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C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells

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Abstract

Malignant melanomas often harbor activating mutations in BRAF (V600E) or, less frequently, in NRAS (Q61R). Intriguingly, the same mutations have been detected at higher incidences in benign nevi, which are largely composed of senescent melanocytes. Overexpression of $BRAF^{V600E}$ or $NRAS^{Q61R}$ in human melanocytes *in vitro* has been shown to induce senescence, although via different mechanisms. How oncogene-induced senescence is overcome during melanoma progression remains unclear. Here, we report that in the majority of analysed $BRAF^{V600E}$- or $NRAS^{Q61R}$-expressing melanoma cells, C-MYC depletion induced different yet overlapping sets of senescence phenotypes that are characteristic of normal melanocytes undergoing senescence due to overexpression of $BRAF^{V600E}$ or $NRAS^{Q61R}$, respectively. These senescence phenotypes were $p16^{INK4A}$- or p53-independent, however, several of them were suppressed by genetic or pharmacological inhibition of $BRAF^{V600E}$ or phosphoino-sitide 3-kinase pathways, including rapamycin-mediated inhibition of mTOR-raptor in $NRAS^{Q61R}$-expressing melanoma cells. Reciprocally, overexpression of C-MYC in normal melanocytes suppressed $BRAF^{V600E}$-induced senescence more efficiently than $NRAS^{Q61R}$-induced senescence, which agrees with the generally higher rates of activating mutations in *BRAF* than *NRAS* gene in human cutaneous melanomas. Our data suggest that one of the major functions of C-MYC overexpression in melanoma progression is to continuous suppress $BRAF^{V600E}$- or $NRAS^{Q61R}$-dependent senescence programs.

Keywords

C-MYC; melanoma; senescence

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Introduction

In normal cells, senescence induced by the aberrant activation of oncogenes represents an important mechanism for suppressing tumor development (Bringold and Serrano, 2000; Mooi and Peeper, 2006). It has been well documented that oncogene-induced senescence (OIS) in normal rodent cells *in vitro* is executed via $p19^{ARF}$-, p53- or $p16^{INK4A}$-pRb pathways (Collado and Serrano, 2006), members of which are often deleted or inactivated in the course of tumor progression (Gil and Peters, 2006; Kastan, 2007). Accordingly, several studies performed in transgenic mice have confirmed an active role of the above pathways in the implementation of senescence in murine tumors (Ventura *et al.*, 2007; Wu *et al.*, 2007; Xue *et al.*, 2007). However, the molecular mechanisms underlying senescence in human tumor cells are less well understood.

Malignant melanoma is one of the most aggressive types of human cancers (Gray-Schopfer *et al.*, 2007). Most frequently arising in the skin, melanomas can evolve from benign or pre-malignant aggregations of melanocytes, termed nevi (Chin *et al.*, 2006). Neoplastic transformation of melanocytes often involves oncoproteins that upregulate the mitogen-activated kinase pathway (Chin *et al.*, 2006). In particular, activating mutations of BRAF (V600E) or NRAS (Q61R) are found in approximately 60% and 20% of melanomas from non-chronically sun-damaged skin, respectively (Maldonado *et al.*, 2003; Curtin *et al.*, 2005), although $BRAF^{V600E}$ mutations in melanomas from chronically sun-damaged skin or from skin relatively unexposed to sun are less common (Maldonado *et al.*, 2003). Intriguingly, $BRAF^{V600E}$ mutations are detected at even higher frequencies in benign acquired nevi (82%) (Pollock *et al.*, 2003), whereas 81% of benign congenital nevi harbor mutations in the *NRAS* gene (Bauer *et al.*, 2007).

Recently, it was shown that melanocytes inside nevi, but not in normal skin, displayed high levels of senescence-associated β-galactosidase activity (SA-β-Gal) (Michaloglou *et al.*, 2005). Moreover, ectopic expression of $NRAS^{Q61R}$ or $BRAF^{V600E}$ in cultured normal human melanocytes is associated with distinct forms of premature cellular senescence (Michaloglou *et al.*, 2005). Taken together, these data suggest that the senescence-inducing activities of $NRAS^{Q61R}$ or $BRAF^{V600E}$ are silenced or reversed in the course of melanoma progression, although molecular mechanisms underlying these events remain unclear.

C-MYC is an oncogenic transcription factor that is frequently upregulated in human malignancies (Nesbit *et al.*, 1999), including melanoma (Ross and Wilson, 1998; Greulich *et al.*, 2000; Kraehn *et al.*, 2001), although its role in melanomagenesis has not been addressed. In normal human cells, overexpression of C-MYC promotes the acquisition of a transformed phenotype in cooperation with other cellular oncogenes (Boehm *et al.*, 2005). In mice, C-MYC upregulation has been shown to induce tumorigenesis in numerous transgenic models (Lutz *et al.*, 2002). Consequently, C-MYC inactivation in the majority of the above mouse models led to tumor regression via several mechanisms, including apoptosis, differentiation or rapid induction of senescence (Arvanitis and Felsher, 2006; Wu *et al.*, 2007). The latter phenotype was shown to be p16- or p53-dependent and mitogen-activated ERK kinase (MEK)-independent (Wu *et al.*, 2007). Accordingly, approximately 50% depletion of C-MYC in normal human fibroblasts overexpressing the telomerase reverse-transcriptase catalytic subunit caused premature senescence, which was also p16-dependent (Guney *et al.*, 2006).

Acute and significant inhibition of C-MYC expression in virtually all human tumor cells tested caused apoptosis or marked proliferation arrest (Vita and Henriksson, 2006; Wang *et al.*, 2008), but has never been associated with the rapid induction of senescence phenotypes.

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Here, we demonstrate that BRAF^{V600E}- or NRAS^{Q61}-dependent senescence programs exist in dormancy in advanced melanoma cells and could be re-activated by the depletion of C-MYC. Interestingly, unlike tumor mouse or normal human cells (Guney *et al.*, 2006; Wu *et al.*, 2007), senescence induced in human melanoma cells was p16- or p53-independent. Accordingly, we demonstrated that senescence induced in normal human melanocytes by BRAF^{V600E} or NRAS^{Q61} also did not depend on p16 or p53 but could be partially overcome by C-MYC overexpression. As senescence induced by BRAF^{V600E} or NRAS^{Q61} is a naturally occurring process in almost all benign nevi, and because a large portion of malignant melanomas originate from nevi, our data suggest that C-MYC upregulation is an important aspect of melanoma evolution, and is therefore necessary for continuous suppression of OIS.

Results

C-MYC is overexpressed in melanoma metastases and in tumor-derived melanoma cells lines

As an initial step in the characterization of C-MYC expression in melanocytic cells, we compared C-MYC levels in benign nevi, metastatic melanoma tissues, metastatic melanoma cell lines and cultured neonatal melanocytes. To this end, lysates from 2 benign congenital nevi and 10 melanoma metastases were subjected to immunoprecipitation with mouse C-MYC-specific antibodies, followed by western blotting with rabbit C-MYC-specific antibodies (to avoid crossreactivity) (Figure 1a).

In parallel, C-MYC levels were determined by western blotting in total cellular extracts from 2 independently isolated populations of neonatal human melanocytes and from 10 metastasis-derived human melanoma cell lines (Figure 1b). As shown in Figure 1, C-MYC protein levels were approximately three- to sevenfold higher in metastatic melanomas as compared with benign nevi. A similar increase in C-MYC levels (3- to 20-fold) was detected in samples from melanoma cell lines, when compared with normal melanocytes. These data demonstrate that C-MYC overexpression observed in cultured melanoma cells reflects upregulation of C-MYC in the course of melanoma progression and suggest that metastatic melanoma cell lines could be an adequate model for studying the functional role of C-MYC in advanced melanomas.

C-MYC depletion in melanoma cells results in the induction of BRAF^{V600E}- or NRAS^{Q61R}-dependent senescence phenotypes

Recently, we demonstrated that acute and efficient short hairpin RNA (shRNA)-mediated inhibition of *C-MYC* in cells from a large number of human tumor lines resulted in proliferation arrest and/or apoptosis (Wang *et al.*, 2008). Here, we confirmed these observations for 10 melanoma cell lines using 2 C-MYC-specific shRNAs: C-MYC-shRNA-1 (M1) and C-MYC-shRNA-2 (M2). As was described previously (Wang *et al.*, 2008), significant inhibition of C-MYC was obtained with each shRNA, although M1 suppressed C-MYC more efficiently than M2 (Supplementary Figure S1a). We performed expression analysis of several *bona fide* targets of MYC in response to its depletion in an arbitrarily chosen melanoma cell line (SK-Mel-94). As shown in Supplementary Figure S1b, expression of genes encoding cyclin B1, ornithine decarboxylase and CDC25A (positively regulated by MYC) was decreased by 2.5- to 4-fold, expression of the gene encoding p27^{KIP1} (suppressed by MYC) was increased by 2-fold, whereas expression of CDK4 (positively regulated by MYC) remained virtually constant. Therefore, C-MYC depletion was accompanied by expression changes of several MYC-target genes.

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Depletion of C-MYC in cells from 8 out of 10 melanoma lines caused substantial reduction in proliferation rates, which eventually culminated in a complete growth arrest between days 4 and 6 (Wang *et al.*, 2008 and Supplementary Figure S1c), whereas C-MYC-depleted cells from lines UACC62 and UACC258 underwent apoptotic death shortly after infection (Wang *et al.*, 2008; data not shown).

We were interested in whether growth arrested C-MYC-depleted cells exhibit features of cellular senescence. To this end, melanoma cells infected with control or C-MYC-shRNAs were tested for the presence of well-established senescence markers: increased SA-β-Gal activity (Itahana *et al.*, 2007) and formation of senescence-associated heterochromatin foci, which could be visualized by staining with DAPI and antibodies to trimethylated lysine 9 of histone H3 (H3K9^{3Me}) (Narita *et al.*, 2003). As shown in Figure 2b, depletion of C-MYC resulted in an approximately 7- to 10-fold increase in the percentage of SA-β-Gal-positive cells in the melanoma lines studied. Moreover, in two arbitrarily chosen BRAF^{V600E}- or NRAS^{Q61R}-expressing melanoma lines, C-MYC inhibition induced an approximately three- to fivefold increase in the proportion of senescence-associated heterochromatin foci-containing cells (Supplementary Figure S2).

As was previously shown, transduction of activated HRAS or NRAS in normal melanocytes induces senescence that is mechanistically distinct from the one caused by BRAF^{V600E} (Denoyelle *et al.*, 2006). In particular, H-RAS^{V12G}- or NRAS^{Q61R}-expressing melanocytes became SA-β-Gal-positive, and flat and extensively vacuolized within 4–6 days after transduction, whereas, melanocytes overexpressing BRAF^{V600E} became SA-β-Gal-positive and developed a more rounded shape without extensive vacuolization only starting 12–15 days after infection (Figure 2a). On the basis of these morphological differences, we noticed that C-MYC-depleted cells from melanoma lines could be segregated into two groups. Cells from the lines of the first group (SK-Mel-19, SK-Mel28, SK-Mel-29, SK-Mel-94, G-361), which express BRAF^{V600E} (Verhaegen *et al.*, 2006), recapitulated the senescence patterns of normal melanocytes overexpressing BRAF^{V600E} (Figure 2a). In contrast, cells from the second group (SK-Mel-103, SK-Mel-147, SK-Mel-2) with NRAS^{Q61R} mutation (Verhaegen *et al.*, 2006) resembled normal melanocytes undergoing senescence in response to the overexpression of NRAS^{Q61R} (Figure 2a). Therefore, our data suggest that C-MYC depletion in melanoma cells induces a cellular senescence program that is dictated by the activated oncoprotein (BRAF^{V600E} or NRAS^{Q61R}) expressed in these cells.

To determine whether senescence caused by C-MYC depletion in melanoma cells depends on constitutively active BRAF^{V600E} or NRAS^{Q61R}, we studied the consequences of inhibiting BRAF^{V600E}- and NRAS^{Q61R}-signaling pathways in the above cells. To this end, control and C-MYC-depleted SK-Mel-94 (expressing BRAF^{V600E}) and SK-Mel-103 (expressing NRAS^{Q61R}) cells were incubated with U0216, an inhibitor of the RAF downstream effector MEK (Gollob *et al.*, 2006), or with LY294002 (an inhibitor of phosphoinositide 3-kinase (PI3K), a downstream target of RAS (Meier *et al.*, 2005)). Inhibitors were added for 48 h starting at day 4 post-infection with C-MYC- or control shRNAs, that is, prior to the emergence of senescence phenotypes in C-MYC-depleted cells. Minimal effective concentrations of the inhibitors in studied melanoma cells have been established previously (Denoyelle *et al.*, 2006; Verhaegen *et al.*, 2006) and were confirmed here by monitoring the inhibition of extracellular signal-regulated kinase (ERK)-1/2 or AKT-1 phosphorylation (Supplementary Figure S3a).

A 48 h treatment with U0216, but not with LY294002, led to an approximately threefold decrease in the number of SA-β-Gal-positive cells in the population of C-MYC-depleted SK-Mel-94 cells. Proliferation rates of control SK-Mel-94 cells were decreased by approximately 50% (Figure 3b), whereas proliferation of the C-MYC-depleted counterparts

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was unaffected, most likely because it had already been severely compromised by the absence of C-MYC. Similar results were obtained with another BRAF^{V600E}-containing melanoma line, SK-Mel-19 (data not shown).

On the other hand, treatment of C-MYC-depleted SK-Mel-103 cells with U0216, while inhibiting ERK phosphorylation (Supplementary Figure S3a), did not affect the number of SA-β-Gal-positive and vacuolized cells (Figure 3a). In contrast, these phenotypes were affected by treating the cells with LY294002, as the proportion of SA-β-Gal-positive and vacuolized cells decreased more than fourfold. Despite an obvious reversal of some senescence phenotypes by LY294002, the proliferation rates of C-MYC-depleted SK-Mel-103 cells were not restored (Figure 3b), again probably owing to the absence of C-MYC expression. Similar results were obtained with another NRAS^{Q61R}-containing melanoma cell line, SK-Mel-147 (data not shown). To further confirm the involvement of activated PI3K pathway in senescence of melanoma cells, we inhibited one of the PI3K downstream effectors, mTOR-raptor (Yang and Guan, 2007), in SK-Mel-103 control and MYC-depleted cells by treating them with 25 nM rapamycin (Yang and Guan, 2007) for 48 h starting at day 4 post-infection. As shown in Supplementary Figure S3d, treatment with rapamycin resulted in a roughly threefold decrease in the numbers of SA-β-Gal-positive and vacuolized cells.

To confirm the above data obtained via pharmacological inhibition of MEK and PI3K, we set up experiments to genetically inhibit activated BRAF or PI3K pathways in the melanoma cells studied. To this end, SK-Mel-94 (BRAF^{V600E}-positive) cells were transfected with control or previously validated small interfering RNA (siRNA) against mutant (BRAF^{V600E}) allele (Sharma *et al.*, 2005), followed by infection with control- or MYC-specific (M1) shRNAs. In agreement with previously published data (Sharma *et al.*, 2005), treatment of SK-Mel-94 cells with BRAF^{V600E} siRNA resulted in inhibition of MEK activity, manifested in a 2.5-fold decrease in the amounts of phosphorylated ERK (Supplementary Figure S3b). Subsequently, depletion of C-MYC in BRAF^{V600E}-siRNA-transfected cells resulted in a roughly 50% decrease in the number of senescent cells, when compared with MYC-depleted cells transfected with control siRNA (Supplementary Figure S3b).

To genetically inhibit PI3K activity in SK-Mel-103 (NRAS^{Q61R}-positive) cells, cDNA for a well-known inhibitor of PI3K, PTEN, was overexpressed in these cells (Haluska *et al.*, 2007). Overexpression of PTEN resulted in 71% reduction of PI3K activity, as was evidenced by the corresponding decrease in the amounts of phosphorylated Akt-1 in cells studied (Supplementary Figure S3c). Accordingly, depletion of C-MYC in PTEN-overexpressing melanoma cells yielded approximately two times fewer senescent cells compared with the population of MYC-depleted cells expressing empty vector (Supplementary Figure S3c).

Taken together, our data suggest that genetic inhibition of BRAF^{V600E} or PI3K pathways (although less efficient than pharmacological inhibition) is sufficient to suppress senescence of melanoma cells caused by the depletion of C-MYC. Therefore, similar to that in normal melanocytes, senescence markers in C-MYC-depleted melanoma cells require constitutively active MEK or PI3K.

Senescence phenotypes induced by C-MYC depletion in melanoma cells or by BRAF^{V600E} and NRAS^{Q61R} overexpression in melanocytes occur independent of p53 or p16^{INK4A}

Oncogene-induced senescence phenotypes in mouse cells have been previously shown to depend on p53 and/or p16 (Ventura *et al.*, 2007; Wu *et al.*, 2007; Xue *et al.*, 2007). However, the precise role of these proteins in OIS of human cells has not been well defined and is likely to be cell type-specific (see Discussion). Therefore, we attempted to identify

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the roles of p53 and p16^{INK4A} in senescence phenotypes caused by C-MYC depletion in melanoma cells and by the overexpression of BRAF^{V600E} or NRAS^{Q61R} in normal melanocytes.

Levels of p53 and p16^{INK4A} in the melanoma cells studied and in normal melanocytes are shown in Figure 4a. As it is well documented in melanomas, all the cell lines studied here, with the exception of SK-Mel-28, contain wild-type p53 (Goding, 2000; Soengas *et al.*, 2001). However, regulation of p53 as well as its downstream pathways might have been altered in the course of tumor progression. To test this possibility, cells from melanoma lines SK-Mel-19, -29, -103 and -147 were infected with control or p53-specific shRNA, as described previously (Wang *et al.*, 2008). Control cells were treated for 16 h with vehicle (dimethylsulfoxide) or 0.3 μg/ml of doxorubicin, a DNA-damaging agent that indirectly induces stabilization of wild-type p53 and activation of its downstream targets (Lowe *et al.*, 1993). After completion of incubation, control cells and untreated p53-shRNA-expressing cells were collected and the expression levels of p53 and its two well-characterized transcriptional targets, p21$^{CIP/WAF}$ (El-Deiry *et al.*, 1993) and p53R2 (a p53-dependent ribonucleoside reductase, subunit 2) (Tanaka *et al.*, 2004), were analysed by western blotting. As shown in Figure 4b, changes in p53, p21$^{CIP/WAF}$ and p53R2 levels correlate well with each other, suggesting that the p53 pathway is at least partially intact in the melanoma cells studied.

As is also characteristic of many melanomas (Sharpless and Chin, 2003), p16^{INK4A} expression is absent in several of the cell lines used by us (Figure 4a). To determine the functional role of p16^{INK4A} or p53 in the mediation of senescence induced by C-MYC depletion, the expression of p53 or p16^{INK4A} was inhibited by the corresponding shRNAs in the melanoma cells SK-Mel-19, -29, -103, -147 or SK-Mel-28, -103, -147, respectively (Supplementary Figure S4), followed by super-infection with control or C-MYC-shRNA-containing lentiviruses. The induction of senescence was monitored by counting SA-β-Gal-positive cells at day 5 post-infection. As shown in Figure 4c, no substantial differences in the proportion of SA-β-Gal-positive cells were detected among cells expressing or not p53 or p16 proteins.

Next, we examined the role of p53 and p16^{INK4A} in BRAF^{V600E}- or NRAS^{Q61R}-induced senescence of normal melanocytes. The levels of p53 or p16 were depleted in normal melanocytes via expression of the above shRNAs followed by super-infection with a control lentivirus vector or vectors expressing BRAF^{V600E} or NRAS^{Q61R} cDNAs (Figure 4d). The proportion of SA-β-Gal-positive cells was determined at days 6 or 10 in NRAS^{Q61R}- or BRAF^{V600E}-expressing melanocytes, respectively. As shown in Figure 4d, suppression of neither p53 nor p16^{INK4A} was sufficient to rescue (even partially) senescence induced by BRAF^{V600E} or NRAS^{Q61R}. Therefore, our data suggest that senescence induced in normal melanocytes by BRAF^{V600E} or NRAS^{Q61R}, as well as senescence caused in melanoma cells by depletion of C-MYC, is p53- or p16^{INK4A}-independent.

C-MYC overexpression partially reverts OIS phenotypes in normal melanocytes

We were interested in whether senescence caused by the overexpression of BRAF^{V600E} or NRAS^{Q61R} in normal melanocytes associates with the inhibition of endogenous C-MYC expression. To answer this question, we assessed by western blotting endogenous C-MYC levels in normal melanocytes at different time points after infection with control vector or vectors expressing BRAF^{V600E} or NRAS^{Q61R} cDNAs. In parallel, senescence in the above cells was monitored by determining the proportion of SA-β-Gal-positive cells (Figures 5a and b).

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Ectopic expression of NRAS^{Q61R} caused an approximately threefold depletion of C-MYC levels by day 10 (Figure 5a), which was associated with the increase in the proportion of SA-β-Gal-positive cells (Figure 5b). In contrast to NRAS^{Q61R}, BRAF^{V600E}-infected melanocytes were previously shown to moderately increase proliferation rates within several days after infection (Denoyelle *et al.*, 2006). In keeping with these findings, C-MYC protein levels in BRAF^{V600E}-expressing melanocytes were increased slightly at day 6 post-infection (Figure 5a). However, starting after day 6, C-MYC levels in these cells were decreasing when compared with control melanocytes (Figure 5a). During this time, the proportion of SA-β-Gal-positive cells gradually increased, reaching maximum at day 18 (Figure 5b). Therefore, C-MYC levels inversely correlate with the number of senescent cells in melanocytes expressing BRAF^{V600E} or NRAS^{Q61R}.

To determine the functional role of C-MYC overexpression in suppressing OIS in normal melanocytes, we co-expressed in these cells *C-MYC* cDNA in combination with cDNAs for either *BRAF^{V600E}* or *NRAS^{Q61R}* (see Figure 5c for schematic representation of infection and Supplementary Figure S5). As shown in Figure 5d, populations of melanocytes co-expressing BRAF^{V600E} and C-MYC contained fewer SA-β-Gal-positive cells compared with populations expressing BRAF^{V600E} and empty vector (20±3% versus 57±9% at day 14 and 29.5%±10.5% versus 71%±11% at day 18). Similarly, melanocytic populations co-expressing C-MYC and NRAS^{Q61R} contained fewer SA-β-Gal-positive cells than their counterparts expressing NRAS^{Q61R} and empty vector (46%±4% versus 59%±6% at day 6 and 51%±4.5% versus 64.5%±6.5% at day 10; Figure 5d). We, therefore, concluded that C-MYC overexpression partially suppresses senescence induced in normal melanocytes by BRAF^{V600E} and, to the lesser extent, by NRAS^{Q61R}.

Discussion

Oncogene-induced senescence in normal cells is emerging as a fail-safe mechanism for suppressing tumor development at a pre-malignant stage (Bringold and Serrano, 2000; Collado and Serrano, 2006; Haluska *et al.*, 2006; Ha *et al.*, 2007). In humans, OIS is probably best exemplified by nevi that represent benign tumors consisting primarily of non-proliferative melanocytes, which often harbor activating mutations in the *BRAF* or *NRAS* gene (Michaloglou *et al.*, 2005). The malignant melanomas that frequently originate from nevi must, therefore, develop mechanisms for escaping OIS (Michaloglou *et al.*, 2005; Mooi and Peeper, 2006).

In answer to this problem, we report here that enforced C-MYC expression in normal melanocytes partially overcomes senescence-inducing effects of BRAF^{V600E} and, to a lesser extent, of NRAS^{Q61R}, whereas acute depletion of C-MYC causes senescence in BRAF^{V600E} and NRAS^{Q61R} melanoma cells. Interestingly, even partial (70%) depletion of C-MYC appears to be sufficient to induce senescence in melanoma cells. Moreover, a simple mathematical analysis of data presented in Figure 1b and Supplementary Figure S1a reveals that senescence in melanoma cells could be induced when C-MYC levels are suppressed approximately to those in normal melanocytes (majority of the studied cell lines), and even when the remaining amounts of C-MYC in melanoma cells are still higher than C-MYC amounts in normal melanocytes (for example, SK-Mel-2 or G361 cells infected with M2-shRNA). Therefore, partial suppression of C-MYC is sufficient to induce senescence in at least several melanoma cell lines.

We have demonstrated that C-MYC-depleted BRAF^{V600E}- or NRAS^{Q61R}-expressing melanoma cells morphologically resemble normal melanocytes that have become senescent as a consequence of BRAF^{V600E} or NRAS^{Q61R} overexpression, respectively. These data suggest that BRAF^{V600E}- or NRAS^{Q61R}-specific senescence programs are latent in

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melanoma cells and subjected to reactivation under certain conditions. Moreover, it appears that one of the major roles of C-MYC overexpression in melanoma cells is to suppress senescence-inducing activity of the above-mentioned oncoproteins. These suggestions are further supported by our findings that pharmacological inhibition of downstream targets of activated BRAF or NRAS in senescent melanoma cells results in suppression of several senescence phenotypes (Figure 3). Interestingly, pharmacological inhibition of PI3K but not MEK was previously reported to decrease vacuolization and the proportion of SA-β-Gal-positive cells in normal melanocytes expressing $HRAS^{V12G}$ (Denoyelle *et al.*, 2006). In fact, additional similarities exist between senescence in melanoma cells caused by depletion of C-MYC and OIS in melanocytes. First, in both cases, senescence phenotypes depended on or were associated with low levels of C-MYC. Indeed, in melanoma cells, C-MYC depletion was the cause of senescence, whereas senescent melanocytes with $BRAF^{V600E}$ or $NRAS^{Q61R}$ had decreased levels of C-MYC (Figure 5).

Second, in all cases, senescence phenotypes were p53- or $p16^{INK4A}$-independent, in contrast to what is commonly seen in rodent cells. In fact, the contribution of these tumor suppressors to the premature senescence of normal human cells is still under debate. For example, normal human fibroblasts deficient in $p16^{INK4A}$ and ectopically expressing the catalytic subunit of the telomerase reverse transcriptase are resistant to the antiproliferative effects of activated HRAS (Drayton *et al.*, 2003). Similarly, oncogenic HRAS may be a poor inducer of premature senescence in freshly isolated human fibroblasts expressing low levels of $p16^{INK4A}$ (Benanti and Galloway, 2004). On the other hand, $p16^{INK4A}$ deficiency did not prevent senescence by $BRAF^{V600E}$ in normal human fibroblasts (Michaloglou *et al.*, 2005). In addition, heterogeneous expression of $p16^{INK4A}$ can be detected in nevi. Thus, SA-β-Gal-positive nevus cells may not necessarily accumulate $p16^{INK4A}$ (Michaloglou *et al.*, 2005). Furthermore, $HRAS^{G12V}$ caused senescence independently of p53 in primary human esophageal keratinocytes (Takaoka *et al.*, 2004), whereas p53 or $p16^{INK4A}$ were shown to be dispensable for the senescence induced in normal melanocytes by $HRAS^{V12G}$ (Denoyelle *et al.*, 2006). Therefore, p53 and $p16^{INK4A}$ may not be universal mediators of OIS in human cells, irrespective of whether they are normal or transformed.

Our data demonstrate that ectopic overexpression of C-MYC can partially rescue the senescence phenotype of melanocytes expressing $BRAF^{V600E}$, and that it does so less efficiently in melanocytes expressing $NRAS^{Q61R}$. Recently, it has been shown that unlike $BRAF^{V600E}$, overexpression of $NRAS^{Q61R}$ in normal melanocytes induces an unfolded protein response, and that shRNA-mediated inhibition of several proteins involved in the unfolded protein response (ATF-4 and ATF-6 (Kim *et al.*, 2006)) suppresses $NRAS^{Q61R}$-induced senescence (Denoyelle *et al.*, 2006). In our experience, C-MYC overexpression in melanocytes does not affect the unfolded protein response pathway and, in particular, expression of ATF-4 and -6 (data not shown), which could account for the inability of C-MYC to successfully overcome senescence induced by $NRAS^{Q61R}$. Interestingly, in melanomas from non-chronically sun-damaged skin, *NRAS* mutations are less common than *BRAF* mutations (Curtin *et al.*, 2005). One of the possible explanations for this observation could be that in the course of melanoma progression, senescence induced by activated NRAS is less likely to be overcome than that induced by activated BRAF. Our *in vitro* data on co-expression of C-MYC with $BRAF^{V600E}$ or $NRAS^{Q61R}$ in normal melanocytes support this scenario.

C-MYC has been implicated in opposing senescence that is not induced by oncogenes. Indeed, a recent study by Guney *et al.* (2006) demonstrated that 50% depletion of C-MYC in normal human fibroblasts ectopically expressing telomerase reverse transcriptase resulted in the premature induction of senescence after five passages. Similarly, continuous partial inhibition of C-MYC in M14 melanoma cells (over a period of seven passages) was reported

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to induce cell crisis associated with telomere shortening, apoptosis and increased activity of SA-β-Gal (Biroccio *et al.* 2003). Accordingly, C-MYC was shown to rescue normal human prostate epithelial cells from replicative senescence (presumably via upregulation of the catalytic subunit of the telomerase reverse transcriptase) (Gil *et al.*, 2005). Importantly, human prostate epithelial-MYC cells were still undergoing senescence-like growth arrest in response to oncogenic Ras (Gil *et al.*, 2005). In addition, when this paper was in preparation, Wu *et al.*, 2007 demonstrated that inactivation of C-MYC resulted in induction of senescence phenotypes in several mouse tumor models, including lymphoma, osteosarcoma and hepatocellular carcinoma. These phenotypes were not suppressed by pharmacological inhibition of MEK, but depended on $p16^{INK4A}$ or p53.

Conversely, in one special case (fibroblasts from Werner syndrome patients that lack a conserved RecQ helicase encoded by WRN gene), C-MYC overexpression was shown to actually induce senescence (Grandori *et al.*, 2003). Therefore, our data and those of others suggest that the role of C-MYC in senescence of human cells is complex and depends on possibly overlapping pathways, the nature of which could be cell type- and/or tumor-dependent.

In summary, our study has identified that $BRAF^{V600E}$- or $NRAS^{Q61R}$-specific senescence programs exist in a dormant state in advanced melanoma cells and that one of the major functions of C-MYC overexpression during melanoma progression is to continuously suppress such senescence.

Materials and methods

Cell lines

Melanoma cell lines were originally obtained from Memorial Sloan Kettering Cancer Center. Cells were cultured in Dulbecco's modified Eagle's essential minimal medium as recommended by the supplier. Supplements included fetal calf serum (10%), 2mM glutamine, and 100 U/ml penicillin G+100 μg/ml streptomycin. All cell culture agents were purchased from Invitrogen Inc. (Carlsbad, CA, USA). Normal melanocytes were isolated from neonatal foreskins as reported before (Denoyelle *et al.*, 2006) and maintained in Medium 254 supplemented with 0.2mM $CaCl_2$, 16nM TPA and melanocyte growth factors (Invitrogen, Portland, OR, USA).

Senescence assay

Cells were plated in 12-well plates at 30% confluence 2 days prior to the assay. Cells were fixed and incubated for different time periods (4 h to overnight, depending on the cell line) at 37 °C with the staining solution containing the X-Gal substrate (BioVision, Mountain View, CA, USA). The development of blue color was detected visually under the microscope.

siRNA and shRNA

Control and $BRAF^{V600E}$-specific siRNAs (Sharma *et al.*, 2005) were manufactured by Invitrogen and transfected according to the manufacturer's recommendations. The lentiviral vectors containing shRNA specific for p53 and $p16^{INK4A}$ were described by Denoyelle *et al.* (2006) and Wang *et al.* (2008), respectively. Lentiviral vector pLKO-1 containing shRNA-1 specific for human C-MYC (C-MYC-shRNA-1) was purchased from Sigma (St Louis, MO, USA). The C-MYC-targeting sequences consisted of nucleotides 1567–1585 of the previously published sequence (GenBank accession no. NM_002467.3). The lentiviral construct H1 was used to express C-MYC-shRNA-2 corresponding to nucleotides 1341–1359 of C-MYC. A control lentiviral vector contained an irrelevant sequence. Lentiviral

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packaging reactions were performed in the 293-FT cell line in the presence of packaging plasmids VSG and DDR (Wang *et al.*, 2008) using Superfect transfection reagent (Qiagen Inc., Chatsworth, CA, USA). Viral supernatants were collected 48 h after transfection, filtered through disposable 0.45 μM cellulose acetate filters (VWR Scientific Inc., West Chester, PA, USA) and frozen in individual aliquots at −80 °C. For infection, cells were plated in 60 or 100 mm tissue culture dishes and allowed to achieve 40–50% confluence before adding the viral supernatant in the presence of 8 μg/ml polybrene (Sigma) for 24 h. Cells infected with C-MYC-shRNA-2 in H1 lentiviral vector were examined by fluorescence microscopy for the expression of EGFP in 48 h.

Immunoprecipitation, immunoblotting and immunofluorescence

Frozen human tumor samples, obtained via the Tissue Procurement Service of the University of Michigan Comprehensive Cancer Center, were embedded in OCT, cryosectioned and stained with hematoxylin to select the appropriate regions for use in the experiments. Typically, these specimens were large enough to obtain all of the material from a single region (for example, 2–3 mm^2). The selected regions were at least 80% tumor cells (tumor cellularity), and tumors with mixed histology were not used. The tumor portions chosen for the experiments were obtained by cutting out that region of the tumor using a pointed scalpel cooled with dry ice. Protein extracts were obtained from frozen sections of melanoma tumors or benign nevi by extracting in F-buffer containing 0.3M NaCl and 0.3% TritonX (Nikiforov *et al.*, 2002). A total of 1 ml of F-buffer concurrently with 20 ml of liquid nitrogen was added directly to the samples followed by grinding using a pestle and a bowl. C-MYC protein levels were assessed by immunoprecipitation from approximately 300 μg of F-buffer protein extracts with bead-conjugated monoclonal mouse C-MYC-specific antibodies (C-33; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by western blotting with rabbit polyclonal C-MYC-specific antibodies (N-262, Santa Cruz Biotechnology). Normalization of the amounts of samples was done by measuring the protein concentration of the extracts in colorimetric assays and verified by probing extract aliquot in western blotting with α-tubulin antibodies. To determine the C-MYC levels in cultured cells, total cell lysates were prepared and immunoblotting was performed, as previously described, using 50 μg of total protein (Nikiforov *et al.*, 2002). The following antibodies were used: human C-MYC-specific C33 mouse monoclonal antibodies or N262 rabbit polyclonal antibodies (Santa Cruz Biotechnology), p53-specific antibodies (Vector Labs, Burlingame, CA, USA), $p16^{INK4A}$-specific antibodies (BD Bioscience, San Jose, CA, USA), ERK1/2, phospho-ERK1/2, AKT-1, phospho-AKT-1 (Cell Signaling Technology, Danvers, MA, USA), p53R2 and $p21^{CIP/WAF}$, tubulin-specific D-10 (all from Santa Cruz Biotechnology). Membranes were developed using alkaline phosphatase-conjugated secondary antibodies and detection/quantification of the signal was performed using STORM PhosphorImager and ImageQuant 2.0 program. Background was calculated from an equivalent area in each lane and subtracted from the value for the protein signal in that lane. For immunofluorescence staining with antibodies specific to trimethylated lysine 9 of histone H3 (Millipore, Billerica, MA, USA), the cells were fixed and processed as previously described (Michaloglou *et al.*, 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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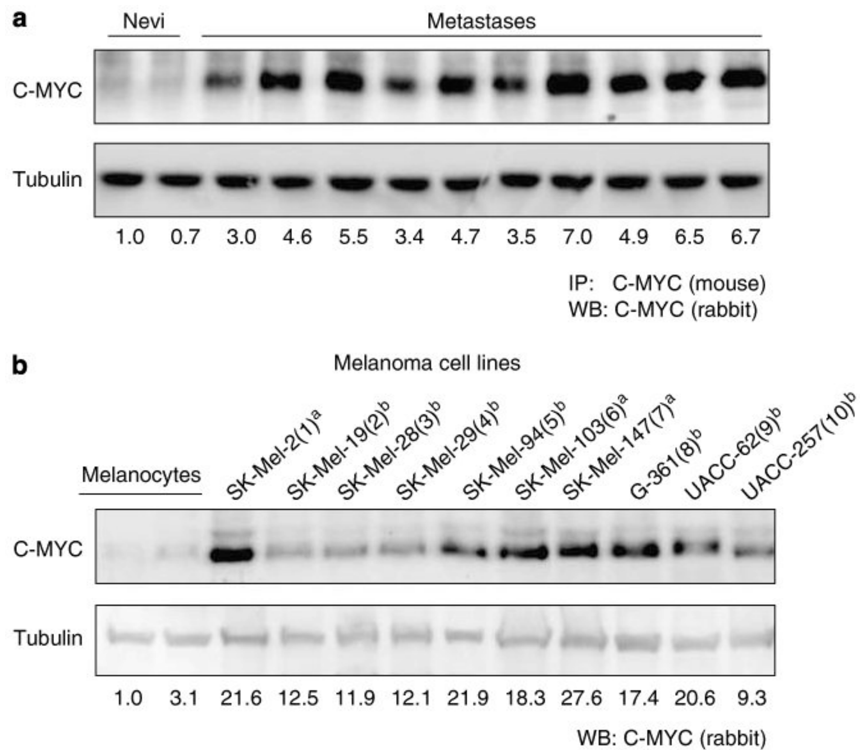

Figure 1.

C-MYC levels are elevated in melanoma tissues and cell lines. (**a**) Human tissue samples were obtained and processed as described in Materials and methods. Immunoprecipitated materials were probed in western blotting with rabbit C-MYC antibodies. Note that the lower panel corresponds to the normalized protein extracts probed with antitubulin antibodies prior to immunoprecipitation. Numbers below the panel indicate fold-increase of C-MYC/tubulin ratio compared with that in the first lane (nevus). See Materials and methods for description of signal quantification. (**b**) Total cellular extracts from two independently isolated populations of normal melanocytes and indicated metastatic melanoma cell lines were probed in western blotting with antibodies designated on the right. Numbers below the panels show fold-increase of C-MYC/tubulin ratio compared with that in the first lane (melanocytes). a, $NRAS^{Q61R}$-expressing cell lines; b, $BRAF^{V600E}$-expressing cell lines. IP, immunoprecipitation; WB, western blotting.

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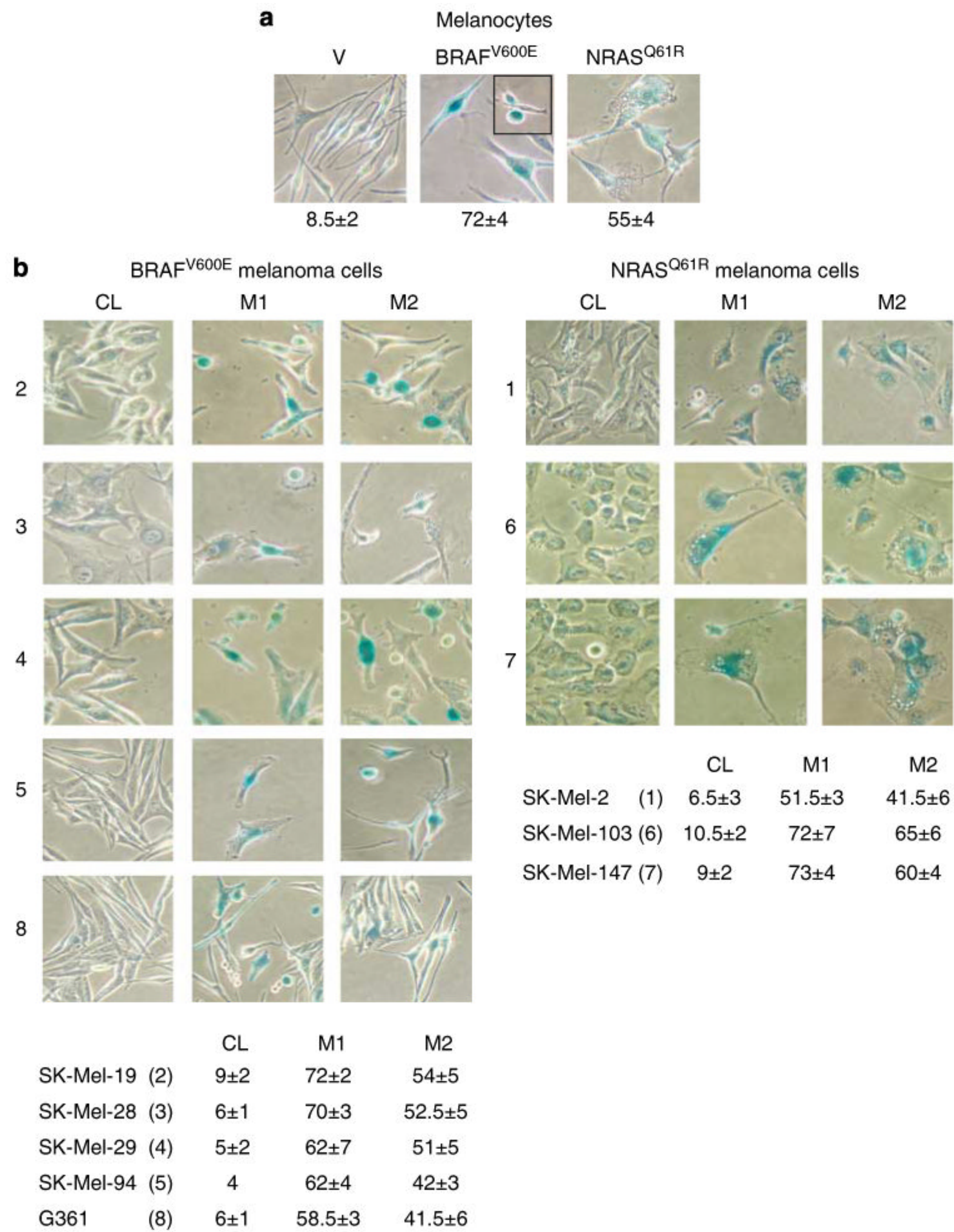

Figure 2.

C-MYC depletion induces senescence-associated β-galactosidase (SA-β-Gal) activity in melanoma cells. (**a**) Normal melanocytes were infected with control lentiviral vector (V) or vectors expressing $BRAF^{V600E}$ or $NRAS^{Q61R}$ cDNAs (indicated at the top of the panel). Cells were fixed and stained for SA-β-Gal activity, according to Materials and methods, after 18 days (control and $BRAF^{V600E}$-infected melanocytes) or after 6 days ($NRAS^{Q61R}$-infected melanocytes). Numbers below the panel indicate percent of SA-β-Gal-positive cells detected in each population (100 cells were counted in each of the duplicate plates). (**b**) Melanoma cells designated on the left from each panel with the number assigned in Figure 1b were infected with control shRNA (CL) or M1- or M2-shRNAs. Cells were fixed and stained for SA-β-Gal activity, according to Materials and methods 6 days after infection.

Numbers below the panels indicate percent of SA-β-Gal-positive cells detected in each population (100 cells were counted in each of the duplicate plates).

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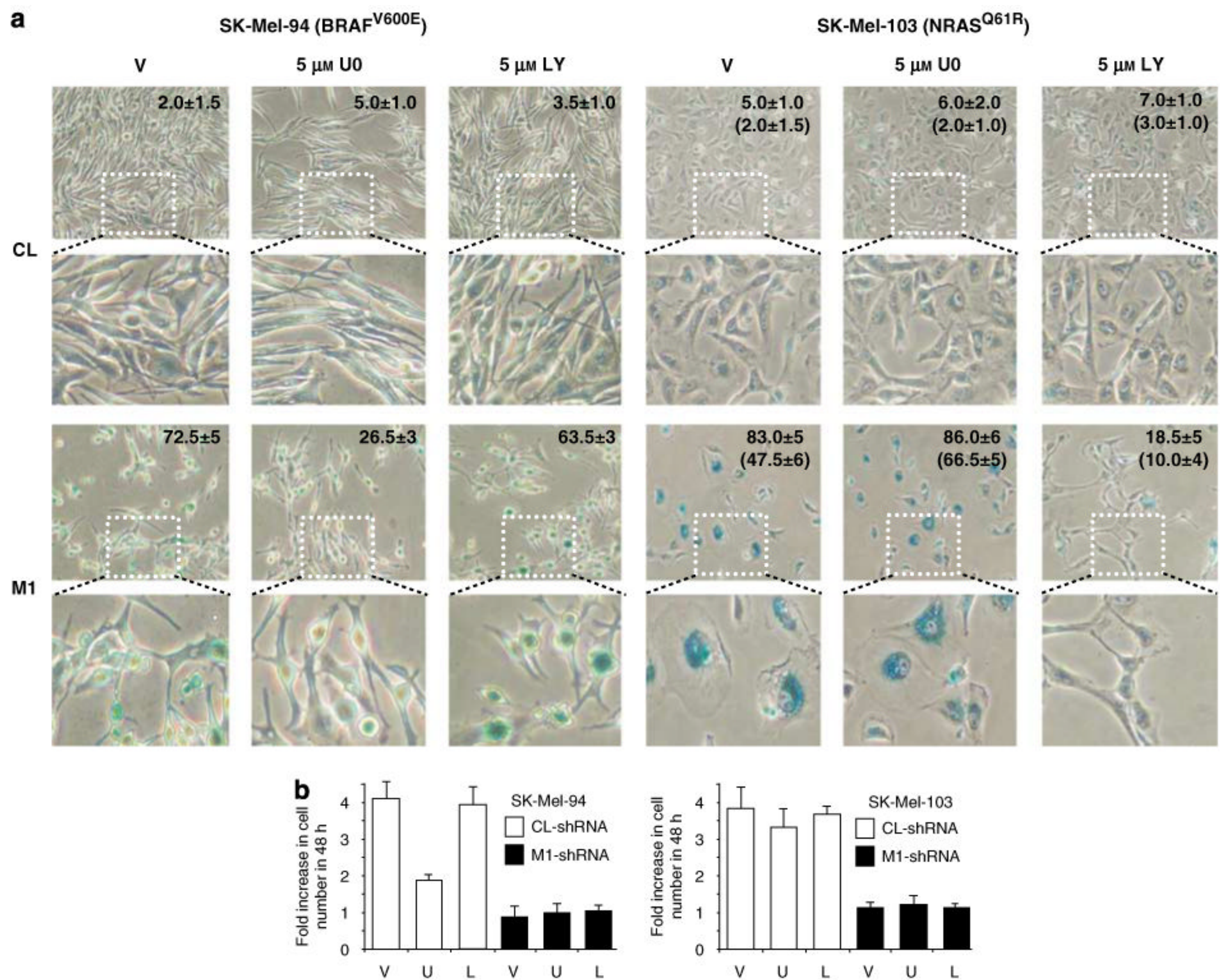

Figure 3.

Pharmacological inhibition of phosphoinositide 3-kinase or MEK suppresses several senescence phenotypes induced by C-MYC depletion in melanoma cells. (**a**) SK-Mel-94 or -103 cells infected with control (CL) or MYC-shRNA-1 (M1) were incubated with vehicle (dimethylsulfoxide) (V), 5 μM U0216 (U0) or 5 μM LY294002 (LY) for 48 h starting at day 4 after infection with lentiviral vectors containing the corresponding shRNAs. Cells were fixed and stained for SA-β-Gal activity as described in Materials and methods. Areas within the white dotted boxes are shown at higher magnification. Numbers outside and within parentheses indicate a percentage of SA-β-Gal-positive cells and vacuolized cells, respectively, and were derived from counting 100 cells in duplicate plates. (**b**) SK-Mel-94 and -103 cells infected and treated as in (**a**) and were counted in duplicates before addition of the drugs and after completion of incubation with the drugs. Fold-increase in cell number was obtained by dividing the number of cells after incubation by the number of cells before the addition of the drugs.

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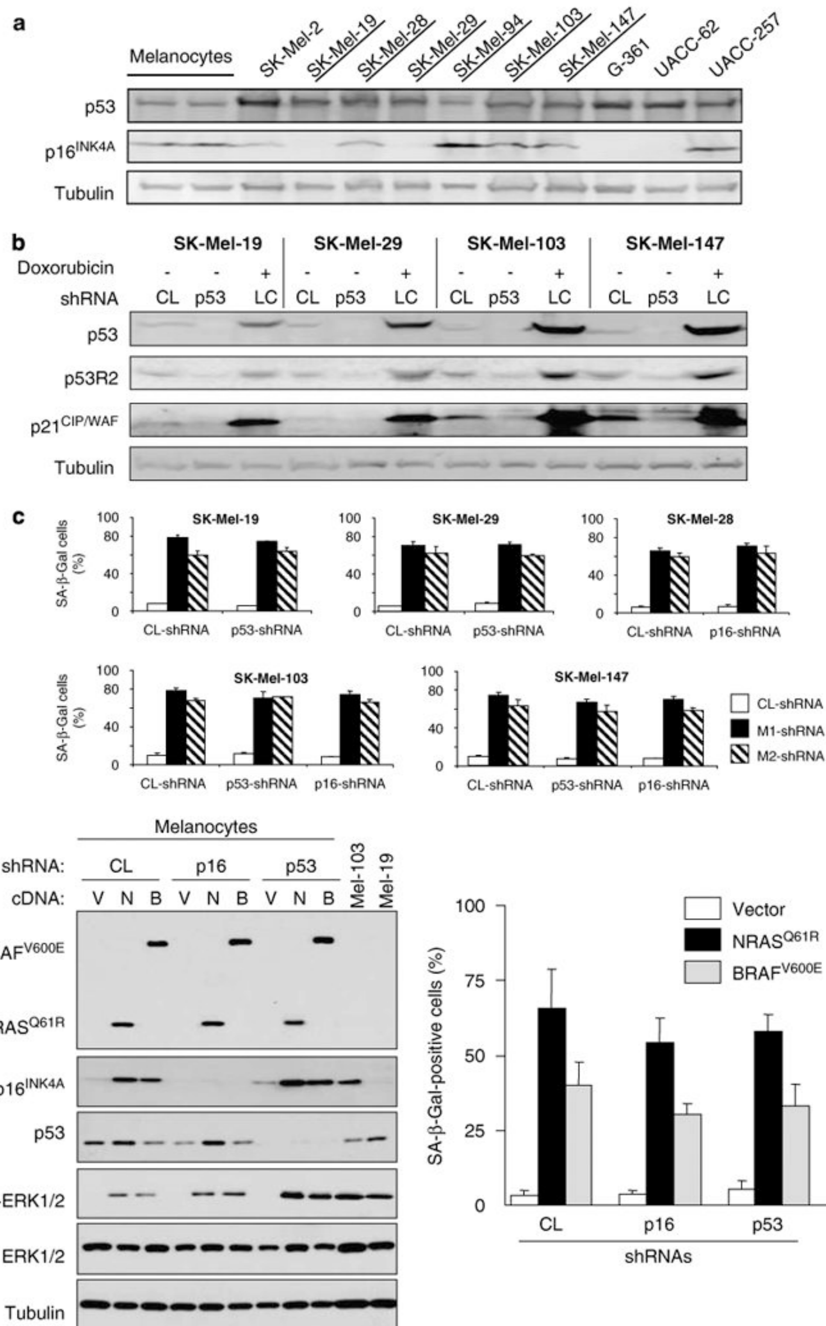

Figure 4.

Inhibition of p53 or p16^{INK4A} does not affect senescence in normal melanocytes and melanoma cells. (**a**) Cells from the designated cell lines were harvested in SDS containing buffer and total cellular extracts were resolved on 4–20% gradient polyacrylamide gel and probed in western blotting with antibodies designated on the left. (**b**) Cells from designated melanoma lines were infected with control lentiviral vector (CL) or with lentiviral vector expressing p53-shRNA. Two days after infection, control cells were incubated with vehicle (dimethylsulfoxide) or 0.3 µg/ml of doxorubicin for 16 h. After incubation, control and untreated p53-shRNA-expressing cells were harvested in SDS containing buffer and total

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cellular extracts were resolved on 4–20% gradient polyacrylamide gel and probed in western blotting with antibodies designated on the left. (**c**) Indicated melanoma cells expressing control (CL), p53- (p53) or p16^{INK4A}- (p16) specific shRNAs were super-infected with control shRNA (CL) or MYC-shRNAs (M1 or M2) 4 days after the primary infection. Cells were fixed and stained for senescence-associated β-galactosidase (SA-β-Gal) activity 6 days after the secondary infection. Numbers on the graphs indicate a percentage of SA-β-Gal-positive cells and were derived from counting 100 cells in duplicate plates. Open boxes, black boxes and crossed boxes correspond to cells infected with control shRNA (CL), MYC-shRNA-1 (M1) and MYC-shRNA-2 (M2), respectively. (**d**) Normal melanocytes were infected with lentiviruses containing shRNAs specific for p53 or p16^{INK4A}. At day 4 after infection, cells were super-infected with control lentiviruses (V) or lentiviruses expressing BRAF^{V600E} (B) or NRAS^{Q61R} (N). Ten days after the secondary infection, cells were harvested and total cellular extracts were resolved on 4–20% gradient polyacrylamide gel and probed in western blotting with antibodies designated on the left. Indicated cells were allowed to propagate for 10 days before fixation and staining for SA-β-Gal activity. Data correspond to the percentage of SA-β-Gal-positive cells determined for each cell population in duplicates. shRNA, short hairpin RNA.

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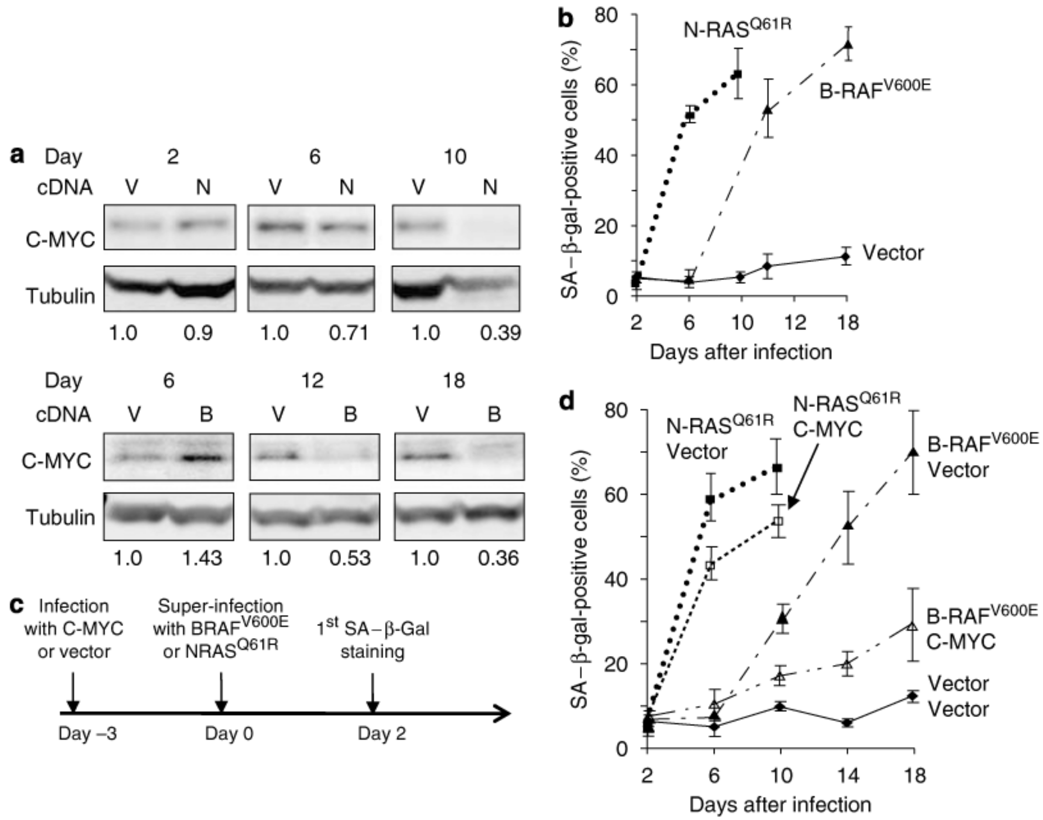

Figure 5.

C-MYC overexpression suppresses senescence in normal melanocytes overexpressing BRAF^{V600E} or NRAS^{Q61R}. (**a**) Normal melanocytes infected with control lentiviral vector (V) or vectors expressing BRAF^{V600E} (B) or NRAS^{Q61R} (N) cDNAs were collected at the designated time points after infection and total cellular extracts from these cells were probed in western blotting with antibodies designated on the left. (**b**) Cells described in (**a**) were stained for senescence-associated β-galactosidase (SA-β-Gal) activity at the designated time points and the percentage of SA-β-Gal-positive cells was plotted (100 cells were counted in duplicate plates). (**c**) Schematic of primary and secondary infections. At day −3, melanocytes were infected with control lentiviral vector or vector expressing *C-MYC* cDNA. At day 0, the above populations were super-infected with control lentiviral vector or vector expressing BRAF^{V600E} or NRAS^{Q61R} cDNAs. Cells were fixed and stained for SA-β-Gal activity at day 2, and every 4 days thereafter for a total of 18 days. (**d**) Cells were infected according to the schematic in (**c**). Populations generated are indicated on the right of the graph: cells were stained for SA-β-Gal activity at the designated time points and the percentage of SA-β-Gal-positive cells was plotted (100 cells were counted in duplicate slides).