Abrogation of BRAF\textsuperscript{V600E}-induced senescence by PI3K pathway activation contributes to melanomagenesis

Liesbeth C.W. Vredeveld,\textsuperscript{1,5} Patricia A. Possik,\textsuperscript{1,5} Marjon A. Smit,\textsuperscript{1,6} Katrin Meissl,\textsuperscript{1,6} Chrysiis Michaloglou,\textsuperscript{1,6} Hugo M. Horlings,\textsuperscript{2} Abderrahim Ajouaou,\textsuperscript{2} Pim C. Kortman,\textsuperscript{3} David Dankort,\textsuperscript{4,7} Martin McMahon,\textsuperscript{4} Wolter J. Mooi,\textsuperscript{3} and Daniel S. Peeper\textsuperscript{1,8}

\textsuperscript{1}Division of Molecular Oncology, \textsuperscript{2}Department of Pathology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands; \textsuperscript{3}Department of Pathology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands; \textsuperscript{4}Helen Diller Family Comprehensive Cancer Center, Department of Cell and Molecular Pharmacology, University of California at San Francisco, San Francisco, California 94158, USA

Human melanocytic nevi (moles) are benign lesions harboring activated oncogenes, including \textit{BRAF}. Although this oncogene initially acts mitogenically, eventually, oncogene-induced senescence (OIS) ensues. Nevi can infrequently progress to melanomas, but the mechanistic relationship with OIS is unclear. We show here that PTEN depletion abrogates BRAF\textsuperscript{V600E}-induced senescence in human fibroblasts and melanocytes. Correspondingly, in established murine BRAF\textsuperscript{V600E}-driven nevi, acute shRNA-mediated depletion of PTEN prompted tumor progression. Furthermore, genetic analysis of laser-guided microdissected human contiguous nevus–melanoma specimens recurrently revealed identical mutations in \textit{BRAF} or \textit{NRAS} in adjacent benign and malignant melanocytes. The PI3K pathway was often activated through either decreased PTEN or increased AKT3 expression in melanomas relative to their adjacent nevi. Pharmacologic PI3K inhibition in melanoma cells suppressed proliferation and induced the senescence-associated tumor suppressor p15\textit{INK4B}. This treatment also eliminated subpopulations resistant to targeted BRAF\textsuperscript{V600E} inhibition. Our findings suggest that a significant proportion of melanomas arise from nevi. Furthermore, these results demonstrate that PI3K pathway activation serves as a rate-limiting event in this setting, acting at least in part by abrogating OIS. The reactivation of senescence features and elimination of cells refractory to BRAF\textsuperscript{V600E} inhibition by PI3K inhibition warrants further investigation into the therapeutic potential of simultaneously targeting these pathways in melanoma.

\textbf{Keywords:} senescence; nevus; melanoma; BRAF\textsuperscript{V600E}; PTEN; AKT; PI3K pathway

Supplemental material is available for this article.

Received January 12, 2012; revised version accepted April 5, 2012.
of OIS of nevus cells may act as a rate-limiting event for melanomagenesis [Bennett 2003; Mooi and Peeper 2006]. Favoring this hypothesis, nevi and melanomas are commonly and significantly histologically associated [Stolz et al. 1989; Smolle et al. 1999, Bevona et al. 2003 and references therein]. Furthermore, melanomas and nevi are found nonrandomly in close proximity [Smolle et al. 1999].

However, the molecular mechanism underlying malignant transformation from nevus to melanoma is not yet resolved. The primary molecular engine driving melanomagenesis is the activation of the ERK pathway, mainly due to oncogenic mutation of BRAF [Davies et al. 2002; Pollock et al. 2003] or NRAS [Raybaud et al. 1988; van’t Veer et al. 1989]. Other frequent genetic events include the loss of CDKN2A and ARF, amplification of CCND1 or CDK4 [Kamb et al. 1994; Nobori et al. 1994, Freeman et al. 2003; Curtin et al. 2005], and, as identified more recently, alterations in MMP8, GRM3, ERBB4, GRIN2A [Palavalli et al. 2009, Prickett et al. 2009, 2011; Wei et al. 2011], and MITF [Garraway et al. 2005; Yokoyama et al. 2011]. Loss of p16INK4A has long been suspected to play a critical role in the abrogation of OIS. However, although its involvement in melanomagenesis is undisputed [Curtin et al. 2005; COSMIC database, http://www.sanger.ac.uk/genetics/CGP/cosmic], and a role in replicative senescence [Gray-Schopfer et al. 2006], the involvement in melanomagenesis is undisputed [Stahl et al. 2004; Davies et al. 2008]. Further- more, AKT3 was shown to cooperate with BRAFV600E in the transformation of murine melanocytes in vitro through mechanisms that involve phosphorylation of BRAFV600E by AKT3 [Cheung et al. 2008].

Here, we put to the test the hypothesis that abrogation of BRAFV600E-induced senescence contributes to melanomagenesis and set out to identify the key players that are mechanistically involved.

Results

PTEN depletion abrogates BRAFV600E-induced senescence in cultured human fibroblasts

To investigate the possible role of PI3K pathway activation in evasion of BRAFV600E-induced senescence, we first focused on any effect of PTEN loss. As expected, PTEN depletion by two independent shRNAs from primary human fibroblasts resulted in activation of a critical downstream effector, AKT [as measured by P-AKT] (Fig. 1A). Consistent with our previous results [Michaloglou et al. 2005], ectopic expression of BRAFV600E in HDF induced a robust cell cycle arrest, which was accompanied by an increase in p16INK4A [Fig. 1A]. Importantly, depletion of PTEN effectively abrogated BRAFV600E-induced senescence [Fig. 1B,C]. We corroborated the participation of PI3K pathway activation in abrogation of BRAFV600E-induced senescence by ectopic expression of either wild-type or activated PIK3CA or activated AKT1 in independent HDF strains. This also effectively prevented BRAFV600E-induced senescence in fibroblasts [Supplemental Fig. 1A–F], indicating a causal role for this signaling route in the growth inhibitory action of BRAFV600E, consistent with previous results [Courtois-Cox et al. 2006]. The increase in cell number seen in proliferation assays was not due to decreased cell death (which is not induced in HDF by BRAFV600E) [data not shown], but reflected decreased senescence and increased DNA replication as a function of PI3K activation, as evident from SA-β-Gal activity and bromodeoxyuridine (BrdU) incorporation assays (Fig. 1C; Supplemental Fig. 1B,C).

PTEN depletion abrogates BRAFV600E-induced senescence in cultured human melanocytes

Given the prominent role of BRAFV600E in nevi and melanomas and the frequent reduction of PTEN levels seen in melanomas [Tsao et al. 2000, 2004; Danio et al. 2004, Lin et al. 2008], we set out to determine the role of PTEN in the senescence program of primary human melanocytes in the context of oncogenic BRAF. These melanocytes were isolated from neonatal foreskins and characterized for melanocytic markers and the ability to undergo OIS [Michaloglou et al. 2005]. Of note, similar to primary mammary epithelial cells [Brenner et al. 1998], primary melanocytes appear to be under selective pressure to lose p16INK4A during in vitro propagation [Bennett 2003]. To avoid spontaneous mutations in p16INK4A or undefined genes, these experiments were performed with p16INK4A-depleted melanocytes [which still undergo BRAFV600E-induced senescence], but similar results were obtained with wild-type melanocytes [data not shown].

Consistent with previous results [Michaloglou et al. 2005; Denoyelle et al. 2006], BRAFV600E efficiently triggered senescence in melanocytes [Fig. 2A–C]. shRNA-mediated PTEN depletion, however, prevented BRAFV600E cells from undergoing OIS. This was illustrated by increased cell proliferation, a significant rise in DNA replication, and decreased SA-β-Gal activity relative to BRAFV600E-expressing melanocytes [Fig. 2A–C].
As expected, BRAFV600E expression resulted in strong ERK activation, while PTEN depletion induced phosphorylation of AKT, which was further enhanced by BRAFV600E (Fig. 2D). Up-regulation of P-AKT was a common feature among BRAF mutant melanoma cell lines but did not always correlate to PTEN levels, consistent with the idea that PTEN-independent signaling also feeds into AKT (Supplemental Fig. 2A). Less expected was the observation that BRAFV600E strongly suppressed the accumulation of AKT3 in human melanocytes. This was at least partially restored in PTEN-depleted melanocytes evading OIS. Of note, we were unsuccessful in over-expressing PI3K or AKT family members (either wild-type or activated forms) in melanocytes, most likely because of toxicity. This is consistent with (but does not formally explain) the observation that PTEN-independent signaling also feeds into AKT (Supplemental Fig. 2A). Less expected was the observation that BRAFV600E strongly suppressed the accumulation of AKT3 in human melanocytes. This was at least partially restored in PTEN-depleted melanocytes evading OIS. Of note, we were unsuccessful in over-expressing PI3K or AKT family members (either wild-type or activated forms) in melanocytes, most likely because of toxicity. This is consistent with (but does not formally explain) the observation that PTEN-independent signaling also feeds into AKT (Supplemental Fig. 2A).

Although consistent with the hypothesis that abrogation of OIS contributes to melanomagenesis, the results above do not necessarily reflect the physiologic situation. Indeed, it is conceivable that PTEN deficiency in vivo occurs after the acquisition of the BRAFV600E mutation rather than prior to it. In an attempt to better mimic such a scenario, we first introduced BRAFV600E to trigger melanocyte senescence. Subsequently, melanocytes were transduced with lentivirus targeting PTEN. Upon its depletion, we again observed a consistent bypass of OIS (Fig. 2E; Supplemental Fig. 2B). Although this effect was moderate in vitro, such a senescence override is likely to be relevant in vivo. We conclude, therefore, that PTEN depletion leads to abrogation of BRAFV600E-induced senescence in human melanocytes.

Abrogation of BRAFV600E-induced senescence by PTEN depletion is dose-dependent

While some studies show that PTEN deficiency induces senescence in fibroblasts (Chen et al. 2005), it appears that its expression levels play an essential role: Slight differences in PTEN expression affect cell survival and proliferation (Alimonti et al. 2010). To address the importance of PTEN expression in melanocytes, we titrated lentiviruses expressing sh-PTEN in the presence or absence of BRAFV600E.

The bypass of BRAFV600E-induced senescence upon PTEN depletion correlated with PTEN expression levels, as judged by the ability to incorporate BrdU and SA-β-Gal activity (Fig. 3A–C). Importantly, determination of cell numbers revealed that there was an optimum: Too much
PTEN depletion no longer resulted in a net increase in cell number, because of cytotoxic effects (Fig. 3D; data not shown). Too little PTEN depletion, on the other hand, failed to impair the senescence program. Thus, there was a window of PTEN expression that allowed BRAFV600E-expressing melanocytes to evade senescence. Furthermore, whereas BRAFV600E overexpression in melanocytes triggered up-regulation of the senescence-associated tumor suppressor p15INK4B, cells bypassing OIS down-regulated expression of p15INK4B. This correlated with both the dosage of PTEN and BrdU incorporation (Fig. 3A,B). We conclude, therefore, that there is an optimal window of PTEN expression allowing BRAFV600E-expressing melanocytes to abrogate OIS bypass: Too high levels do not allow for proliferation, whereas too low levels are cytotoxic.

Acute in vivo PTEN depletion in BRAFV600E-expressing nevi drives tumor formation

To validate the role of PTEN in the context of BRAFV600E in an in vivo setting, we made use of a knock-in BRAFV600E mouse model that allows for melanocyte-specific and inducible expression of BRAFV600E (Tyr::CreER, BRafCA) (Dankort et al. 2009). Upon 4-OHT (4-hydroxytamoxifen) treatment, these mice develop nevus-like lesions that are characterized by hyperplasia of melanocytes in the dermis and hair follicles with the presence of melanophages (Fig. 4A; Dankort et al. 2009). This represents a physiologically relevant setting to investigate whether, also in mice, PTEN depletion triggers proliferation of BRAFV600E-expressing melanocytes. To this end, we injected a lentivirus carrying sh-PTEN into these lesions. This commonly led to tumor formation at the sites of injection (Fig. 4B; Supplemental Fig. 3A). Focal pigmentation areas (dendritic melanocytes and melanophages) were observed and confirmed by H&E staining (Fig. 4A,C) and electron microscopy [data not shown]. Histologically, these tumors consisted predominantly of oval to elongated cells with oval monochromatic nuclei and ill-defined pale cytoplasm. A minority of cells contained microgranular melanin pigment, which highlighted dendritic processes in some.
Scattered groups of larger, polygonal, pigmented cells were seen as well. Necrosis was absent. The tumor cells infiltrated diffusely into the cutaneous and subcutaneous tissues, including striated muscle.

Of note, the lesions developed within the first month after injection of sh-PTEN virus, but subsequently slowed down without progressing further. Indeed, mitotic figures were noted at that stage only occasionally. Mice that had received control vector showed no lesions at all or very small lesions. Confirming BRAF\textsuperscript{V600E} expression in these sh-PTEN-induced lesions, rearrangement of the BRaf\textsuperscript{CA} allele was detected by PCR [Supplemental Fig. 3B]. Lesions of mice treated with sh-PTEN showed enrichment of the BRAF\textsuperscript{V600E}-encoding allele compared with control-treated mice, suggesting that cells expressing BRAF\textsuperscript{V600E} constitute the lesions. These results indicate that PTEN depletion can cause BRAF\textsuperscript{V600E}-expressing nevus cells to resume proliferation, providing a time window for tumorigenic expansion in vivo.

Significant conservation of BRAF and NRAS mutations during progression from nevi to melanomas

We next wished to extend our findings obtained in vitro and in mice to humans. Specifically, we examined the possibility that also in human melanocytic lesions, reduction in PTEN levels, resulting in activation of the PI3K pathway, represents a rate-limiting step for the outgrowth of BRAF\textsuperscript{V600E}-expressing melanocytes. Therefore, we investigated a series of 21 human tissue sections with nevi in direct contiguity with melanomas. Because a nevus-to-melanoma progression model predicts conservation of the driver mutation, we first examined whether the cells of the nevus and the contiguous melanoma in each specimen were clonally related. Genetic evidence has been presented previously in support of a progression model [NRAS, KRAS, and BRAF mutations [Demunter et al. 2001; Yazdi et al. 2003; Dadzie et al. 2009] and LOH of 9p21 markers [Bogdan et al. 2003]], but, importantly, no
statistical analysis was used to rule out random co-occurrence of mutations within these lesions.

We first attempted to identify the "driver" mutations in the nevus and melanoma cell compartments. We focused on the likely suspects \(\text{BRAF}\) and \(\text{NRAS}\), as they are most commonly activated in melanomas as well as nevi. Seventeen specimens were considered suitable for laser capture microdissection, after which the mutational status of \(\text{BRAF}\) exon 15 and \(\text{NRAS}\) exon 3 (in which most activating mutations reside) (Forbes et al. 2006; COSMIC database, http://www.sanger.ac.uk/genetics/CGP/cosmic) was analyzed by genomic DNA PCR amplification and sequencing. In addition, areas with normal tissue in these sections were microdissected in all cases and processed along with the nevi and melanomas as controls for positive identification of the wild-type \(\text{NRAS}\) and \(\text{BRAF}\) alleles and for excluding contamination problems or the occurrence of polymorphisms.

We found the common \(\text{BRAF}^{V600E}\) mutation (encoding \(\text{BRAF}^{V600E}\)) in eight samples, seven of which showed co-occurrence of this mutation in both the nevi and their flanking melanomas (Fig. 5A, red bars, Supplemental Table 1). Interestingly, in one sample, we identified a rare \(\text{BRAF}^{G1799–1800AA}\) double mutation (also encoding \(\text{BRAF}^{V600E}\)) in the melanoma as well as the associated nevus (Fig. 5B–D, Supplemental Fig. 4A–D for detailed information). In addition, activating \(\text{NRAS}\) mutations were detected in four samples, two of which showed co-occurrence in nevi and their contiguous melanomas. The presence of a \(\text{BRAF}\) or \(\text{NRAS}\) mutation in three melanomas but not in their contiguous nevi suggests that either the lesions within these specimens are clonally unrelated or the lesions harbor driver mutations other than those located in \(\text{BRAF}\) exon 15 and \(\text{NRAS}\) exon 3, with \(\text{BRAF}\) or \(\text{NRAS}\) occurring later, during melanoma progression. Finally, we identified four specimens with wild-type \(\text{BRAF}\) exon 15 and \(\text{NRAS}\) exon 3 sequences in all lesions. We cannot exclude, however, that some of these results are false negatives due to the very small lesion size in some cases.

To evaluate the statistical significance of these findings, we collected from the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic) the percentages of each mutation identified in the above-described specimens (Fig. 5E) and calculated for all cases the probability that identical mutations in nevi and melanomas could have occurred by chance (Supplemental Table 1). For each mutation identified in the 17 specimens, we calculated the expected frequencies of random co-occurrence in nevi and matched melanomas [i.e., Fig. 5A, gray bars labeled "Expected"] and compared these with the observed co-occurrence frequency obtained from actual
mutational analysis (Fig. 5A, red bars labeled “Observed”). The predicted chance of random co-occurrence based on the percentages from the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic) was then compared with the observed results of the mutational analysis. We found co-occurrence of BRAF and NRAS mutations in flanking nevi and melanomas that was statistically significant in 10 out of 17 specimens (P-value < 0.05), arguing that at least a considerable proportion of melanomas in this series are clonally related to their contiguous nevi.

**Decrease in PTEN and increase in AKT3 and P-AKT expression in melanomas relative to their contiguous nevi**

To investigate the role of PTEN reduction and PI3K pathway activation in human melanomagenesis in vivo, we analyzed the relative expression levels of PTEN by immunohistochemical staining of our series of contiguous nevus–melanoma specimens. As we had noted that BRAFV600E suppresses AKT3 accumulation in cultured melanocytes (Fig. 2D), we also included immunostaining for this factor as well as for P-AKT. Five out of 21 samples displayed reduced PTEN immunostaining in the melanoma compared with the associated nevus, including the sample with the rare BRAF double mutation (Fig. 6A–C; Table 1; Supplemental Fig. 5A–F). Conversely, an increase in AKT3 staining in the melanoma compared with the nevus was detected in nine specimens (Fig. 6D–F; Table 1; Supplemental Fig. 5G–I). In two specimens, both PTEN reduction and AKT3 increase were seen. Correspondingly, in the majority of the specimens analyzed, we detected induction of P-AKT in the melanoma relative to the nevus (Fig. 6G–I; Table 1). These analyses reveal recurrent activation of the PI3K pathway in melanomas.
relative to their contiguous nevi, with alterations in PTEN and AKT accounting for 12 out of 21 cases.

**Pharmacologic PI3K inhibition induces cell cycle arrest in melanoma cells**

In view of the strong evidence linking PTEN reduction and PI3K pathway activation to melanoma, PI3K is receiving increasing interest as a possible therapeutic target. As our results indicate that activation of the PI3K pathway overrides BRAFV600E-induced senescence and represents a frequent event during melanomagenesis, we next investigated whether PI3K pathway inhibition could reactivate aspects of the senescence program in melanoma cells. We treated BRAFV600E melanoma cell lines with increasing concentrations of Pi-103, an inhibitor of PI3K and mTOR kinases. Cells treated with Pi-103 showed a profound decline in proliferative capacity, as measured by cell proliferation and BrdU incorporation assays (Fig. 7A,B), consistent with previous data (Marone et al. 2009). Correspondingly, the expression levels of p15INK4B increased upon Pi-103 treatment (Fig. 7C). Occasionally, but not consistently, we observed induction of SA-β-Gal activity (data not shown), suggesting that PI3K inhibition reactivates senescence incompletely, perhaps owing to the large number of genetic alterations typically seen in melanoma.

**Pharmacologic PI3K inhibition enhances cytotoxicity of BRAFV600E inactivation**

An exciting recent clinical development is the availability of mutant BRAF-specific inhibitors. Although, initially, dramatic responses are seen in melanoma patients, eventually, most of them show tumor progression. Intense investigations have revealed that tumor relapse occurs owing to a variety of resistance mechanisms, some of them implicating activation of the PI3K pathway as a key player in resistance to BRAF or MEK inhibition (Shao and Aplin 2010; Atefi et al. 2011; Paraiso et al. 2011). Therefore, there is a dire need to find combination therapy to delay or avoid resistance. In view of the results obtained for pharmacologic PI3K inhibition, we explored the possible therapeutic benefit of combining PI3K and BRAFV600E inhibition in melanoma cells.

As expected, treatment with Pi-103 and PLX4720 [a BRAFV600E inhibitor] inhibited the activity of AKT and ERK, respectively (Fig. 7D). More importantly, Pi-103 treatment profoundly cooperated with BRAFV600E inhibition in dose response curves (Fig. 7E; Supplemental Fig. 6A). Interestingly, whereas melanoma cells mainly arrested upon Pi-103 treatment, combination with PLX4720 induced cell death, as demonstrated by the cooperative induction of cleaved caspase 3 (Fig. 7F; Supplemental Fig. 6B). This was accompanied by the neutralization of p15INK4B induction by Pi-103, suggesting cross-talk between these two pathways (Fig. 7C).

We noted that the response to PLX4720 treatment varied among melanoma cells, and in some cases, a population of cells survived even high doses of this drug (Fig. 7G). We therefore treated also these resistant cell lines with increasing concentrations of PLX4720 and analyzed the possible additive effect of Pi-103. Interestingly, combined inhibition of the PI3K and BRAFV600E kinases efficiently eliminated the population of resistant melanoma cells (Fig. 7G, Supplemental Fig. 6C).
conclude from these results that while BRAF and PI3K pathway activation collaborate in melanomagenesis, pharmacologic PI3K inhibition reactivates senescence features and enhances the cytotoxicity of targeted mutant BRAF inhibition.

Discussion

To understand the molecular basis of melanomagenesis, it is imperative to identify the genetic changes that facilitate the different steps in progression from normal melanocytes to nevus and melanoma. A large proportion of melanomas harbor alterations in both the BRAF and PTEN genes, leading to their constitutive activation and inactivation, respectively. As we and others have previously postulated that abrogation of OIS in human nevi contributes to melanomagenesis, we here put to the test the hypothesis that reduction of PTEN plays a rate-limiting role in the progression from nevus to melanoma.

We employed four independent settings for this investigation: cultured human fibroblasts, cultured human melanocytes, a murine nevus model, and human contiguous nevus–melanoma specimens. First, we determined whether down-regulation of PTEN affects the senescence program that is elicited by BRAF<sup>V600E</sup> in vitro. In both primary human fibroblasts and melanocytes, PTEN silencing abolished BRAF<sup>V600E</sup>-induced senescence. These results are in agreement with and extend models previously proposed by us and others. For instance, senescent neurofibromas initiate a negative feedback loop in response to oncogenic stress, which inhibits AKT activation and results in the induction of senescence (Courtois-Cox et al. 2006). Furthermore, activation of the PI3K pathway by expression of myristoylated AKT1 attenuates RAS-induced senescence (Kennedy et al. 2011). These results suggest that this mechanism is conserved across cell types and shared among different OIS triggers. Hence, the abolishment of OIS by activation of the PI3K pathway may play a role also in tumor types other than melanoma.

In human tissue sections, it has been shown that normal melanocytes and most nevi exhibit PTEN expression, which is down-regulated in approximately a third of primary melanomas and becoming even more pronounced in melanoma metastases (Zhou et al. 2000; Whiteman et al. 2002; Tsao et al. 2003). This has led to the idea that PTEN reduction may be involved in melanoma progression rather than initiation (Whiteman et al. 2002). We analyzed PTEN expression in human contiguous nevus–melanoma specimens to investigate its role in melanoma initiation. By determining the mutational status of BRAF and NRAS in the benign and malignant melanocytic lesions within these specimens, we first investigated whether the nevi that are in direct contiguity with melanomas were clonally related. Indeed, we found co-occurrence of several different mutations in nevi and their associated melanomas. This includes the

<table>
<thead>
<tr>
<th>Number</th>
<th>PTEN reduction in melanoma relative to nevus</th>
<th>AKT3 induction in melanoma relative to nevus</th>
<th>P-AKT induction in melanoma relative to nevus</th>
<th>Mutation in nevus and melanoma conserved</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>V</td>
<td>V</td>
<td>?</td>
<td>?</td>
<td>Both N and M BRAF exon 15 and NRAS exon 3 wild type</td>
</tr>
<tr>
<td>4</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>?</td>
<td>Both N and M BRAF exon 15 and NRAS exon 3 wild type</td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>ND</td>
<td>V</td>
<td>V</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>V</td>
<td>V</td>
<td>ND</td>
<td>ND</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>?</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>?</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>?</td>
<td>Both N and M BRAF&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M NRAS&lt;sup&gt;A182G&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>V</td>
<td>ND</td>
<td>V</td>
<td>ND</td>
<td>Both N and M NRAS&lt;sup&gt;A182G&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>V</td>
<td>ND</td>
<td>V</td>
<td>ND</td>
<td>Both N and M NRAS&lt;sup&gt;A182G&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M NRAS&lt;sup&gt;A182G&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M NRAS&lt;sup&gt;A182G&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M NRAS&lt;sup&gt;A182G&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>V</td>
<td>V</td>
<td>ND</td>
<td>ND</td>
<td>Both N and M NRAS&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M NRAS&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>Both N and M NRAS&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>19</td>
<td>V</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Both N and M NRAS&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Both N and M NRAS&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
<tr>
<td>21</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Both N and M NRAS&lt;sup&gt;T1799A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(V) Present; [ND] not determined because specimen was not suitable for laser capture microdissection. BRAF exon 15 and NRAS exon 3 are wild-type sequences; the remainder of the gene was not sequenced. (?) The driver mutation is unknown; [N] nevus; [M] melanoma.
PI3K inhibition induces cell cycle arrest in melanoma cells and enhances cytotoxicity of BRAFV600E inhibition. Melanoma cell lines were treated with Pi-103 and/or PLX4720. (A) Samples were kept in medium containing Pi-103 for 7 d and fixed and stained with crystal violet. (B) Samples were analyzed for BrdU incorporation 4 d after treatment with increasing concentrations of Pi-103 (0.25, 0.5, 1, and 2 μM). Error bars represent SEM (standard error of the mean) of three independent experiments. For 453A0 melanoma cells, a representative experiment is shown. (C) Samples were analyzed by Western blotting for p15INK4B 2 d after treatment with 0.25 μM Pi103 and/or 1 μM PLX4720. β-Actin served as a loading control. (D) Melanoma cell lines were treated with 0.5 μM Pi-103 and/or 2 μM PLX4720 for 4 h. Samples were analyzed by Western blotting for the indicated proteins. β-Actin served as a loading control. (E) Melanoma cell lines were treated with a dilution series of Pi-103 either alone or in combination with the BRAFV600E inhibitor PLX4720 at a concentration of 3 μM [D10] or 1 μM [453A0] for 3 d. Total cell numbers were determined with a cell titer blue assay. The Y-axis represents the percentage of living cells. (F) Cells were treated with 0.25 μM Pi-103 and/or 1 μM PLX4720 for 1 d [D10] or 3 d [453A0]. Samples were analyzed by Western blotting for cleaved caspase 3. Apoptotic cells in the supernatant were included in the analysis. β-Actin served as a loading control. (G) Cells were treated with a dilution series of PLX4720 either alone or in combination with 0.5 μM Pi-103. Total cell numbers were determined with a cell titer blue assay. The Y-axis represents the percentage of living cells.
BRAFV600E mutation that is commonly found in both nevi and melanomas but was found co-occurring in our data set in a significantly higher number of cases than expected by chance. Furthermore, we found rare mutations (NRAS<sup>C181A</sup> and BRAF<sup>T1799A–1800AA</sup>) occurring in adjacent nevi and melanomas that are highly unlikely to have occurred independently. Although this is not formal proof, these results strongly suggest that at least a proportion of melanomas emerge from nevi.

More than half of the lesions we analyzed displayed activation of the PI3K pathway, as judged by loss of PTEN or gain of AKT3 immunopositivity in the melanoma compared with the nevus compartment. Of note, PTEN levels were reduced but appeared to be not completely shut down. This is consistent with our in vitro PTEN RNAi results and suggests a window for PTEN reduction that allows for melanoma development, in agreement with observations on PTEN dosage in cellular senescence and tumor development by others [Chen et al. 2005; Alimonti et al. 2010]. As expected, in most of these cases, this was accompanied by increased P-AKT staining in the melanoma compartment. The observation that identical BRAF and NRAS mutations are present in both the nevi and flanking melanomas [at least in 10 out of 17 cases], with concomitant PTEN reduction or AKT activation in the melanoma, is compatible with a model in which BRAF/NRAS mutations precede the PTEN/AKT alterations. This is further supported by our in vitro observation that senescent human melanocytes expressing BRAF<sup>V600E</sup> are able to re-enter the cell cycle upon PTEN depletion. Together, our results suggest that not only in vitro, but also in lesions in vivo, activation of the PI3K pathway contributes to the abrogation of the OIS program of human melanocytes, thereby promoting the progression of nevi to melanomas. Clearly, although diminished PTEN expression or increased AKT3 production in melanomas was seen in roughly half of the cases, other pathways are likely to contribute to melanoma progression as well. We propose that alterations at several levels in the PI3K pathway represent a common feature of progression from nevus to melanoma.

We also observed that AKT3 expression levels were down-regulated upon BRAF<sup>V600E</sup> expression in human melanocytes. This is consistent with the previous suggestion that AKT3 is the critical AKT isoform in melanoma cell lines [Stahl et al. 2004] and that overexpression of activated AKT3 enhances anchorage-independent growth of melanoma cells [Cheung et al. 2008]. Conversely, specific inhibition of AKT3 has been reported to induce apoptosis and inhibit the tumorigenic capacity of melanoma cells in a xenograft model [Stahl et al. 2004]. Although they do not rule out contributions by the other AKT family members, our in vitro and in vivo data are consistent with these observations and support a prominent role of AKT3 in melanomagenesis.

Our finding that PTEN depletion and consequent activation of the PI3K pathway abrogates BRAF<sup>V600E</sup>-induced senescence is in agreement with and extends previous data on two BRAF<sup>V600E</sup> mouse models. Dhomen et al. [2009] showed that the induction of BRAF<sup>V600E</sup> in melanocytes results in senescent melanocytes displaying elevated SA-β-Gal activity, low Ki67 expression, and formation of [blue] nevi. After a year, half of these mice developed melanoma, presumably by acquiring additional [yet to be defined] mutations. When the BRAF<sup>V600E</sup> nevus-carrying mice were bred onto a p16<sup>INK4A</sup> null background, they produced more tumors, which developed earlier, but nonetheless, these mice continued to develop nevi [Dhomen et al. 2009]. This is consistent with the mosaic pattern of p16<sup>INK4A</sup> immunopositivity frequently seen in human nevi [Michaloglou et al. 2005] and the observation that p16<sup>INK4A</sup>-deficient individuals have nevi in addition to an increased propensity to develop melanoma [Pavel et al. 2003], arguing that also p16<sup>INK4A</sup>-independent mechanisms contribute to melanomagenesis. Goel et al. [2009] observed similar features in melanocytes residing in the skin of BRAF<sup>V600E</sup> transgenic mice, including increased levels of p16<sup>INK4A</sup>, p53, and PTEN and elevated SA-β-Gal activity. Consistent with our model, transgenic BRAF<sup>V600E</sup> mice that develop melanoma strongly up-regulate P-AKT levels [Goel et al. 2009].

Also, Dankort et al. [2009] found murine benign melanocytic hyperplasia driven by an endogenous BRAF<sup>V600E</sup>-encoding allele. These lesions were highly stable and failed to progress to melanoma for at least up to 20 mo. However, upon simultaneous activation of BRAF<sup>V600E</sup> and genetic inactivation of PTEN, mice quickly developed metastatic melanoma [Dankort et al. 2009]. In our in vivo nevus injection model, we also observed cooperation between BRAF<sup>V600E</sup> expression and PTEN depletion in tumor formation, in accordance with Dankort et al. [2009]. We used the same conditional BRAF<sup>V600E</sup> mouse model, but instead of concomitant induction of mutant BRAF expression and PTEN depletion, we first allowed the mouse to develop nevus-like lesions, which were later treated with sh-PTEN-carrying lentivirus. This strategy allowed us to analyze the order of events and demonstrate that tumors can emerge from BRAF<sup>V600E</sup>-arrested lesions in vivo in a PTEN-dependent manner. We cannot rule out that PTEN depletion in stromal cells contributed to this effect. In aggregate, these results support a model in which mutant BRAF and subsequent activation of the PI3K pathway cooperate in melanomagenesis in cultured melanocytes, in murine nevus cells, and in human lesions, with PTEN reduction and PI3K/AKT activation serving, at least in part, to abrogate OIS.

In recent years, several groups have detected senescence biomarkers in several preneoplastic lesions in mice and humans [Kuilman et al. 2010]. Commonly, an initial “driver” mutation—either the activation of an oncogene or the loss of a tumor suppressor allele—triggers the establishment of a benign lesion. After this initial phase of cell proliferation, a senescence program is executed, causing the termination of tumor expansion. Only upon the emergence of additional tumorigenic alterations can malignant progression occur. OIS is now widely considered to represent a bona fide tumor suppressor mechanism, acting alongside cell death programs. We believe, therefore, that the field is now entering a second phase, in which we need to delineate the fundamental mechanistic
aspects of OIS. As we show here, the nevus–melanoma model is ideally suited to unmask at least some of the key players involved. Similarly, BRAF<sup>V600E</sup>-driven nevus models in zebrafish and mice allow for the identification of melanoma progression genes (Ceol et al. 2011; Karreth et al. 2011). The factors and pathways identified in this way will likely be relevant for other tumor settings as well, as OIS is seen in many tissue types (Collado and Serrano 2010). One question that is particularly relevant in this context is the cell of origin within the nevus—or similar benign lesions, for that matter—that gives rise to the tumor. Is it a fully senescent cell that somehow acquired a secondary mutation, a cell that has stalled halfway through the senescence program, or perhaps a rare senescent (nevus) cell with special (stem cell) properties? This and related questions can be addressed in advanced animal models engineered for lineage tracing.

The critical role for the PI3K pathway in melanoma development may be explored for the development of more effective therapeutic approaches. Our results predict that inhibitors of the PI3K pathway may restrain the expansion of tumors with mutations in BRAF at least in part through restoration of OIS features, a concept that is receiving increasing interest (Nardella et al. 2011). Furthermore, in agreement with previous data (Shao and Aplin 2010; Atefi et al. 2011; Paraiso et al. 2011), we show that inhibition of PI3K can enhance the cytotoxicity of targeted BRAF<sup>V600E</sup> inhibition. In particular, the observation that a PI3K inhibitor can eliminate melanoma cells that resist killing by a BRAF<sup>V600E</sup> inhibitor is of obvious interest and merits further investigation.

Finally, the elucidation of the pathobiology of nevi will increase our understanding of the role of OIS in melanomagenesis and may identify biomarkers that allow a better distinction from melanoma in problem cases. There is a need to identify senescence biomarkers in benign lesions such as nevi; in particular, factors that do not merely accompany OIS, but contribute to the execution of the program. Most markers currently used, such as SA-β-Gal, are associated with the process but likely do not contribute to rate-limiting steps. Identification of new biomarkers is important not only to deepen our understanding of the role of OIS (abrogation) in tumorigenesis, but also for translation of fundamental findings to the clinic, for example, to create new diagnostic opportunities. Indeed, some nevi are difficult or impossible to distinguish with certainty from melanomas and vice versa. We show here that there is a common activation of the PI3K pathway in situ comparing nevi with their contiguous melanomas. These results merit further investigation into the possibility of using biomarkers like PTEN reduction and increases in AKT3 and P-AKT to allow a better distinction between nevi and melanomas in difficult cases.

Materials and methods

Cell culture and viral transduction

The HDF cell lines TIG3 and HCA2 (coexpressing the ectopic receptor and hTERT) as well as Phoenix and HEK293T cells were maintained in DMEM (Gibco) supplemented with 9% fetal bovine serum (Greiner Bio-One), 2 mM glutamine, 100 U mL<sup>−1</sup> penicillin, and 0.1 mg mL<sup>−1</sup> streptomycin (all Gibco). The HDF cell line IMR90 (expressing the ectopic receptor, hTERT, and shRNA for p16<sup>INK4A</sup>) was maintained in MEM + Earle’s salts (Gibco) containing all supplements mentioned above and non-essential amino acids, 0.15% sodium bicarbonate, and 1 mM sodium pyruvate (all Gibco). Human melanocytes were isolated from the epidermis of neonatal foreskins (Michaloljou et al. 2005) and maintained in Medium 254 (Cascade Biologicals) supplemented with melanocyte growth supplement (HMGS, Cascade Biologicals), 100 U mL<sup>−1</sup> penicillin, and 0.1 mg mL<sup>−1</sup> streptomycin (Gibco). Melanoma cell lines (A375, D10, SKMEL28, 453AD, MEL888, and A875) were maintained in DMEM supplemented with 9% fetal bovine serum, 2 mM glutamine, 100 U mL<sup>−1</sup> penicillin, and 0.1 mg mL<sup>−1</sup> streptomycin.

Retroviral transductions were performed using Phoenix cells as producers. Retrovirus production employing Phoenix cells was performed as described [http://www.stanford.edu/group/nolan/retroviral_systems/phx.html]. For production of lentiviruses, HEK293T cells were refreshed with complete medium containing 25 mM chloroquine, transfected with 8 μg of lentiviral construct and 3 μg of each of the helper plasmids pMDLgIPRRE, pHCMV-G, and pRSVrev and washed and refreshed in complete medium after 6 h. Lentivirus was frozen and diluted for transduction. For TIG3, HCA2, and IMR90 cell proliferation assays, cells were transduced with shRNA- or cDNA-encoding retrovirus and checked for successful proviral integration (by puromycin selection or GFP expression). Cells were plated at equal densities, subsequently transduced with BRAF<sup>V600E</sup>-encoding retrovirus, and selected [Blasticidin]. DNA replication was measured by incubating cells for 3 h with BrdU 3 d post-infection. Cells were also seeded at equal numbers and fixed and stained with crystal violet, which was extracted by incubation with 10% acetic acid, and relative absorbance was measured with a TECAN infinite M200 scanner 8 d post-infection. Samples were harvested for Western blot analysis 8 d post-infection. Activity of SA-β-Gal was also measured 8 d post-infection (SA-β-Gal staining kit, Cell Signaling). Melanocytes were transduced with shRNA- or cDNA-encoding lentivirus, cultured for 1 wk with Medium 254 supplemented with HMGS-2 [–PMA, Cascade Biologicals], and subsequently transduced with BRAF<sup>V600E</sup>-encoding lentivirus and subjected to Blasticidin selection. Cells were seeded at equal numbers, analyzed for BrdU incorporation, and fixed and stained with crystal violet, analyzed for SA-β-Gal activity, and harvested for Western blot analysis 15 d post-infection. Alternatively, cells were first transduced with BRAF<sup>V600E</sup>-encoding lentivirus, selected for 1 wk with Blasticidin, and subsequently transduced with sh-PTEN-encoding lentivirus. Cells were analyzed 8 d post-infection for BrdU incorporation and Western blot.

Plasmids

pRSF<sub>PGK-sh-PTEN</sub>[#1 and #2], pMX<sub>GFP</sub>-PK3CA<sup>INV</sup>, K545, R1047, CAAX<sup>INV</sup>, and pMSCV<sub>BLAST</sub>-BRAF<sup>V600E</sup> were used for retroviral transduction. KH<sub>1</sub><sub>GFP</sub>-sh-p16<sup>INK4A</sup>, KH<sub>1</sub><sub>GFP-sh-PTEN</sub>[#1 and #3], and HIV-CSC<sub>BLAST</sub>-BRAF<sup>V600E</sup> were used for lentiviral transductions. Corresponding empty vectors were used as controls. Sequences for sh-PTEN are described in the Supplemental Material.

Antibodies

Antibodies used for Western blot were against BRAF [sc-5284, Santa Cruz Biotechnology], PTEN [sc-7974, Santa
In vivo administration of lentivirus
The dorsal skin of Tyr::CreER, BrAfc−/− mice [Dankort et al. 2009] was treated with 4-OHT (20 mg ml−1 in EtOH) each second day during the first week after birth. Adult mice received a subcutaneous total body irradiation (5 Gy) 3 d prior to lentivirus application. shRNA targeting PTEN [sh-PTEN#1 and sh-PTEN#3] was delivered by intradermal injection of lentivirus into the dorsal skin of 4-OHT-treated Tyr::CreER, BrAfc−/− mice. The animal experiments were performed following local and international regulations and ethical guidelines and have been authorized by the local experimental animal committee at The Netherlands Cancer Institute. PCR detection of BRAF allele rearrangements was performed as described [Dankort et al. 2007].

Immunohistochemistry
Twenty-one formalin-embedded human specimens harboring nevi in direct contiguity with melanomas were immunostained for PTEN (clone 6H2.1, DAKO), and P-AKT [Ser 473] (736E11, Cell Signaling), cleaved caspase 3 (Asp175, Cell Signaling), and β-actin [AC-74 and A5316, Sigma].

Drug treatments
Melanoma cell lines were treated with indicated concentrations of PI-103 [Echelon Biosciences] and/or PLX4720 [Selleck]. For dose response curves, samples were measured after 3 d of treatment with a cell titer blue assay (Promega), and fluorescence was measured with a TECAN infinite M200 scanner. For proliferation assays, cells were either analyzed for BrdU incorporation 4 d after treatment or seeded at equal densities and fixed and stained with crystal violet 7 d after treatment. Samples were harvested for Western blot analysis on the indicated days.

Acknowledgments
We thank N. Armstrong and M.L. Yurda for statistical analysis, Ji-Ying Song for scientific support, R. Kortlever, M. Voorhoeve, and K. Berns for kindly providing constructs, T. Kullman for critical reading of the manuscript, and all members of the Peper laboratory for their valuable input. This work was supported by a Melanoma Research Alliance grant to D.D. and M.M., grants from the Dutch Cancer Society [KWF Kankerbestrijding, including a Queen Wilhelmina Award program], and Vidi and Vici grants from the Netherlands Organization for Scientific Research [NWO] to L.C.W.V., P.A.P., C.M., M.A.S., and D.S.P.

References
Vredeveld et al.


Abrogation of BRAF<sup>V600E</sup>-induced senescence by PI3K pathway activation contributes to melanomagenesis

Liesbeth C.W. Vredeveld, Patricia A. Possik, Marjon A. Smit, et al.

*Genes Dev.* 2012, 26: originally published online May 1, 2012
Access the most recent version at doi:10.1101/gad.187252.112

**Supplemental Material**
http://genesdev.cshlp.org/content/suppl/2012/04/23/gad.187252.112.DC1

**References**
This article cites 64 articles, 16 of which can be accessed free at:
http://genesdev.cshlp.org/content/26/10/1055.full.html#ref-list-1

**License**

**Email Alerting Service**
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).