFP was a gift from D. Peep (The Netherlands Cancer Institute, Amsterdam, the Netherlands; ref. 5). Short hairpin RNA (shRNA)-targeting mouse p53 sequence 5′-GTACTCTCCTCCCCTCAAT-3′ was generated in the pMKO.1 retroviral vector.

UV exposure

For irradiation of cultured cells, cells were washed with PBS and subsequently exposed to UV radiation using UVB lamps (280–314 nm; UVP, Inc.) at the indicated dose. UV emittance was measured with the use of a UV photometer (model ILT1400A; UVP, Inc.).

For in vivo tumor induction, animals were irradiated under the UVB bulbs at a dose of 750 mJ/cm<sup>2</sup> at neonatal day 3.5. Following UV exposure, mice were carefully monitored for the degree of erythema and/or desquamation. Severe erythema or desquamation resulted in sacrifice of the animal but was rarely observed. The majority of animals displayed mild to moderate erythema and was monitored for tumor formation as indicated.

Histology

Tissue samples were fixed in 10% buffered formalin overnight and stored in 70% ethanol before paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining (by the Rodent Histopathology Core, Harvard Medical School, Boston, MA). Immunohistochemistry was conducted with the following antibodies: Ki-67 (SP6; Thermo Scientific), p16 (M-156; Santa Cruz Biotechnology), p53 (Thermo Scientific), interleukin (IL)-6 (ab6672; Abcam), Trp2 (ab74073; Abcam), S100 (Ab-2; Thermo Scientific), Melan A (C-20; Santa Cruz Biotechnology), and HMB45 (ab732; Abcam). Staining was carried out with Vector NovaRED Substrate Kit (Vector Laboratories). Negative control was done by replacing the primary antibody with species-matched total immunoglobulin G (IgG).

Senescence-associated β-galactosidase staining

Skin samples were fixed in 4% buffered paraformaldehyde at room temperature for 30 minutes, followed by a wash with 50 mmol/L glycine in PBS. Dehydration was carried out by subsequently soaking the samples in 20% sucrose overnight and 30% sucrose until the samples settle at the bottom. Dehydrated samples were embedded in optimal cutting temperature compound, frozen, and sectioned. The staining for perinuclear senescence-associated β-galactosidase (SA-β-gal) activity was carried out according to protocol described previously (29, 30).

Immunoblotting and immunoprecipitation

For immunoblotting, tissue samples or cultured cells were homogenized and/or lysed with radioimmunoprecipitation assay (RIPA) buffer containing 50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mmol/L sodium pyrophosphate, 10 mmol/L β-glycerophosphate, 0.2 mmol/L Na<sub>2</sub>VO<sub>4</sub>, 10 mmol/L NaF, 1 mmol/L dithiothreitol (DTT), and Complete-Mini protease inhibitor cocktail (Roche). Primary antibodies used included p19 (ab880; Abcam), p16 (F-12; Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MAB374; Chemicon), p53 (Thermo Scientific), p21 (ab61902; Abcam), p27 (ab299; Abcam), and p16 (ab9034; Abcam). Immunoprecipitation was carried out with Protein A purified rabbit anti-p19 (ab14592; Abcam) for p19, and rabbit anti-p53 (ab1353; Abcam) for p53. Western blotting was carried out with anti-p19 (ab14592; Abcam), and rabbit anti-p53 (ab1353; Abcam) for p53. Immunoprecipitation was carried out with Protein A purified rabbit anti-p19 (ab14592; Abcam) for p19, and rabbit anti-p53 (ab1353; Abcam) for p53. Western blotting was carried out with anti-p19 (ab14592; Abcam), and rabbit anti-p53 (ab1353; Abcam) for p53.
(F-5; Santa Cruz Biotechnology), phosphor-p53-Ser15 (9284; Cell Signaling Technology), pERK1/2 (4376; Cell Signaling Technology), and extracellular signal–regulated kinase 2 (ERK2; C-14; Santa Cruz Biotechnology).

Chromatin immunoprecipitation was conducted as described by Dominguez-Brauer and colleagues (21). The antibodies used were p19 (ab80; Abcam), E2F4 (C-20; Santa Cruz Biotechnology), and DP1 (hybridoma supernatant kindly provided by J. Lees, Massachusetts Institute of Technology, Cambridge, MA).

Quantitative real-time PCR
Total RNA was extracted by TRIzol (Invitrogen) and converted into cDNA using either iScript (Bio-Rad) or SMART-Script (Clontech) cDNA synthesis kit. Gene expression was quantified using Quantitect SYBR Green (Qiagen) or SYBR Advantage (Clontech).

Genomic DNA (gDNA) was extracted by phenol–chloroform, followed by methylation-sensitive enzyme HpaII digestion for 3 hours. Quantitative PCR (qPCR) was conducted with 1 μL of the HpaII-digested heat-inactivated gDNA and with the XPC promoter-specific and control primers (Supplementary Table S1). The sequences between XPC forward and reverse primers harbor multiple HpaII recognition sites, thereby will be digested if not methylated; the sequences between the control primers do not have any HpaII sites, and thus served as an internal control.

CPD immunoblot
Heat-denatured gDNA was dot-blotted onto a nitrocellulose membrane and was blocked with 5% milk overnight. The membrane was then incubated with anti-CPD antibody (provided by R. Cui, Boston University, Boston, MA) for 30 minutes at room temperature.

Growth curve and soft agar colony-formation assay
Primary mouse melanoma cells were stably infected with dominant-negative p53 (DNp53) or empty vector and seeded in 6-well plates at a density of 1 × 10⁶/well in triplicate. Cell number was counted at indicated time points.

For anchorage-independent growth assays, 2 × 10⁴ cells were suspended in medium containing 0.4% agarose and plated onto a solidified layer of medium-containing 0.8% agarose in 6-well plates. Colonies were quantified in 5 fields in each well 3 weeks after seeding.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChiP) was conducted according to the protocol described previously (31) using E2F4 antibody (C-20; Santa Cruz Biotechnology). Real-time PCR (RT-PCR) was conducted according to Dominguez-Brauer and colleagues (21). ChiP data were normalized to input chromatin.

Results

ARF is a suppressor of BRAFV600E-driven murine melanoma
ARF can be induced by oncogenes such as c-Myc and Ras in cultured cells (17). To determine whether oncogenic BRAFV600E can trigger ARF expression in vivo, we conducted immunoblots on skin lysates from either WT or melanocyte-specific BRAFV600E transgenic mice (3). ARF, but not p16INK4a, was detectable in the BRAFV600E skin from adult mice (Fig. 1A), suggesting that ARF induction is a significant cellular response to chronic BRAF activation. Therefore, we introduced the germline ARF-specific deletion allele (p16INK4a is intact) into BRAFV600E mice (strain 476; ref. 3) and monitored tumor development. Neither BRAFV600E nor BRAFV600E;ARF−/− mice developed tumors for up to 5 months, whereas BRAFV600E;ARF−/− mice developed tumors relatively rapidly, with a mean latency for survival of 85 days of age (Fig. 1B). Forty percent of the BRAFV600E;ARF−/− mice were sacrificed because of progressive melanoma, as indicated by asterisks in the curve (Fig. 1B and Supplementary Fig. S1A); the remaining mice were sacrificed as a result of sarcoma and lymphoma, the prominent tumor types in germline ARF-null animals (32). Surprisingly, during the experimental period, sarcomas and lymphomas were also observed in the BRAFV600E;ARF−/− mice but not in the control (except in one BRAFV600E;ARF−/− mouse; Fig. 1B), suggestive of reduced latency for these ARF-null tumors in the tyrosinase-driven BRAFV600E background. Given that expression of the BRAFV600E transgene was not readily detected in the sarcomas and lymphomas examined (Supplementary Fig. S1B), it is unlikely that these tumors resulted from expression of BRAFV600E in the affected tissues. Considering the existence of elevated amounts of inflammatory cytokines (Fig. 3B and C), it is possible that alteration of the microenvironment by the BRAFV600E transgene facilitates the development of other tumors.

Histologically, ARF−/− skin was comparable with WT skin (Fig. 1C). Expression of BRAFV600E caused melanocytic proliferation presenting as large nests of epithelioid cells in the deep dermis and subcutis (Fig. 1C, triangles; ref. 3). Knockout of ARF in BRAFV600E mice increased the apparent hyperplasia of melanocytes, with increased deposits in the dermis and subcutis and the appearance of a Schwannian differentiated phenotype (Fig. 1C, arrows; ref. 3). Melanomas that formed in the BRAFV600E;ARF−/− mice showed a transition from pigmented to unpigmented, suggesting progression from a differentiated to an undifferentiated phenotype (Fig. 1C). The tumors were often very invasive, penetrating through the muscle layer underneath the skin (Fig. 1C, arrowheads), and showed a high proliferation index seen in the form of mitotic figures (Fig. 1C, inset) and Ki-67 staining (Fig. 1D). Interestingly, this proliferation was observed despite the fact that a substantial number of cells positive for nuclear p16INK4a were detected in the tumor sections (Fig. 1D). Analysis of lungs from 6 melanoma-bearing mice revealed pigmented micrometastasis in 2 cases (Fig. 1C). Taken together, these data indicate that deletion of ARF accelerates invasive melanoma formation driven by BRAFV600E, and thus acts as a melanoma suppressor in this context.

ARF loss does not abrogate p53 activation in BRAFV600E;ARF−/− murine melanomas
ARF is a well-characterized p53 stabilizer, therefore abrogation of ARF is expected to dampen p53 activation.
Surprisingly, we detected strong p53 expression in the BRAFV600E:ARF−/− precancerous skin and tumors (Fig. 2A and Supplementary Fig. S2A). p53 was active based on its phosphorylation at Ser15 site and expression of the downstream target p21 (Fig. 2B), suggesting that loss of ARF might not affect the function of p53 induced by chronic BRAFV600E stimulation in this model. To confirm that p53 in BRAFV600E:ARF−/− tumor cells is functional, we knocked down p53 and found that p21 level was reduced (Fig. 2C). In addition, we restored ARF expression in BRAFV600E:ARF−/− tumor cells (Supplementary Fig. S2B), and challenged the cells with UVR. Upon UV induction, p53 was rapidly phosphorylated regardless of ARF status (Fig. 2D), further supporting the contention that p53 in ARF-null melanoma cells is functional.

Given that deletion of ARF does not alter p53 functionality, we reasoned that repressing the function of p53 would have an additive effect on ARF loss to enhance tumor formation. To test this, we generated BRAFV600E:ARF−/− tumor cells stably expressing either dominant-negative p53 (DNp53; Supplementary Fig. S2C) or mouse p53-specific shRNA. Proliferation assays showed that DNp53-expressing cells grew significantly faster than cells with vector control (Fig. 2E). Soft agar colony formation assays confirmed that DNp53-expressing cells produced more and larger colonies than control cells (Fig. 2F). Results using p53-shRNA were similar (Supplementary Fig. S2D and S2E). Taken together, the data suggest that loss of ARF affects targets other than p53 to increase melanoma formation, as p53 is active despite ARF deficiency.

**Loss of ARF cannot suppress BRAFV600E-induced senescence in vivo**

It has been shown that loss of ARF bypasses senescence in cultured primary melanocytes (14). To examine whether ARF deficiency can repress oncogene-induced senescence in vivo, we conducted SA-β-gal staining on skin sections from age-matched mice. This approach indicated the presence of senescence in BRAFV600E skin; however, to our surprise, strong SA-β-gal staining can also be detected in the double-mutant skin (Fig. 3A), suggesting senescent cells...
are still abundant in the ARF-null background. Quantitation of senescent cells in the skin based on SA-β-gal staining is complicated by dye retention in hair follicles and sebaceous glands. Therefore, we analyzed the expression of genes encoding members of the senescence-associated secretome as an additional marker. Quantitative real-time PCR (qRT-PCR) revealed that most of the secretome factors tested were upregulated in BRAFV600E skin and considerably more highly expressed in BRAFV600E;ARF+/C0/C0 skin (Fig. 3B). Furthermore, immunohistochemical (IHC) staining confirmed increased expression of IL-6 adjacent to putative senescent cells (Fig. 3C). The presence of an equal or increased number of senescent cells observed in the BRAFV600E:ARF−/− skins is consistent with the observation that both p16INK4a and active p53 are present in situ (Fig. 3D). Therefore, the persistent expression of senescence activators and the presence of a copious number of senescent cells despite the lack of ARF strongly argue that direct evasion of senescence is not the mechanism by which loss of ARF accelerates BRAFV600E-driven melanoma formation.

**Loss of ARF sensitizes BRAFV600E mice to UV-induced melanomagenesis**

The data described earlier are consistent with the notion that increased and/or persistent cellular stress in BRAFV600E;ARF−/− melanocytes leads to genetic mutations that bypass senescence and lead to melanomagenesis. To determine if cellular changes elicited by combined BRAF and ARF mutation render melanocytes sensitive to DNA damage, we studied the response of mutant cells and animals to UVR, as solar UVR is the main etiologic factor for melanoma. To this end, we irradiated neonatal mice of different genotypes with a single dose of UVB (750 mJ/cm2; Fig. 4A). During the period of study (100 days), none of the WT, BRAF V600E, or ARF+/C0/C0 mice succumbed to melanoma following UVB exposure (Fig. 4B). Extending the observation period to 300 days also failed to reveal UV-induced tumors in WT and BRAFV600E mice, in agreement with a previous report focusing on Tyr-NRas mice (13). Significantly, every UVB-irradiated BRAF V600E;ARF+/C0/C0 mouse developed multiple melanomas, with 50% of mice developing tumors by day 70 post-UV (73 days of age; Fig. 4B). Thus, UVB irradiation promotes melanomagenesis in BRAFV600E;ARF+/C0/C0 mice. Histopathologic analysis of the tumors confirmed most of them were amelanotic melanomas, as they did not secrete melanin, but stained positive for melanoma markers Trp2, S100, Melan A, and HMB45 (Fig. 4C). Consistent with our observation in spontaneous melanomas, UVB-induced melanomas also preserve active p53 (Fig. 4C, bottom). Therefore, although UVB irradiation is not sufficient to induce melanosomes in mice expressing melanocytic...
BRAFV600E, loss of ARF sensitizes BRAFV600E mice to UVB-induced melanomagenesis.

Loss of ARF impairs DNA damage repair by transcriptionally repressing XPC

UVR results in bulky DNA adducts such as CPD and 6,4-photoproducts, which can be repaired by nucleotide excision. In the absence of repair, mutagenic events may become fixed in the genome and lead to cellular transformation. Because UVB can increase melanoma development in BRAFV600E;ARF−/− mice, we wanted to know whether the combination of those 2 mutations has any effect on DNA repair capability. Because the xeroderma pigmentosum, complementation group C gene (XPC) is a key component in the NER pathway responsible for recognizing DNA adducts, we tested the expression of XPC in primary melanocytes with different genotypes by qRT-PCR. Single- or double-mutant melanocytes expressed a significantly lower level of XPC mRNA, with the lowest level seen in the double-mutant cells (Fig. 5A). The reduced XPC mRNA level correlated with impaired DNA repair capability as shown by a CPD removal assay (Fig. 5B): CPD accumulated dramatically 3 hours following UVB irradiation and was successfully cleared in WT melanocytes by 24 hours post-UVB, but persisted in cells with BRAFV600E and ARF loss. It has been reported that in mouse fibroblasts, ARF enhances XPC
expression by inhibiting the transcriptional repressor activity of E2F4 via disruption of the E2F4–DP1 interaction (21). To determine whether this mechanism might be active in melanomas, we subjected BRAF V600E;ARF−/− melanoma cells with or without ARF reconstitution to UVB irradiation and measured the XPC mRNA level. Upon UVB, XPC mRNA decreased dramatically in the absence of ARF, whereas the presence of ARF maintained the expression of XPC mRNA (Fig. 5C). Furthermore, we confirmed that ARF is able to bind DP1, depleting the fraction of DP1 bound to E2F4 (Fig. 5D). ChIP of E2F4 confirmed the association of this transcriptional repressor with the XPC promoter in both unirradiated and irradiated melanoma cells (Fig. 5E). Interestingly, E2F4 association with the XPC promoter decreased following UVB irradiation (Fig. 5E) concomitant with reduction in XPC mRNA (Fig. 5C), suggesting that UVB irradiation may increase the association of E2F4 with corepressive factors such as p130 (21), thus repressing promoter activity more efficiently despite reduced promoter occupancy. Importantly, reconstitution of melanoma cells with ARF further decreases the association of E2F4 with the XPC promoter and results in increased transcription, suggesting that ARF acts to counteract E2F4-mediated repression of XPC in UVB-irradiated melanoma cells, consistent with the reported role of ARF in mouse embryo fibroblasts (MEF; Fig. 5E; ref. 21).

BRAFV600E inhibits the NER pathway by promoting methylation of the XPC promoter

The qRT-PCR result in Fig. 5A shows that BRAF V600E mutation alone can reduce the level of XPC mRNA. It has been reported that in lung cancer, the promoter region of XPC is highly methylated (33). Furthermore, perturbation of Ras signaling can regulate DNA methylation (34). Because BRAF functions downstream of Ras, it is reasonable to postulate that activation of BRAF could also influence promoter methylation. To test the relevance of this mechanism to XPC regulation, we first analyzed the CpG island in the mouse XPC promoter that spans −1000 to −1 relative to the transcriptional start site. As is the case in the human XPC...
promoter, this region contains multiple CpG islands, as predicted by CpG island searcher software (Fig. 6A; ref. 35). Three HpaII restriction enzyme recognition sites are located within the XPC promoter. Therefore, we conducted HpaII-based qPCR to determine XPC methylation in melanocytes (33). PCR products can be detected in HpaII-digested gDNA from BRAFV600E and BRAFV600E;ARF−/− cells, with a higher amount in the BRAFV600E;ARF−/− DNA (Fig. 6B), indicating that the XPC promoter is hypermethylated in cells of these genotypes. Consistent with this, treatment of melanocytes with the DNA demethylating agent 5′-aza-2′-deoxycytidine can increase the XPC mRNA level only in cells harboring the BRAFV600E mutation (Fig. 6C), suggesting a link between BRAF mutation and XPC hypermethylation.

To examine whether BRAFV600E directly stimulates XPC promoter hypermethylation, we acutely expressed BRAFV600E in BRAF WT CHL1 melanoma cells (Fig. 6D and Supplementary Fig. S3A). Consistent with a previous report (36), introduction of BRAFV600E instigated senescence, presumably due to activation of the DNA damage response mainly in the form of...
double-strand breaks (DSB; Supplementary Fig. S3B). The expression of XPC was significantly inhibited in response to BRAF\(^{V600E}\) addition (Fig. 6E), correlating with a dramatic induction of methylation in the XPC promoter region (Fig. 6F). Abrogating mutant BRAF activity by selective inhibition with PLX-4032 was able to not only restore XPC expression but also impaired promoter methylation (Fig. 6E and F), suggesting that BRAF kinase activity is required for maintaining XPC promoter hypermethylation. Interestingly, PLX-4032 caused ERK activation in CHL1 cells with WT BRAF (Fig. 6D; ref. 37), which also resulted in XPC reduction and promoter methylation (Fig. 6E and F), further supporting the involvement of BRAF-mediated signaling in XPC promoter hypermethylation.

Discussion

In this study, we confirmed ARF is a melanoma suppressor that can cooperate with activated BRAF and showed that loss of ARF is able to sensitize BRAF\(^{V600E}\) mice to neonatal UVB-induced melanoma. Oncogenic BRAF\(^{V600E}\) instigates senescence in melanocytes, which cannot be bypassed by ARF deletion in vivo. Mechanistically, we showed that ARF loss did not abrogate p53 activity, but instead reduced NER by elevating the inhibitory effect of E2F4–DP1 on XPC expression, as first identified in fibroblasts (21). In addition, BRAF\(^{V600E}\) also impaired XPC expression by increasing promoter hypermethylation. Interestingly, XPC has been shown to play a role in melanoma photocarcinogenesis (38). Therefore, XPC expression seems to be a nexus for both oncogenic events studied here, and the combined effects of BRAF mutation and ARF deletion act synergistically to inhibit DNA repair and enhance melanomagenesis in the presence of UVB irradiation. We surmise that increased melanomagenesis in BRAF\(^{V600E}\); ARF\(^{−/−}\) mice that have not been exposed to UVR also results from decreased DNA repair capacity, rendering melanocytes sensitive to the increase in DSBs that accompany BRAF\(^{V600E}\) expression. Interestingly, chronically reduced XPC expression has been linked to a reduced ability to repair DSBs (39), but effects of BRAF\(^{V600E}\) and ARF loss on other forms of
DNA repair may also contribute to the high rate of mutations observed in melanoma cells (40).

The efficient formation of melanosomes in BRAFV600E;ARF−/− mice despite the persistence of high levels of senescence in the skin strongly suggests that the elevated mutation rate leads to selection for events that bypass senescence in individual cells that then emerge as tumor foci. The gene encoding p16INK4a is an obvious candidate, yet BRAFV600E;ARF−/− tumors retained p16INK4a expression, excluding the possibility of concurrent loss of p16INK4a as a cause. Indeed, although deletion of p16INK4a undoubtedly can predispose to melanoma formation, preservation of this protein in established melanomas is, nevertheless, not uncommon (4). Identification of mutational events that cooperate with BRAFV600E to bypass senescence and drive melanoma formation or progression is of obvious importance therapeutically, and the system described here could be useful for studying these mutations. In addition to unbiased screens for cooperating alleles, approaches to this problem include further analysis of likely candidates such as components of the retinoblastoma (Rb) pathway (12), as well as several genes newly identified as susceptible to UV signature mutations in human tumors (41).

One candidate that seems surprisingly excluded from alteration in these melanomas is p53, whose UV-signature mutants are commonly linked to nonmelanoma skin tumors (42). Furthermore, although ARF has been long appreciated as a p53 stabilizer, in our model, we did not observe p53 activity owing to ARF deletion (7). Indeed, contrary to other human malignancies, p53 in melanomas commonly remains WT at the genomic level (16), and also tends to be overexpressed at the protein level (43). Although p53 targeting, in combination with lineage-specific oncogenes activation, has been proven to drastically induce melanoma formation (3, 6, 13, 14), BRAFV600E;TP53−/− mouse melanomas show a distinct transcript profile from that of BRAFV600E;CDKN2A−/− tumors, which closely resemble human melanomas (Haluska; unpublished data). This in turn suggests that the TP53-null melanoma might derive from a distinct population from that giving rise to human melanoma. It is well established that ARF possesses functions independent of p53 (17), and selection against these functions of ARF seem to predominate in melanoma. However, it is likely that alterations in the p53-independent targets of ARF, the actions of hyperactivated BRAF, and selected genetic and epigenetic events combine to offset the effects of activated p53 in nascent melanomas.

The presence of p53 in precancerous BRAFV600E;ARF−/− skin correlates well with our observation that senescence persists in the same tissues, although a previous report indicates ARF ablation bypasses senescence in cultured melanocytes (14). A potential explanation for this discrepancy lies in the difference of senescence triggers: oncogenic BRAFV600E stimulation in melanocytes in situ versus primary melanocytes passaged in culture. Because we have not tested the stability of p53 in the context of ARF loss in this model, it is also possible that without ARF, the turnover of p53 is augmented; however, constitutive oncogenic signaling may effectively counterbalance this, leading to the observed elevation of active p53. On the other hand, the fact that senescence persists in the skin of BRAFV600E;ARF−/− animals that eventually succumb to tumors does not challenge the tumor-suppressive effect of senescence intrinsically, but instead, supports the notion that massive senescence might be protumorigenic extrinsically. Senescent cells secrete numerous factors including inflammatory cytokines, growth factors, and extracellular matrix remodeling enzymes that are collectively called the senescence-associated secretome (44). Such secretome factors make the surrounding milieu protumorigenic, facilitating malignant progression of individual cells that have bypassed senescence due to further mutations gained spontaneously or UVB-induced. This could also potentially explain why BRAFV600E;ARF−/− mice display a decrease in the latency of sarcoma and lymphoma when compared with ARF−/− counterparts.

In addition to increased mutation events resulting from impaired XPC expression, a broader array of epigenetic changes may also lead to bypass of tumor suppressive influences such as p53 function and senescence. Indeed, epigenetic alterations have emerged as an important mechanism underlying BRAFV600E-driven tumorigenesis. For example, BRAFV600E can contribute to methylation alterations in single genes (45, 46) as well as to broader changes in methylation patterns in the genomes of melanomas and papillary thyroid cancers (47, 48). Interestingly, one report failed to associate BRAF mutations with hypermethylation of 15 cancer-linked genes in melanomas (49), suggesting that important BRAF methylation targets in melanomas remain to be discovered. Here, we provide evidence that promoter hypermethylation of XPC is such an event, but this gene is unlikely to be solely responsible for BRAFV600E-mediated changes in mutation sensitivity. On the basis of bioinformatic analysis, CpG islands are clearly evident in other NER genes, such as XPA and ERCC5, suggesting BRAFV600E-mediated methylation of those promoters may also occur. In addition, the mechanism behind BRAFV600E-induced methylation remains to be elucidated. Upregulation of DNA methyltransferase 1 by BRAFV600E is a promising candidate, as has been reported (47). Interestingly, Ras-mediated methylation requires numerous factors including E2F1 (50), and this is likely to be the case for BRAFV600E as well. Because the activity of E2F1, like E2F4, requires the association of DP1, and this association can also be influenced by ARF (20), it would be intriguing to investigate whether ARF loss facilitates BRAFV600E-mediated promoter methylation, in addition to altering the expression of individual genes in an E2F-dependent manner. Clearly, the ability to reverse BRAFV600E-induced epigenetic changes that then lead to an increased incidence of genetic changes could have an important impact on both tumor progression and development of
resistance to therapy. The induction of XPC observed here upon PLX-4032 treatment offers a glimmer of hope that such processes remain targetable in malignant melanomas, and are therefore an important area for future study.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Luo, M.G. Hu, R. Cui, P.N. Tsichlis, P.W. Hinds
Development of methodology: C. Luo, F.G. Hahska, G.-F. Hu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Luo, J. Sheng, P.N. Tsichlis, G.-F. Hu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Luo, G.-F. Hu
Writing, review, and/or revision of the manuscript: C. Luo, G.-F. Hahska, Z. Xu, P.N. Tsichlis, P.W. Hinds
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Luo, M.G. Hu
Study supervision: C. Luo, P.W. Hinds

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References
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Chi Luo, Jinghao Sheng, Miaofen G. Hu, et al.


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