CDKN2B Loss Promotes Progression from Benign Melanocytic Nevus to Melanoma

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ABSTRACT
Deletion of the entire CDKN2B-CDKN2A gene cluster is among the most common genetic events in cancer. The tumor-promoting effects are generally attributed to loss of CDKN2A-encoded p16 and p14ARF tumor suppressors. The degree to which the associated CDKN2B-encoded p15 loss contributes to human tumorigenesis is unclear. Here, we show that CDKN2B is highly upregulated in benign melanocytic nevi, contributes to maintaining nevus melanocytes in a growth-arrested premalignant state, and is commonly lost in melanoma. Using primary melanocytes isolated directly from freshly excised human nevi naturally expressing the common BRAFV600E-activating mutation, nevi progressing to melanoma, and normal melanocytes engineered to inducibly express BRAFV600E, we show that BRAF activation results in reversible, TGFβ-dependent, p15 induction that halts proliferation. Furthermore, we engineer human skin grafts containing nevus-derived melanocytes to establish a new, architecturally faithful, in vivo melanoma model, and demonstrate that p15 loss promotes the transition from benign nevus to melanoma.

SIGNIFICANCE: Although BRAFV600E mutations cause melanocytes to initially proliferate into benign moles, mechanisms responsible for their eventual growth arrest are unknown. Using melanocytes from human moles, we show that BRAF activation leads to a CDKN2B induction that is critical for restraining BRAF oncogenic effects, and when lost, contributes to melanoma. Cancer Discov; 5(10); 1072–85.

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INTRODUCTION

The CDKN2A locus on chromosome 9p21 encodes two tumor suppressors: the cyclin-dependent kinase (CDK) inhibitor p16INK4A and the MDM2 regulator p14ARF, which together help maintain functional RB and p53. Homozygous deletion of this locus is among the most common genetic events in human cancer across tissues, with the resultant selective advantage to cancer cells generally attributed to the loss of CDKN2A proteins. However, in greater than 90% of cancer tissues harboring CDKN2A deletion, the adjacent CDKN2B gene encoding the p15INK4B CDK inhibitor is also deleted (1, 2). The role of p15 in normal or neoplastic human tissues has not been clearly established. Here, we use primary cells isolated directly from normal human skin and benign melanocytic nevi, along with genetically defined human skin tissue xenografts engineered in vivo, to establish p15 induction as a key element arresting common nevi in their premalignant state. p15 depletion in benign nevi promotes progression to melanoma, establishing the functional importance of CDKN2B loss in human cancer.

Melanoma is a potentially fatal skin cancer arising from the pigment-producing melanocyte cells within the basal layer of the human epidermis. Most melanomas are driven by an acquired, activating V600E mutation in the BRAF proto-oncogene, and are attributed to nucleotide sequence errors incorporated during imperfect repair of ultraviolet DNA damage (3). This same BRAFV600E mutation is also responsible for the formation of the majority of common benign melanocytic nevi (moles; refs. 4, 5). Nevi typically develop in the first 25 years of life, when constitutive BRAFV600E signaling causes melanocytes to transiently proliferate, forming the common brown skin papule present on most adults. Nevus growth typically halts after reaching 3 to 5 mm in diameter, where melanocytes then remain quiescent for the remainder of a person’s life, despite continued expression of the BRAFV600E oncogene (6). Mechanisms responsible for proliferation arrest at this stage have not been previously established, but are likely to include induction of CDK inhibitors, as bypass of RB-mediated cell-cycle restraints via CDK4 activation occurs in human melanoma, and was shown previously to promote melanoma formation in a human model driven by virally overexpressed active RAS or BRAF (7). Although most nevi remain innocuous, up to half of malignant melanomas arise from preexisting nevi, suggesting that the growth arrest is likely reversible (8–10). However, no previous work has established the reversibility of the growth arrest observed in human nevus melanocytes, nor demonstrated that BRAFV600E nevus melanocytes within common benign human moles are capable of progressing to malignancy. Determining the mechanisms through which benign nevus cells ultimately restrain BRAF proliferative signaling, and how this capacity is lost in the progression to malignant melanoma, may be crucial for the development of new melanoma therapeutics.

To delineate the signaling events responsible for the genesis of nevi and their subsequent growth arrest, we first developed methods to isolate primary BRAFV600E-expressing melanocytes directly from freshly excised benign human nevi,
a significant technical advance that has not been demonstrated previously. Nevi are naturally occurring “knockin” experiments where melanocytes harbor a single copy of the BRAFV600E gene regulated by the endogenous promoter, in the context of native human stroma, and in most adult cases have been growth arrested in vivo for decades. By examining these nevus melanocytes, as well as normal primary melanocytes engineered to inducibly express BRAFV600E, we were able to link BRAF activation to a TGFβ-dependent p15 induction that arrests the cell cycle and antagonizes oncogenic BRAF signaling. We demonstrate that the growth arrest is reversible and use primary nevus cells to establish a new genetically defined human melanoma xenograft model in vivo, which maximizes medical relevance by supporting tissues in their native three-dimensional environment (11). With this approach, we show that p15 loss is a major factor promoting the transition from benign nevus to melanoma.

RESULTS

BRAFV600E Nevus Melanocytes Are Growth Arrested

To establish mechanisms promoting growth arrest in BRAFV600E nevi, we isolated primary melanocytes (nevus-MC) from clinically benign nevi excised from 27 human donors (Fig. 1A). Only long-standing nevi with benign appearance and no history of recent morphologic changes were collected. Nevi were microdissected from surrounding tissue, and melanocytes were separated from stroma and other skin cell types using physical and enzymatic dissociation followed by culture in selective media. Cultured nevus-MCs were morphologically indistinguishable from melanocytes isolated from unremarkable skin (Fig. 1B). Sequencing BRAF cDNA from cultured nevus-MCs revealed overlapping, equal-intensity peaks at nucleotide 1799, corresponding to expression of both wild-type (1799T) and mutant V600E (1799T→A) BRAF alleles (Fig. 1C). Consistent with the high frequency of BRAF mutation in benign nevi, every nevus-MC culture we successfully initiated harbored an identical BRAFV600E mutation on a single allele. Keratinocytes isolated from epidermis associated with the nevi expressed only wild-type BRAF (Fig. 1C).

Although nevus-MCs remained viable in vitro, they failed to proliferate, independent of cell plating density, reflecting their in vivo growth arrest. Consistent with this, the Ki67+ proliferative index of nevus-MCs was only 4%, compared with 38% for normal melanocytes (Fig. 1D and Supplementary Fig. S1). The significance of the few, weakly Ki67-positive nevus-MCs is unclear, but as Ki67 is expressed in all proliferative...
phases of the cell cycle, cells arrested beyond G0 may retain some Ki67 expression, without actually completing cell division (12).

To determine whether BRAF V600E expression was sufficient to drive melanocytes’ proliferative arrest in the absence of additional in vivo cues, we engineered normal primary melanocytes to express doxycycline-inducible BRAF V600E (diBRAF V600E). Consistent with a key role for BRAF V600E in nevus-MC growth arrest, doxycycline-treated diBRAF-MCs ceased to proliferate, whereas untreated diBRAF cells proliferated normally (Fig. 1E).

The CDK Inhibitor p15 Is Induced in Nevi

To elucidate the mechanisms underlying BRAF V600E-induced growth arrest, we compared expression of cell-cycle regulatory genes in nevus-MCs versus normal MCs by quantitative reverse transcriptase (qRT) PCR analysis. This revealed altered expression of multiple cell-cycle regulators, the most prominent being CDKN2B (p15), which was induced 138-fold (Fig. 1F). Consistent with this finding, benign human nevi in vivo express high levels of p15 (Fig. 2A and B and Supplementary Fig. S1). Epidermal melanocytes in adjacent tissue, not associated with the nevus, appeared p15 negative (Fig. 2C and D). As p15 inhibits cyclin D–CDK4/6 complexes and promotes RB-dependent maintenance of the G1–S cell-cycle checkpoint (13), we hypothesized that p15 plays a role in arresting nevus growth, and may cooperate with other upregulated cell-cycle regulators, including p14, p16, and p21, to maintain proliferative arrest in vivo. In line with this hypothesis, previous efforts to elucidate the basis for BRAF-mediated growth arrest in human cells using viral-driven transgene expression of BRAF V600E in fibroblasts, or NRAS Q61K in melanocytes, noted p16 induction, but concluded that other factors must contribute, as p16 antagonism was insufficient to rescue proliferation (6, 14).

To test the hypothesis that p15 and/or p16 induction is a necessary component for BRAF V600E-induced growth arrest, and to determine whether the growth arrest is reversible, we first sought to circumvent p15/p16 function through expression of CDK4 R24C in arrested nevus and diBRAF V600E melanocytes. The activating R24C mutation, which occurs in human melanoma (15, 16), rendered CDK4 resistant to p15/p16 and restored proliferation in both nevus and engineered diBRAF V600E melanocytes (Supplementary Fig. S2A–S2C).

p15 Induction by BRAF Is Both Sufficient and Necessary for Melanocyte Growth Arrest

To test whether expression of BRAF V600E is sufficient on its own to upregulate p15 and p16, we compared their expression in doxycycline-treated diBRAF V600E-MCs, nevus-MCs, and normal melanocytes. Whereas normal melanocytes expressed very little p15 or p16, doxycycline-treated diBRAF-MCs and nevus-MCs both expressed p15 and p16 at high levels in primary culture and in vivo (Fig. 3A and Supplementary Fig. S3). Extended culture of primary nevus-MCs in vitro showed that elevated levels of p15 and p16 were maintained along with the growth-arrested phenotype. Importantly, p15 induction and the associated growth arrest are both dependent on the level of BRAF V600E expression. Levels of p15 initially increase proportionally with increasing BRAF V600E protein. However, at the highest doxycycline-induced BRAF levels, or with high-level BRAF expression driven by a conventional lentivirus using a constitutive PGK promoter, p15 protein induction is attenuated, and melanocytes continue to proliferate (Fig. 3A and data not shown). This highlights the need to consider the BRAF protein level when using model systems, and led us to rely on the native nevus cells for functional in vivo experiments.

To determine whether p15 is sufficient to cause melanocyte growth arrest without simultaneous induction of other growth regulators, including p16, we transduced primary...
melanocytes with p15 or p16. Melanocytes expressing p15 halted proliferation, whereas melanocytes expressing p16 continued to divide, albeit more slowly than controls (Fig. 3B and Supplementary Fig. S4A). Through virus titering and quantitative PCR, we demonstrated that these proliferation differences did not result from relatively lower levels of p16 gene expression. The p16 virus titer was actually higher than that of the p15 virus used, and this corresponded to higher pRB levels (Supplementary Fig. S4B and S4C). Although p15 and p16 share structural homology through their ankyrin repeat domains, there are significant regions of divergent sequence, particularly in the N-terminus, that may serve to make p15 a more efficient CDK inhibitor (reflected by lower pRB).

In addition, previous work using a series of cell lines and chimeric p15/p16 domain swap constructs established that sequences unique to the p15 N-terminus confer p15 with the ability to influence cell cycle beyond RB, and that p16 does not possess this activity (17). Although the specific interacting proteins and pathways mediating this additional p15 activity are not yet established, they are likely functionally important in nevus melanocytes.

To test whether BRFV600E-induced growth arrest requires p15 or p16, we transduced diBRAFV600E cells with shRNAs targeting p15 (p15i) or p16 (p16i; Fig. 3C and D). Without doxycycline, diBRAFV600E cells harboring p15i or p16i proliferated normally as expected. With doxycycline, proliferation of diBRAFV600E cells expressing control or p16i slowed dramatically, whereas p15i cells continued to proliferate. Together, these data indicate that p15 is both necessary and sufficient for BRFV600E-mediated growth arrest. To ensure that the observed proliferation phenotype of the p15 knockdown was due to loss of p15 and not an off-target effect of the shRNA, we restored p15 expression in p15i shRNA knockdown, and demonstrated that cells again growth arrested as expected (Supplementary Fig. S5C and S5D).

Figure 3. p15 is necessary and sufficient to arrest melanocyte proliferation. A, Western blot analysis of BRAF, p15, and p16 expression in diBRAFV600E melanocytes in response to increasing levels of doxycycline. β-Actin was used as a loading control. B, proliferation of normal human melanocytes engineered to express p15 or p16. C, proliferation of diBRAFV600E melanocytes with nonsilencing control (NSi), p15 (p15i) or p16 (p16i) shRNA knockdown, without (left) or with (right) 0.25 µg/mL doxycycline. Data, average ± SEM for B and C, n = 3 biologic replicates for each group. D, Western blot analysis of BRAF, BRAFV600E, p15, and p16 in diBRAFV600E cells with nonsilencing control (NSi), p15 (p15i), and p16 (p16i) shRNA knockdowns. β-Actin was used as a loading control. Western blot analysis results and growth curves were representative of three biologic replicates composed of distinct melanocyte populations.
As CDKN2A and CDKN2B are frequently simultaneously deleted in melanoma, we next questioned whether simultaneous loss of p15 and p14/16 activity had an additive effect on overcoming BRAFV600E-mediated growth arrest. We used the melanoma-associated dominant-negative p53 (dnp53) mutant (R248W) to recapitulate the functional effects of p14 loss. Accordingly, we compared the relative proliferation of diBRAFV600E-MCs expressing shRNAs targeting p15 to matched cells with the p16 shRNA and dnp53, or the combination of p15 and p16 shRNAs and dnp53. In the absence of BRAFV600E expression, all cell populations proliferated normally, indicating that no off-target effects compromised general cell-cycle regulation. As seen previously, melanocytes expressing BRAFV600E growth arrested, whereas BRAFV600E cells with p15 knockdown proliferated, albeit more slowly than normal controls lacking BRAFV600E. BRAFV600E-expressing cells with p16 knockdown and dnp53 expression failed to proliferate. However, cells with the combination of BRAFV600E, p15/p16 knockdown, and dnp53 proliferated faster than the cells with p15 knockdown alone (Supplementary Fig. S6). Therefore, the combinatorial loss of p15, p14 (through dnp53), and p16 activity promoted proliferation in the face of BRAFV600E to a greater extent than the loss of the individual elements. These results are consistent with our data showing that although each of the proteins encoded by the CDKN2A–CDKN2B locus are important in melanomagenesis, p15 is an especially important mediator of BRAF-induced growth arrest.

p15 Is Diminished in Melanoma

To discover whether p15 helps restrain the oncogenic activity of BRAFV600E in vivo, we examined informative clinical cases in which invasive melanoma arose in clear continuity with a preexisting benign nevus (Fig. 4A). p15 protein was diminished in the malignant melanomas compared with adjacent nevi (Fig. 4B and C). To determine the general relevance of this finding, we examined a series of 12 randomly selected malignant melanomas and 15 benign nevi, along with five nevus-melanoma cases. The majority of nevi strongly expressed both p15 and p16, whereas the majority of melanomas showed very low to undetectable p15 and p16 (Supplementary Fig. S7A). This pattern of coordinate regulation of p15 and p16 is consistent with the latest Cancer Genome Atlas (TCGA) analysis of 278 melanomas in which 93% of melanomas with homozygous deletion of CDKN2A (p16) also harbor homozygous deletions of CDKN2B (p15; Supplementary Fig. S7B and S7C; refs. 1, 2). Simultaneous loss of these two cell-cycle inhibitors likely reflects the close proximity of the adjacent CDKN2A–CDKN2B loci on chromosome 9p21.3. Importantly, the TCGA series includes a tumor in which CDKN2B is lost but p16 remains intact, a genetic event that has also been described in other series (18, 19). Consistent with these previous reports, we also identified a clinical case in our sample group in which a p16-positive melanoma developed in the epidermis overlying a benign nevus (Supplementary Fig. S8). Although the nevus-MCs robustly expressed both p15 and p16, the melanoma retained only p16 and lacked p15 expression. Together, these findings highlight the functional importance of p15 loss in human melanoma and suggest that p15 expression may serve as a clinically useful tool to help distinguish between benign and malignant melanocytic neoplasias that are otherwise histologically ambiguous.

BRAFV600E Activates p15 Expression through TGFβ Signaling

In other cell contexts, p15 is induced via TGFβ/SMAD activity (13, 20). Increased TGFβ secretion in response to BRAF has not been previously demonstrated in melanocytes, but has been observed in immortalized rat thyroid epithelial cells (21). We questioned whether BRAFV600E-induced melanocyte TGFβ expression. BRAFV600E increased both TGFβ mRNA and protein secretion in nevus-MCs and diBRAFV600E-MCs (Fig. 5A and B). To determine whether TGFβ was sufficient to recapitulate the antiproliferative effects of BRAFV600E, we added TGFβ to primary melanocyte cultures. As expected, this induced p15 and arrested growth (Fig. 5C). p15 induction resulting from either exogenous TGFβ or BRAFV600E is dependent on the TGFβ receptor, as the TGFβ receptor inhibitor SB-431542 blocked p15 induction in both cases (Fig. 5D). These data are consistent with human nevus tissue, which displays robust SMAD activation (22).

The specific transcription complexes downstream of BRAF responsible for directly activating TGFβ transcription are not definitively established, but are likely to include EGR1. In other human cell contexts, EGR1 binds to the regulatory regions of the TGFβ promoter to drive transcription (23, 24), and EGR1 is a firmly established ERK1/2 target (25). Consistent with these prior studies, EGR1 is upregulated in BRAFV600E nevus melanocytes (Supplementary Fig. S9A). Also consistent with this ERK-dependent p15 induction, inhibition of ERK signaling by the MEK...
inhibitor U0126 blocked BRAF-driven p15 induction (Supplementary Fig. S9B).

We next sought to determine whether inhibiting BRAF

V600E itself would restore proliferation in melanocytes harboring constitutively active BRAF. We treated both our engineered doxycycline-inducible diBRAF

V600E melanocytes and primary nevus-derived V600E melanocytes with the selective BRAF

V600E small-molecule inhibitor PLX4720. As expected, PLX4720 treatment, when delivered with doxycycline to diBRAF

V600E cells, prevented both p15 induction and the corresponding growth arrest. In contrast, when the inhibitor was delivered to diBRAF cells in which growth arrest had been previously established by antecedent doxycycline administration, cells remained growth arrested, and p15 levels, although lower than those observed without PLX4720, remained higher than in normal cells (Supplementary Fig. S10A). This suggests that under in vitro culture conditions, BRAF activity and the corresponding p15 induction are necessary for establishing the growth arrest, but that additional events may also serve to reinforce and maintain it. These other events are unclear, but are likely to involve epigenetic transcriptional memory influencing core cell-cycle regulators. This finding of persistent growth arrest despite BRAF

V600E inhibition is consistent with clinical observations, in which vemurafenib-treated patients do not typically display obvious morphologic changes in pre-existing benign nevi. Also consistent with this is our observation that PLX4720 does not reverse growth arrest in primary melanocytes isolated directly from nevus specimens (Supplementary Fig. S10B). To examine this issue further, we used an alternative approach to “turn off” BRAF

V600E. Melanocytes expressing inducible BRAF

V600E that underwent growth arrest after 3 days of doxycycline administration were subsequently cultured in media lacking doxycycline. Those cells remained growth arrested (Supplementary Fig. S10C and S10D), reinforcing the idea that melanocytes may use additional BRAF-independent mechanisms that maintain the growth-arrested state, once established.

BRAF

V600E Alters the Epigenetic Chromatin Landscape at the CDKN2A and CDKN2B Loci

CDKN2A and CDKN2B are normally suppressed in proliferating melanocytes, suggesting that the loci are epigenetically repressed. Protein induction in response to BRAF activation would therefore require not only engagement of TGFβ-activated transcription complexes, but also removal of repressive epigenetic chromatin marks. Chromatin immunoprecipitation targeting the H3K27me3 and H3K9me3 repressive epigenetic chromatin marks. Chromatin immunoprecipitation targeting the H3K27me3 and H3K9me3 repressive histone modifications showed that BRAF

V600E induction is inhibited by 50 μmol/L SB-431542 (SB4) TGFβ receptor blockade in human melanocytes.

Figure 5. TGFβ secretion induces p15 expression and drives melanocyte growth arrest. A, relative transcription of TGFβ1, TGFβ2, and TGFβ3 in nevus-MCs and normal melanocytes determined by TaqMan qPCR. n = 3 biologic replicates for normal melanocytes and n = 4 biologic replicates for nevus-MCs. *, P < 0.05. B, TGFβ levels in culture media from diBRAF

V600E or normal melanocytes. Data, average ± SEM; n = 3 biologic replicates for diBRAF

V600E melanocytes and normal melanocytes. C, proliferation of luciferase-transduced melanocytes with or without 100 pmol/L exogenous TGFβ. Data, average ± SEM; n = 3 biologic replicates for each group. D, 100 pmol/L TGFβ or BRAF

V600E-driven p15 induction is inhibited by 50 μmol/L SB-431542 (SB4) TGFβ receptor blockade in human melanocytes.
Although CDK4 activation overcame nevus-MC growth arrest in culture, it was insufficient to sustain proliferation in vivo. Melanocytes survived in the orthotopic location, but demonstrated a low Ki67+ index (Fig. 6C). As a cell’s ability to progress through the cell cycle depends on the relative balance of proliferative versus antiproliferative signals, this difference between in vitro and in vivo proliferation likely results from a relative lack in vivo of the mitogenic growth factors incorporated into melanocyte tissue culture media that cooperate with activated BRAF and CDK4 to overcome the antiproliferative effects of TGFβ. These data are consistent with the observation that although normal interfollicular melanocytes proliferate under culture conditions optimized to support normal melanocyte cell division, the mitotic rate of normal melanocytes in vivo is extremely low (27), and underscores the importance of assays that mimic the in vivo setting. These data also suggest that additional genetic events promote melanoma cell-cycle progression in vivo. As most melanomas express increased levels of telomerase (28), and telomerase upregulation has been shown to decrease the requirements for exogenous mitogens to maintain human cell growth in culture (29) in addition to maintaining telomere length, we next transduced nevus-MCs with both human telomerase reverse transcriptase (hTERT) and CDK4R24C prior to xenografting. These cells displayed a 4-fold increase in telomerase activity (Supplementary Fig. S12A), consistent with the magnitude of the increase seen in spontaneous melanoma. Tissue grafts with nevus-MC expressing hTERT and CDK4 contained nests of pigmented melanocytes similar in architecture to those seen in benign human nevi (Fig. 6C), indicating that after a brief proliferative period, the nevus-MCs ceased to divide. The initial proliferation increase stimulated by the introduction of hTERT prompted us to ask whether TERT may play a role in downregulating p15 expression in melanocytes; however, hTERT expression did not inhibit p15 induction in BRAFV600E melanocytes (Supplementary Fig. S12B). Therefore, nevus-MCs expressing the combination of hTERT, CDK4, and BRAFV600E still lack elements required to fully overcome the senescence-like program in vivo and progress to melanoma. To determine whether these growth-arrested melanocytes (shown in Fig. 6C) also expressed other traditional “senescence” markers in addition to absence of Ki67, we next examined the tissues for melanocyte expression of p16, which was highly expressed in all melanocytic nests, but largely absent in single nevus-derived melanocytes that failed to proliferate, even transiently, in xenografts (Supplementary Fig. S13A). As p16 is generally expressed in response

Figure 6. In vivo melanoma model using nevus-derived BRAFV600E melanocytes. A, in vitro organotypic skin tissue prior to skin grafting. B, human skin xenograft harboring BRAFV600E-expressing melanocytes. C, hematoxylin and eosin (H&E) staining, with Melan-A, microphthalmia-associated transcription factor (MITF), and Ki67 immunohistochemistry (columns, left to right) from xenografts incorporating nevus-MC expressing CDK4R24C (row 1), CDK4R24C and hTERT (row 2), or CDK4R24C, hTERT, and dnp53R248W (row 3). Images are representative of 3 biologic replicates. hTERT, human telomerase reverse transcriptase. Scale bars, 100 μm.
to proliferation and mitogenic stimulation, the growth-arrested nevus cells that remain as single cells likely do not reach the mitogenic threshold in vivo required to trigger p16 expression. The functional significance of these variably positive senescence markers is somewhat unclear, as previous efforts have determined that there are currently no available senescence markers that reliably distinguish between normal melanocytes, nevi, and melanoma (30). To determine whether cell death was increased in the growth-arrested nests, we next analyzed the sections by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL). As a positive control, we used skin that had been acutely exposed to UVB radiation, which triggers an apoptotic response in the epidermis. Although many apoptotic cells were seen in controls, there were very few, if any, apoptotic cells noted in the xenografts (Supplementary Fig. S13B). In addition to p15 induction, nevus-MCs displayed increased levels of the p53-regulated CDK inhibitor p21 (Supplementary Fig. S14). Most melanomas lack functional p53 as a result of either p14ARF loss or dominant-negative p53 mutation (31). To recapitulate this functional loss, we engineered xenografts with nevus-MCs expressing dominant-negative p53R248W together with CDK4R24C and hTERT (row 1), and nevus-MC expressing shRNA against p15 (p15i), dnp53R248W, and hTERT (row 2). Tissue from human skin xenografts was harvested at either 60 days (left) or 100 days (right) and is representative of 3 biologic replicates. Arrows indicate upward epidermal pagetoid scattering of neoplastic melanocytes. Scale bars, 100 μm.

**Figure 7.** p15 loss reverses nevus-MC growth arrest and promotes progression to melanoma in vivo. Hematoxylin and eosin (H&E) staining with Melan-A immunofluorescence of nevus-MC expressing control shRNA (NSi), dnp53R248W, and hTERT (row 1), and nevus-MC expressing shRNA against p15 (p15i), dnp53R248W, and hTERT (row 2). Tissue from human skin xenografts was harvested at either 60 days (left) or 100 days (right) and is representative of 3 biologic replicates. Arrows indicate upward epidermal pagetoid scattering of neoplastic melanocytes. Scale bars, 100 μm.

**p15 Loss Reverses Nevus-MC Growth Arrest In Vivo and Promotes Progression to Melanoma**

To determine whether p15 loss functionally substitutes for CDK4 activation in vivo to promote progression from benign nevus to melanoma, nevus-MCs transduced with shRNA targeting p15 (p15i), or control shRNA (NSi), dnp53, and hTERT were seeded along with unremarkable keratinocytes on native human dermis. Xenografts were harvested at 60 and 100 days. At 60 days after grafting, nevus-MCs harboring anti-p15 shRNAs had clearly overcome their growth-arrested state and proliferated to form nests of melanocytes at the dermal–epidermal junction. Conversely, nevus-MCs harboring the control hairpin survived the grafting process, but were present only as single cells at regular intervals along the basement membrane of the tissue and did not proliferate into melanocytic nests (Fig. 7). At 100 days after grafting, nevus-MCs harboring control shRNAs maintained their growth-arrested state, whereas those with p15 knockdown proliferated into large nests and displayed hallmark features of melanoma in situ, including large histologically atypical melanocytes with high mitotic index and pagetoid cells migrating upward through epidermis (Fig. 7).

To determine whether loss of p16 was also sufficient to reverse the BRAFV600E-induced growth arrest of nevus-MCs in vivo, we repeated the experiment with nevus-MCs harboring shRNAs targeting p16. Consistent with our in vitro data, we found that p16 knockout nevus-MCs proliferated in the xenograft setting, but did so less robustly than melanocytes lacking p15 (Supplementary Fig. S15A). Loss of p16 led to modest increases in melanocyte density that were intermediate between the controls and that of the p15 knockout tissues (Supplementary Fig. S15B).

**DISCUSSION**

The initial identification of activating BRAFV600E mutations in common acquired melanocytic nevi provided an explanation for how nevi originate, as BRAF activation engages MAPK signaling to drive cell-cycle progression. However,
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identifying the mechanistic basis for the subsequent growth arrest has remained elusive. A striking clinical feature is that most acquired nevi, independent of body site, sex, or age of onset, are similarly sized, suggesting that nevi engage a common feedback mechanism that ultimately restrains BRAF signaling, and that this process is related to lesion size. Telomere erosion with replicative senescence could potentially limit nevus size; however, telomere length in nevi is not shortened (6). Our results identify a novel mechanism for nevus growth arrest in which BRAFV600E activation induces expression of secreted TGFβ via signaling through the canonical MEK/ERK MAPK cascade. TGFβ signaling, in turn, upregulates p15 and halts proliferation. The involvement of a secreted molecule, TGFβ, may provide a mechanistic explanation for why most human nevi fall within the narrow size range. Because TGFβ is secreted as an inactive precursor molecule that requires proteolytic processing in the extracellular space in order to activate the TGFβ receptor, the local tissue concentration of TGFβ would need to be high enough to signal back through the TGFβ receptor to halt proliferation. Small lesions may not produce sufficient TGFβ to reach the threshold level required to counteract BRAF mitogenic activity. In this model, TGFβ’s rate of secretion, diffusion, and half-life in skin would together dictate the number and density of melanocytes necessary for growth arrest. It seems likely that for human nevi, this critical mass often corresponds to a 3 to 5 mm sphere, although the geometry of the nascent nevus nest and the local ratio of receptor to ligand may contribute to the variations in three-dimensional nevus architecture observed in human skin.

Previous studies exploring the genesis of melanocytic nevi and subsequent progression to melanoma have relied on either murine models or viral transduction of human cells with activated BRAF. Although these efforts have yielded useful insights, their medical relevance is questionable, as mice do not naturally form nevi, and most mouse skin contains melanocytes only within hair follicles. In contrast, human skin contains melanocytes both within hair follicles and also along the basal layer of the intervening epidermis, where they are exposed to higher levels of ultraviolet radiation within a three-dimensional environment architecturally distinct from the hair follicle. Using the naturally occurring human BRAFV600E nevus cells in the context of native human tissue also avoids the potential of being misled by murine-specific oncogene effects. This is an important point to consider when developing models for testing potential therapeutics, as murine and human cells can differ in their response to oncogenes activating the MAPK pathway (34).

In characterizing nevus-MCs and primary melanocytes engineered to express inducible or constitutive BRAFV600E, we noted that the proliferative capacity depends on the level of BRAFV600E protein. Increasing BRAFV600E expression initially correlates with proportionally increased p15 expression. However, at the highest levels of BRAF expression (achieved with either dI-BRAF cells treated with high doxycycline doses, or cells harboring a constitutive BRAFV600E gene driven by a standard PGK promoter), p15 protein levels were attenuated and melanocytes continued to proliferate. In this case, it appears that supraphysiologic BRAF activity overcomes the TGFβ-mediated growth arrest observed at lower BRAF levels. Although an exact mechanism underlying the reduction of p15 expression at the highest levels of BRAFV600E signaling remains elusive, increased MAPK flux and potential cross-talk with the PI3K–AKT pathway may influence core cell-cycle machinery and lead to negative feedback on p15. This finding provides direct evidence that the effects of oncogenes on human cells are highly dependent on their absolute expression level, and underscores the importance of controlling oncogene expression level. A similar observation was recently made in mice, where relatively modest changes in KRAS translation, driven by alternative codon usage, altered its oncogenic potential (35).

To our knowledge, this is the first research that formally demonstrates a proliferation arrest of human melanocytes in response to BRAFV600E. Previous studies using viral-oncogene expression in primary cells did not include melanocyte growth curves, and instead relied on senescence-associated β-galactosidase, Ki67 positivity, or decreased growth in another cell type (fibroblasts) as proliferation surrogates (6). Indeed, our initial experiments with constitutive BRAF-expressing vectors failed to arrest growth, and it was for that reason that we developed the tunable doxycycline-inducible approach.

Our data from in vivo xenograft experiments demonstrate that human nevus melanocytes are not irreversibly growth arrested and can become malignant with the acquisition of a minimal set of melanoma-associated secondary mutations, including CDKN2A/B loss (or CDK4 activation), dominant-negative p53, and telomerase upregulation. This malignant combination of genetic insults counteracts the cell-cycle inhibition, resulting in conversion from benign nevus to melanoma. The melanoma-promoting effects of hTERT, CDK4, and dominant-negative p53 were shown in a prior xenograft model (7), but that study did not use nevus melanocytes, used high-level oncodriver expression using constitutive retroviral promoters, and did not examine the role of p15. The functional consequence of CDKN2A/B loss in human tissue has not previously been examined directly, but our observation that CDKN2B was induced 138-fold in nevus versus normal melanocytes suggested that p15 is likely functionally important. Indeed, all of the benign nevi tested displayed high p15 expression, whereas malignant tissues were frequently p15-negative, indicating that p15 expression may be a useful marker to help distinguish benign versus malignant melanocytic tumors. These clinical data are consistent with our functional studies in vitro, as well as with our genetically defined human skin tissue in vivo, demonstrating clearly that p15 loss promotes progression from benign nevus melanocyte to melanoma.

In human cancer, nonsense and missense mutations are more commonly observed in CDKN2A than in CDKN2B. This likely reflects the fact that CDKN2A mutations frequently compromise function of the overlapping p14ARF gene (36), decreasing p53 activity and conferring added proliferative advantage to melanocytes harboring these mutations. Conversely, CDKN2B mutations do not directly affect ARF. The most recent TCGA melanoma data include 36 of 278 cases (13%) in which CDKN2A contains nonsense, missense, or frameshift mutations, most of which likely negatively affect p16 activity (1, 2). The vast majority of these cases (31 of 36; 86%)
are in exons shared with ARF. Exon 1 from p16, which does not have overlapping sequence with ARF, is mutated in only 5 of 278 cases. Remarkably, 3 (60%) of those cases with CDKN2A exon 1 mutations also harbor inactivating TP53 mutations, which are otherwise rare in melanoma. (The other two cases are tumors driven by RAS, not BRAF.) This indicates that p16 loss on its own is likely not sufficient to provide a significant fitness advantage to human melanocytes in vivo, and suggests that CDKN2A mutations may be more common than CDKN2B mutations, at least in part because of the associated ARF effects. The idea that p53/ARF effects may play a relatively larger role than p16 effects in melanoma is consistent with a mouse melanoma model driven by HRAS where tumors form more quickly on a germline ARF-null background than on a p16-null background (37), and in murine melanoma models driven by BRAFV600E with intact p16, where ARF loss promotes melanoma formation without altering the initial growth-arrested senescent phenotype in vivo (38).

Together, our in vitro and in vivo data indicate that although each of the proteins encoded by the CDKN2A–CDKN2B locus are important in melanomagenesis, p15 is an especially important element and a critical mediator of BRAF growth arrest. Combined antagonism of p15, p16, and p14 (via dn53) promoted proliferation of diBRAFV600E melanocytes beyond that seen with the individual elements. In contrast, we have been unable to restore proliferation in primary BRAFV600E nevus melanocytes in vitro using individual or combinatorial loss of p15, p16, and dn53. This likely reflects technical differences encountered when moving nevus cells from the three-dimensional in vivo to the two-dimensional in vitro environment, and is consistent with the results from the PLX4720 and doxycycline withdrawal experiments where additional factors beyond BRAF and CDKN2A/B seem to maintain the previously established growth arrest in vitro.

Our data establishing that p15 is important for restraining human BRAF oncogenic activity is consistent with the existence of benign nevi in p16-null humans who retain p15 (39), indicating that melanocytes are capable of using additional mechanisms beyond p16 to restrain BRAF proliferative signals. Also suggestive of an important role for p15 is the previous identification of inactivating p15 mutations in spontaneous melanomas in which p16 is preserved (40).

Although it is likely that most BRAFV600E nevi undergo proliferative arrest through a common pathway involving p15 induction, there are likely multiple mechanisms through which the growth arrest can be subsequently bypassed in conversion to melanoma. In addition to the p15 loss demonstrated here in human tissue, recent murine models have suggested that mTOR activation may play a role in some cases, as STK11 loss (which occurs in about 5% of human melanoma and leads to increased mTOR activity) promotes BRAF-driven mouse melanoma (41). PTEN loss or AKT3 activation, which both increase mTOR signaling, are also frequent events in nevus-associated human melanoma (42).

Although our studies suggest that melanoma can develop from growth-arrested precursor cells, we cannot formally exclude the possibility that there are rare nevus-associated melanocytes that are not growth arrested and that serve as melanoma-initiating cells. However, mitotically active melanocytes are seen very rarely in benign nevi in adults. Furthermore, we have maintained primary nevus-derived melanocytes in culture for up to 2 months and have not observed emergence of proliferative clones. As melanocytic nevi are present on nearly all adults, future melanoma studies will likely benefit from this new technical capacity to directly examine primary human nevus melanocytes, in order to gain insight into mechanisms promoting their malignant progression. The in vivo melanoma tissue grafts developed here recapitulate progression from benign nevus to melanoma in an architecturally faithful tissue environment that provides a human alternative to murine or melanoma cell-line surrogates for mechanistic studies and the testing of potential therapeutics.

METHODS

Additional experimental details are included in the Supplementary Methods.

Isolation of Melanocytes from Human Nevi

Benign human melanocytic nevi were excised with informed consent from patient donors seen in the Dermatology clinic at the Hospital of the University of Pennsylvania (Philadelphia, PA) according to an Institutional Review Board–approved protocol. Patient studies were conducted in accordance with the Declaration of Helsinki. A portion of each specimen was processed for routine histologic examination to confirm the clinical diagnosis of benign nevus. The tissue samples were microdissected to isolate nevus from surrounding normal tissue. Nevus tissue was then mechanically separated into fine pieces, and enzymatically dissociated in a mixture of dispase and collagenase for 2 hours. Melanocytes were further isolated from contaminating fibroblasts by selective trypsinization and 4 day exposure to 100 μg/mL G418.

Quantification of Immunohistochemistry Staining

Tissue sections from human nevi, melanomas, and melanomas arising in continuity with existing nevi were stained for p15 or p16 expression using methods described in the immunohistochemistry section. Photomicrograph images (>10) of representative tissue sections were taken using the Zeiss microscope. TIF files of the images were saved and transferred to Adobe Photoshop, where pixels corresponding to p15 or p16 IHC staining were selected using the color selection tool. Images corresponding to the single specific color were then analyzed using Fiji (ImageJ) to determine the number of pixels in each sample. The total number of pixels comprising the IHC stain and the DAPI counterstain was also obtained so that the levels of p15 and p16 expression could be normalized to the total amount of label in each section. Final ratios of p15 and p16 expression were calculated by dividing the number of p15 or p16 pixels by the total pixels in each section.

Generation of Lentiviral Vectors

The following lentiviral plasmids were used to express the corresponding human genes: diBRAF, p15, p16, CDK4R24C, dominant-negative p53R172H, hTERT, and luciferase. The human BRAFV600E gene was inserted immediately after the Tetr operator in a modified version of the doxycycline-inducible lentiviral pSTRIP2 vector (Thermo Scientific), in which the shRNA hairpin sequences were deleted. Human p15 (Addgene 16454), CDK4R24C, p53R172H, and hTERT (7) were cloned into the pRRL lentivector (43). Human p16 was expressed using Addgene plasmid #22263 (Eric Campeau).
shRNAs were expressed from pLKO.1 and are available through OpenBiosystems.

qRT-PCR

mRNA was extracted from melanocytes according to the RNeasy Mini Kit protocol (Qiagen), and reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative PCR of the resulting cDNA was carried out using Power SYBR Green Master Mix (Applied Biosystems) and gene-specific primers, in triplicate, on a Viia 7 Real-Time PCR System (Life Technologies). Relative expression was determined using the 2-ΔΔCT method followed by normalization to the wild-type melanocyte transcript levels.

Preparation of 3-D Organotypic Skin Culture

Organotypic skin grafts containing melanocytes were established using modifications to previously detailed methods (7, 11). The Keratinocyte Growth Media (KGM) used for keratinocyte-only skin grafts was replaced with Melanocyte Xenograft Seeding Media (MXSM). MXSM is a 1:1 mixture of KGM and Keratinocyte Media 50/50 (Gibco) containing 2% FBS, 1.2 mM/L calcium chloride, 100 mM/L Et-3 (endothelin 3), 10 ng/mL rhSCF (recombinant human stem cell factor), and 4.5 ng/mL r-basic FGF (recombinant basic fibroblast growth factor). Melanocytes (1.5 × 10^5) and keratinocytes (5.0 × 10^4) were suspended in 80 μL MXSM, seeded onto the dermis, and incubated at 37°C for 4 days at the air-liquid interface.

Mouse Xenografting

Organotypic skin tissues were grafted onto 5- to 7-week-old female ICR SCID mice (Taconic) according to an International Animal Care and Use Committee–approved protocol at the University of Pennsylvania. Mice were anesthetized in an isoflurane chamber. Murine skin was removed from the upper dorsal region of the mouse. Reconstituted skin grafts was replaced with Melanocyte Xenograft Seeding Media (MXSM). MXSM is a 1:1 mixture of KGM and Keratinocyte Media 50/50 (Gibco) containing 2% FBS, 1.2 mM/L calcium chloride, 100 mM/L Et-3 (endothelin 3), 10 ng/mL rhSCF (recombinant human stem cell factor), and 4.5 ng/mL r-basic FGF (recombinant basic fibroblast growth factor). Melanocytes (1.5 × 10^5) and keratinocytes (5.0 × 10^4) were suspended in 80 μL MXSM, seeded onto the dermis, and incubated at 37°C for 4 days at the air–liquid interface.

Chromatin Immunoprecipitation Followed by Quantitative PCR (ChIP-qPCR)

Cells in 10 cm² dishes were fixed in 1% formaldehyde for 5 minutes and fixation was quenched with the addition of glycerin to 125 mM/L for an additional 5 minutes. Cells were harvested by scraping from plates, and washed twice in 1× PBS before storage at -80°C. ChIP was performed as previously described (44) except that extracts were sonicated six times for 7.5 minutes each round (30 seconds sonication with intermediate incubation of 30 seconds per round) using a Bioruptor (Diagenode). All ChIPs were performed using 150 μg of extract and 2 μg of antibody per sample. Protein G Dynabeads (30 μL), Invitrogen 100.02D were used per ChIP. Antibodies included anti-histone H3 (Abcam ab1791), anti-histone H3 (trimethyl K9; Abcam ab8898), anti-histone H3 (trimethyl K4; Abcam ab8580), and anti-histone H3 (trimethyl K27; Abcam ab6002). Following elution, ChIP DNA was analyzed by standard qPCR methods on a 7900HT Fast-Real-Time PCR (ABI). Primer sequences are available upon request.

Viralt Transfection and Transduction

Viralt transfections were performed as described previously (7, 11, 26). HEK293FT cells were cultured to 40% confluency and incubated in DMEM supplemented with 5% FBS and 1% antibiotic and antimycotic in 6-well plates. For each well, 1.22 μg lentiviral vector was mixed with viral packaging plasmids pCMVΔR8.91 (0.915 μg) and pUC-MDG (0.305 μg). This plasmid solution and 7.2 μL of Fugene 6 Transfection Reagent (Promega) were added to 96 μL of

CDKN2B and pUC-MDG (0.305 μg). This plasmid solution and 7.2 μL of

supplement-free DMEM. The resulting mixture was incubated at room temperature for 15 minutes and then added into the HEK293T culture media. At 16 hours after transfection, 10 mM/L sodium butyrate was added onto the cells. At 24 hours, the culture media was replaced and the cells were incubated at 32°C. At 45 hours, viral supernatant was collected by filtering the media through a 0.45-μm syringe filter (Argos). Melanocytes seeded at 2.5 × 10^5 cells per well were incubated in viral supernatant in the presence of 5 μg/mL polybrene and centrifuged at 300 × g for 60 minutes at room temperature. After incubating the transduced melanocytes for 15 minutes at 37°C, viral supernatant was removed and replaced with growth media.

CRISPR-Cas9 Cloning and Transduction

Guide RNAs were designed using software tools developed by the Zhang laboratory (45). Guide RNAs were subsequently cloned into lentiCRISPRv2 (Addgene #52961). Guide RNA sequences are as follows: lentiCRISPR GFP 5’-GAA GTT CGA GGG CGA CAC CC-3’, lentiCRISPR p15.2 5’-CCC GAA ACG GAT GCC TTG-3’. Lentiviral transductions in human melanocytes were conducted as previously described for the other lentiviral constructs used in this study.

Statistical Analysis

Mean values were compared using an unpaired Student two-tailed t test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


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