Cancer Research

Activation of Forkhead Box O Transcription Factors by Oncogenic BRAF Promotes p21^{cip1}-Dependent Senescence

Peter L.J. de Keizer[1], Leisl M. Packer[3], Anna A. Szypowska[1], Paulien E. Riedl-Polderman[1], Niels J.F. van den Broek[1], Alain de Bruin[2], Tobias B. Dansen[1], Richard Marais[3], Arjan B. Brenkman[1], and Boudewijn M.T. Burgering[1]

Abstract

Oncogene-induced senescence (OIS) is a potent tumor-suppressive mechanism that is thought to come at the cost of aging. The Forkhead box O (FOXO) transcription factors are regulators of life span and tumor suppression. However, whether and how FOXOs function in OIS have been unclear. Here, we show a role for FOXO4 in mediating senescence by the human BRAF^{V600E} oncogene, which arises commonly in melanoma. BRAF^{V600E} signaling through mitogen-activated protein kinase/extracellular signal-regulated kinase kinase resulted in increased reactive oxygen species levels and c-Jun NH_2 terminal kinase–mediated activation of FOXO4 via its phosphorylation on Thr^{223}, Ser^{226}, Thr^{447}, and Thr^{451}. BRAF^{V600E}-induced FOXO4 phosphorylation resulted in p21^{cip1}-mediated cell senescence independent of p16^{ink4a} or p27^{kip1}. Importantly, melanocyte-specific activation of BRAF^{V600E} *in vivo* resulted in the formation of skin nevi expressing Thr^{223}/Ser^{226}-phosphorylated FOXO4 and elevated p21^{cip1}. Together, these findings support a model in which FOXOs mediate a trade-off between cancer and aging. *Cancer Res; 70(21); 8526–36. ©2010 AACR.*

Introduction

Activating mutations in the Ser/Thr kinase BRAF are observed in ~7% of all human tumors with high occurrence in thyroid carcinoma, colorectal cancer, ovarian cancer (1), and especially melanoma (~70%; ref. 2). The predominant BRAF mutation present in these cases is a substitution of Val600 for Glu (BRAF^{V600E}), which causes increased downstream signaling toward mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK; ref. 2). Although BRAF-activating mutations initially stimulate proliferation, cell cycle progression is ultimately arrested through induction of senescence (3–5). Oncogene-induced senescence (OIS) can be facilitated through the individual activities of p16^{ink4a} and p21^{cip1} (6, 7), and also in case of BRAF^{V600E}, these cell cycle inhibitors are thought to regulate senescence (4, 8, 9).

Reactive oxygen species (ROS) propagate cellular signaling induced by growth factors and thereby regulate a variety of cellular processes including proliferation (10, 11). However, when ROS levels increase above a certain threshold, sometimes called oxidative stress, ROS react with and damage the cellular interior. Additionally, excessive ROS can induce cellular senescence (12), and as such, they are considered to accelerate aging and age-related pathologies (13, 14). ROS are known to signal to a plethora of downstream targets, and it is currently elusive which of these regulate the induction of senescence.

Forkhead box O (FOXO) transcription factors are the mammalian orthologues of the *Caenorhabditis elegans* protein DAF-16, which functions as an important determinant of life span (15). FOXOs were originally identified as downstream components of insulin/insulin-like growth factor signaling through phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB/AKT; refs. 16, 17). In mice, FOXOs act as functionally redundant tumor suppressors (18), and in cell systems, FOXOs can either mediate apoptosis or quiescence in response to growth factor deprivation (19). In contrast to insulin signaling, which represses FOXO activity, cellular ROS can activate FOXOs (20, 21). Regulation of FOXOs by ROS occurs through numerous posttranslational modifications (22), rendering FOXOs sensors of cellular ROS (23). Consequently, FOXO activation increases resistance to oxidative stress through transcription of enzymes as MnSOD (24) and catalase (25) through a negative feedback loop. Increased FOXO activity is associated with longevity in model organisms (15)

Authors' Affiliations: [1]Department of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center Utrecht; [2]Department Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands; and [3]Signal Transduction Team, Cancer Research UK Centre of Cell and Molecular Biology, Institute of Cancer Research, London, United Kingdom

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A.B. Brenkman and B.M.T. Burgering contributed equally to this work.

Current address for P.L.J. de Keizer: Buck Institute for Age Research, Novato, CA.

Current address for N.J.F. van den Broek and A.B. Brenkman: Department of Metabolic and Endocrine Diseases and Netherlands Metabolomics Center, University Medical Center Utrecht, the Netherlands.

Corresponding Author: Boudewijn M.T. Burgering, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands. Phone: 31-88-7568918; Fax: 31-88-7568101; E-mail: b.m.t.burgering@umcutrecht.nl.

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and humans (26), which lends credit to the hypothesis that excessive ROS accelerate aging. Thus, FOXOs are regulated by ROS, play a role in both tumor suppression and aging, and thereby provide an important paradigm to understanding the relation between aging and disease such as cancer.

Materials and Methods

Additional information is available in Supplementary Materials and Methods.

Antibodies

The antibodies against FOXO4 (834), HA (12CA5), phosphorylated Thr^{447}, and phosphorylated Thr^{451} have been described before (21, 27). The following antibodies were purchased: phosphorylated Thr^{183}/Tyr^{185}–c-Jun NH_2 terminal kinase (JNK) and phosphorylated Thr^{202}/Tyr^{204}-ERK (Cell Signaling); Thr^{28}–phosphorylated FOXO4 (Upstate); MnSOD (Stressgen); trimethyl-H3K9 and FOXO3a (Upstate); p27^{kip1} and p21^{cip1} (BD Pharmingen); p16^{ink4a} (ab-2; Neomarkers); p21^{cip1} (M19), BRAF (C19), FOXO4 (N19), FOXO1 (N18), proliferating cell nuclear antigen (PCNA; PC10), and p53 (DO-1; Santa Cruz); and tubulin (Sigma). Antibodies against phosphorylated Thr^{223} and phosphorylated Thr^{223}/Ser^{226} were generated by immunizing rabbits with the keyhole limpet hemocyanin–conjugated peptides CKAPKKKPSVLPAPPEGA-pT-PTSPVG and CKAPKKKPSVLPAPPEGA-pT-PT-pS-PVG, respectively, wherein pT and pS present phosphorylated threonine and serine. Produced antibodies were subjected to positive and negative affinity purification according to manufacturer's protocol (Covance).

Constructs and RNA interference

The following constructs have been described before: pbabe-puro, pMT2-HA-FOXO4, and pRP261-GST-FOXO4-ΔDB (16); 6× FOXO DNA-binding element (DBE)–firefly luciferase, MnSOD-firefly luciferase, and TK-*Renilla* luciferase (24); pEFm-BRAF^{V600E} (2); and p21^{cip1}-luciferase (28). pSuper-p21^{cip1} was a kind gift from Mathijs Voorhoeve (29). A detailed explanation on the generation of HA-FOXO4-4A/E and pSuperior-shFOXO1/3 and 4 is available in Supplementary Materials and Methods. Smartpool oligos against FOXO1, 3a, and 4; BRAF; or scrambled oligos (Dharmacon) were transfected at a final concentration of 100 nmol/L each (300 nmol/L for scrambled) using oligofectamine according to the manufacturer's protocol (Invitrogen).

Immunofluorescence, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining, and BrdUrd incorporation

Immunofluorescence was performed as described (27) using antisera against FOXO4 (834 and monoclonal antibody), HA (12CA5), PCNA, H3K9-Me(III), and pT^{223}/S^{226}. BrdUrd incorporation and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining were performed according to the manufacturer's protocols (Roche). For the mouse sections, anti-p21^{cip1} M19 was used.

Cellular ROS measurements with H_2DCFDA

HEK293T cells were transfected with pcDNA3 or a plasmid encoding BRAF^{V600E} (2 μg), in parallel with pbabe-puro (500 ng). At 16 hours posttransfection, cells were selected with 2 μg/mL puromycin for 36 hours and subsequently left untreated or pretreated for 24 hours with 4 mmol/L *N*-acetyl cysteine (NAC) or 10 μmol/L U0126, washed with PBS, and incubated for 10 minutes with 1 mL 10 μmol/L H_2DCFDA (Invitrogen). Following recovery for 4 hours in medium with or without NAC or U0126, cells were pretreated with or without 200 μmol/L H_2O_2 for 45 minutes and collected by trypsinization. Centrifuged cells were incubated with 0.02 mg/mL propidium iodide (PI), and live cells were analyzed by FACS for DCF fluorescence. CHL and WM266.4 cells were treated similarly, but without puromycin and PI selection.

Colony formation assay and senescence-associated β-galactosidase staining

A14 or U2OS cells were transfected as indicated together with pbabe-puro (500 ng). At 24 hours posttransfection, cells were subjected to puromycin selection (2 μg/mL). Following 2.5 days of selection, one set of cells was lysed and analyzed by immunoblotting for protein expression. At 10 days posttransfection, cells were fixed in methanol and stained with 0.5% crystal violet in 25% methanol. Plates were dried, and colony formation was quantified by destaining in 10% acetic acid and measuring absorbance at 560 nm. CHL, PMWK, Colo829, and A375 cells were treated similarly but transfected with 500 ng FOXO4 and 250 ng pbabe-puro. Senescence-associated β-galactosidase (SA-β-GAL) staining was performed 9 days posttransfection as described (30).

Results

Ectopic introduction of FOXO4 induces cellular senescence in BRAF^{V600E}-expressing Colo829, A375, and SK-mel28 melanoma cells

To study the involvement of FOXOs in BRAF^{V600E}-dependent cellular responses, we ectopically expressed FOXO4 in the human melanoma-derived cell line, Colo829, harboring an endogenous BRAF^{V600E} mutation. This resulted in reduced colony formation along with diminished PCNA and BrdUrd positivity (Fig. 1A) but without significant TUNEL staining (Supplementary Fig. S1).

FOXOs repress oxidative stress (21), and increased oxidative stress is suggested to cause cellular senescence (12). Surprisingly, however, ectopic FOXO4 expression rendered Colo829 cells positive for SA-β-GAL activity (Fig. 1B). Also detection of two other independent markers of senescence (4, 31), senescence-associated heterochromatin foci (SAHF) and H3K9-trimethylation, was significantly enhanced by FOXO4 (Fig. 1C), suggesting that this indeed is a senescence response.

To exclude artifacts of a single cell type, we also expressed FOXO4 in other melanoma cell lines that express endogenous BRAF^{V600E}, A375, and SK-Mel28, or wild-type BRAF, CHL, and PMWK. Whereas FOXO4 induced SA-β-GAL expression in A375 and SK-Mel28, no positivity was observed in CHL or PMWK cells (Supplementary Fig. S2 and data not shown).

Thus, in endogenous BRAF^{V600E}-expressing Colo829, A375, and SK-Mel28 melanoma cells, expression of FOXO4 induces a growth arrest through cellular senescence.

BRAF^{V600E} induces phosphorylation of FOXO4 on JNK target sites

The MEK-ERK pathway is a primary signaling output for normal and oncogenic BRAF. In addition to MEK-ERK signaling, BRAF^{V600E} expression is reported to promote activation of JNK (32) which we confirmed (Supplementary Fig. S3). Previously, we showed that FOXO4 is a JNK target and identified Thr^{447} and Thr^{451} through mutation analysis as a subset of the phosphorylated acceptor sites (21). We therefore wondered whether BRAF^{V600E} could signal through JNK toward FOXO4 to promote senescence. To fully address this question, we first determined all possible JNK sites of *in vitro* phosphorylated FOXO4 by liquid chromatography–tandem mass spectrometry analysis (Supplementary Data). In addition to the previously characterized Thr^{447} and Thr^{451}, this revealed two novel residues, Thr^{223} and Ser^{226} (Supplementary Fig. S4). We generated phosphospecific antisera against these sites, including dually phosphorylated Thr^{223}/Ser^{226}. *In vitro*

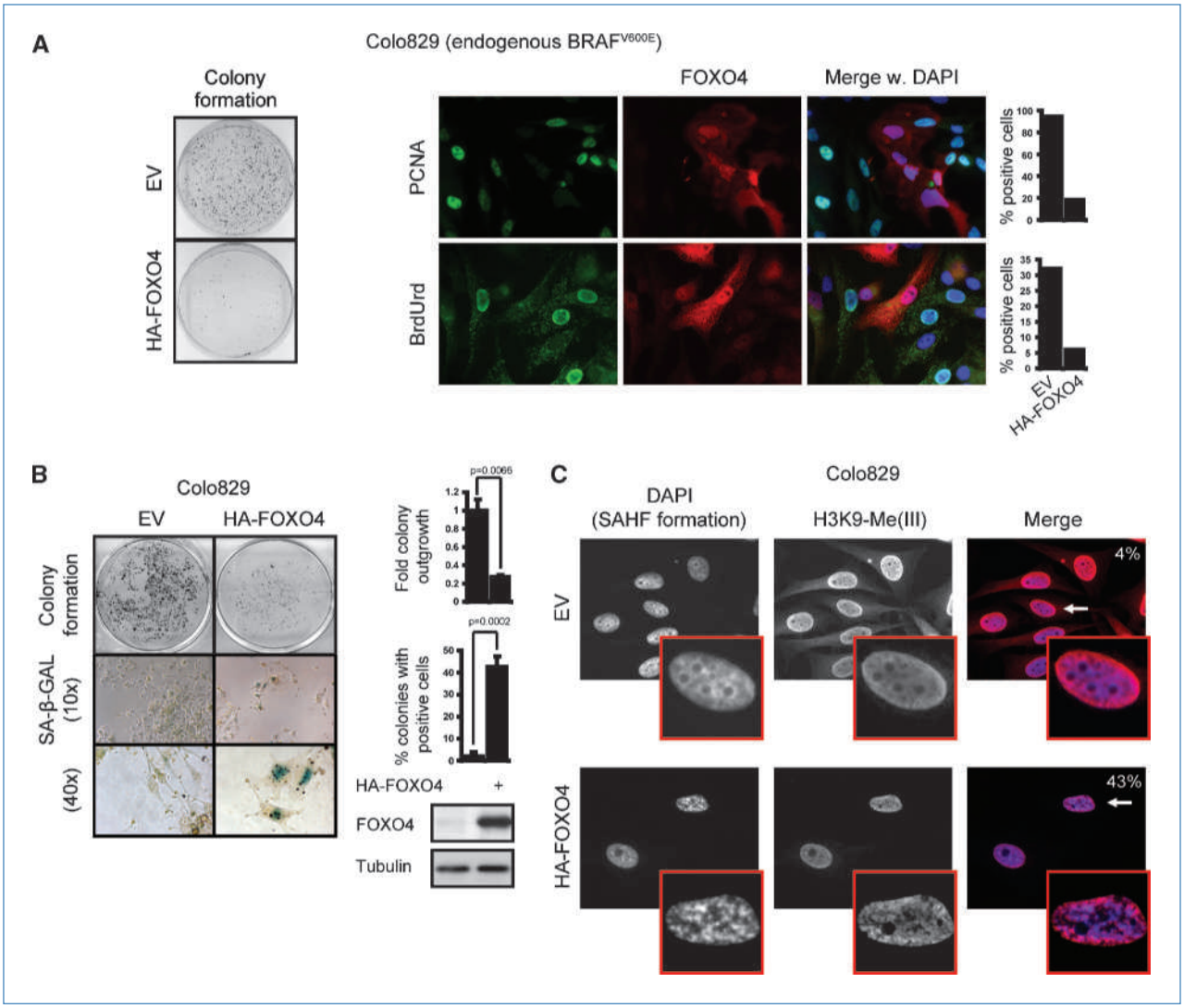

Figure 1. FOXO4 induces cellular senescence in endogenous BRAF^{V600E}-expressing Colo829 and A375 cells. A, ectopic FOXO4 expression reduces proliferation of Colo829 cells. Colo829 cells transiently expressing HA-FOXO4 were subcultured in puromycin containing selection medium and stained for colony outgrowth. Additionally, a set of cells were stained at 2.5 d posttransfection with anti-PCNA or analyzed for BrdUrd incorporation. Two hundred fifty nontransfected and 50 transfected cells were quantified. Similar results were obtained in A375 melanoma cells. EV, empty vector. B, ectopic FOXO4 expression induces SA-β-GAL positivity in Colo829 cells. Colo829 cells expressing HA-FOXO4 were selected with puromycin and stained for colony formation or SA-β-GAL. Protein samples were obtained at 2.5 d posttransfection and analyzed by immunoblotting. Fifty colonies were quantified for positive cells. C, FOXO4 expression in Colo829 cells induces senescence. Colo829 cells were transfected as in A and at 5.5 d posttransfection stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize SAHF formation in parallel with anti-H3K9-Me(III) for H3K9-trimethylation. One hundred cells were quantified, and the percentage of double-positive cells was indicated.

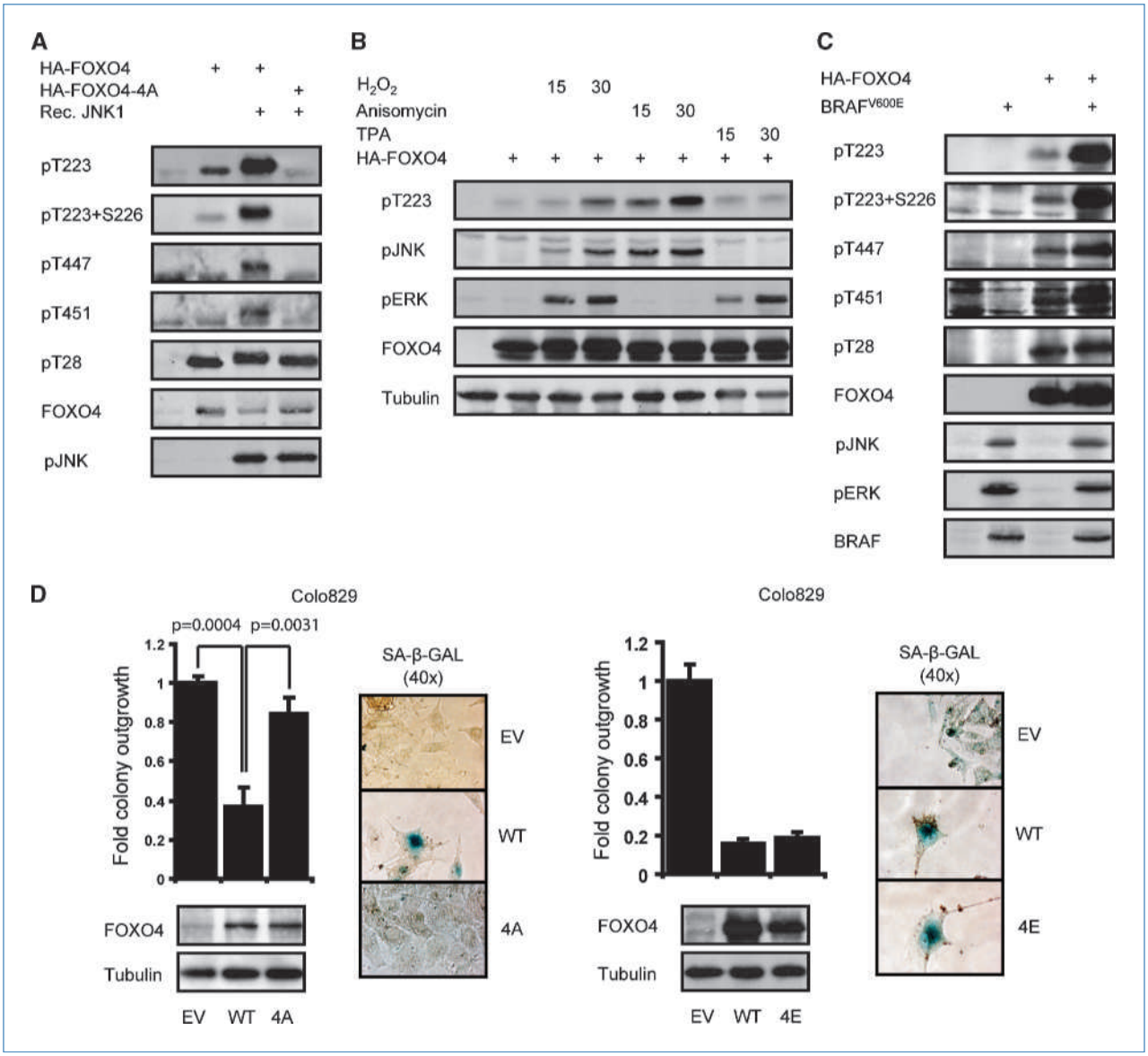

Figure 2. FOXO4 is a downstream target of BRAF^{V600E} through JNK-mediated phosphorylation. A, Thr^{223}, Ser^{226}, Thr^{447}, and Thr^{451} of FOXO4 are JNK sites *in vitro*. Phosphorylation status of immunoprecipitated HA-FOXO4 or HA-FOXO4-4A isolated from HEK293T cells was determined on *in vitro* phosphorylation by recombinant JNK1. B, phosphorylation of FOXO4 on Thr^{223} correlates with activation of JNK, not ERK. HEK293T cells transiently expressing HA-FOXO4 were treated with 200 μmol/L H_2O_2, 10 μg/mL anisomycin, or 100 ng/mL TPA and analyzed for activation of ERK and JNK as well as FOXO4 phosphorylation on Thr^{223}. C, BRAF^{V600E} induces phosphorylation of FOXO4 on JNK sites. The phosphorylation of ectopically expressed FOXO4 in HEK293T cells was determined in the presence or absence of BRAF^{V600E}. D, mutation of the JNK sites to Ala, but not Glu, impairs senescence induction by FOXO4 in Colo829 cells. Colo829 cells were transfected with HA-FOXO4, HA-FOXO4-4A, or HA-FOXO4-4E in which the JNK target sites Thr^{223}, Ser^{226}, Thr^{447}, and Thr^{451} are mutated to Ala (left) or Glu (right), respectively. Colony formation and SA-β-GAL assays were performed as in Fig. 1B.

phosphorylation by JNK significantly increased detection of wild-type FOXO4 by these respective antisera, especially the newly discovered Thr^{223} and Ser^{226}, whereas FOXO4-4A in which these residues are mutated to Ala (Fig. 2A) was not detected. This indicates that Thr^{223}, Ser^{226}, Thr^{447}, and Thr^{451} are JNK-phosphorylated acceptor sites.

Because BRAF^{V600E} signaling induces activation of both ERK and JNK, we next determined whether phosphorylation of the identified sites in cultured cells is mediated by either of these kinases. H_2O_2, which activates both, indeed resulted in the phosphorylation of Thr^{223} of FOXO4. Additionally, stimuli that exclusively activate either ERK [12-*O*-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor] or JNK (anisomycin) showed that Thr^{223} phosphorylation correlates with activation of JNK, not ERK (Fig. 2B; Supplementary Fig. S5).

In agreement with JNK activation, BRAF^{V600E} induced a significant increase in phosphorylation on all JNK sites, but

not on the PKB/AKT site Thr^{28} (Fig. 2C). Furthermore, treatment of cells with the JNK inhibitor SP600125 not only inhibited $BRAF^{V600E}$-induced JNK autophosphorylation in a dose-dependent manner, but also Thr^{223} phosphorylation of FOXO4 (Supplementary Fig. S6). Together, these results indicate that $BRAF^{V600E}$ promotes JNK-mediated phosphorylation of FOXO4.

To address whether phosphorylation of FOXO4 on the JNK sites is required for FOXO4 to be able to induce senescence in $BRAF^{V600E}$-expressing melanoma cells, we expressed the FOXO4-4A mutant next to wild-type FOXO4. FOXO4-4A neither significantly repressed colony formation nor induced SA-β-GAL positivity (Fig. 2D). In contrast, a mutant of FOXO4 that mimics phosphorylation on JNK sites, FOXO4-4E, induced a senescence response similar to wild-type FOXO4 (Fig. 2D). Altogether, these data indicate that FOXO4 is a downstream target of $BRAF^{V600E}$ through JNK-mediated phosphorylation and that phosphorylation on the JNK target sites is required for FOXO4 to promote senescence in response to $BRAF^{V600E}$.

$BRAF^{V600E}$ signaling elevates cellular ROS levels, which promote FOXO4 phosphorylation by JNK

JNK activity is regulated through a large variety of signaling pathways, and we therefore next addressed the molecular mechanism through which $BRAF^{V600E}$ regulates JNK and thereby FOXO4 activity. Elevations in cellular ROS generated through H_2O_2 treatment of cells can directly invoke senescence (12), and senescence induction in, for instance, melanocytes has recently been correlated with increased ROS (33). Moreover, OIS can be bypassed by ROS scavenging compounds such as NAC (34, 35). Hence, we investigated the possibility that $BRAF^{V600E}$ signaling affects cellular ROS levels by loading cells with the ROS detecting probe H_2DCFDA (DCF). $BRAF^{V600E}$ expression significantly increased cellular ROS levels as detected by DCF fluorescence (Fig. 3A). The $BRAF^{V600E}$-induced increase in cellular ROS could be further increased by treatment with H_2O_2 (45 minutes, 200 μmol/L) but was impaired on preincubation with NAC. Downstream signaling through MEK seems at least partially required, because preincubation with the MEK inhibitor U0126 reduced DCF fluorescence. These data indicate that ectopic $BRAF^{V600E}$ expression leads to the generation of cellular ROS through downstream MEK signaling. In agreement herewith, melanoma cells expressing $BRAF^{V600E}$ showed higher basal ROS levels compared with wild-type BRAF-expressing cells (Supplementary Fig. S7). Elevations in ROS are sufficient for phosphorylation of FOXO4 by JNK, as treatment of cells with H_2O_2 resulted in a time-dependent increase of both JNK activation and Thr^{223} phosphorylation (Supplementary Fig. S8; Fig. 2B). Moreover, $BRAF^{V600E}$-mediated JNK activation and FOXO4 phosphorylation were repressed on pretreatment of cells with NAC or U0126 (Fig. 3B).

Prolonged treatment with U0126 induces apoptosis in Colo829 cells (36), making it impossible to interpret the effect of this inhibitor on FOXO4-induced senescence. Therefore, Colo829 cells were treated with NAC to reduce cellular ROS. This resulted in reduced colony formation of Colo829 cells (Fig. 3C), most likely due to the fact that proliferation per se requires low amounts of ROS (14). Importantly, however, NAC impaired the ability of FOXO4 to induce senescence in these cells. Altogether, these data point to a pathway in which $BRAF^{V600E}$ induces FOXO4 phosphorylation by JNK through a MEK-regulated elevation of intracellular ROS, and in line with that, ROS are essential for FOXO4 to induce senescence in the presence of $BRAF^{V600E}$.

$p21^{cip1}$ mediates the cell cycle arrest and senescence response by $BRAF^{V600E}$-FOXO4 signaling

Next, we addressed the mechanism downstream of how FOXO4 promotes $BRAF^{V600E}$-induced senescence. $p27^{kip1}$ is an important mediator of FOXO-induced G_1 arrest and subsequent quiescence response in the absence of growth factors (19). Therefore, we reasoned a role for $p27^{kip1}$. FOXO4-induced $p27^{kip1}$ expression, however, was counteracted rather than enhanced by coexpression of $BRAF^{V600E}$ (Fig. 4A; data not shown). Thus, we conclude that the FOXO4-mediated cell cycle arrest, in response to $BRAF^{V600E}$ signaling, is unlikely to be regulated through $p27^{kip1}$.

Next, we addressed the importance of another cyclin-dependent kinase (CDK) inhibitor, $p16^{ink4a}$, which has been implicated in senescence. $p16^{ink4a}$ levels do not seem to increase on FOXO4 and $BRAF^{V600E}$ coexpression (Fig. 4A; data not shown). Also, in Colo829 cells in which FOXO4 induces senescence (Fig. 1), a premature stop mutation is present in the *CDKN2A* gene resulting in loss of $p16^{ink4a}$ expression (37). These data also argue against involvement of $p16^{ink4a}$ in FOXO4-mediated OIS driven by $BRAF^{V600E}$.

Because $p21^{cip1}$ and $p16^{ink4a}$ seem functionally redundant in OIS (6, 7, 9), we next analyzed a role for $p21^{cip1}$. Interestingly, $BRAF^{V600E}$ cooperated with FOXO4 to induce $p21^{cip1}$ expression (Fig. 4A), and in correlation with the induction of senescence, FOXO4 expression increased $p21^{cip1}$ expression in Colo829 cells (Supplementary Fig. S9). Similar effects were observed on $p21^{cip1}$ mRNA expression determined by quantitative real-time PCR. Moreover, $BRAF^{V600E}$ and FOXO4 expression resulted in a synergistic activation of a luciferase-reporter gene driven by the $p21^{cip1}$ promoter (Fig. 4B). This level of synergy was also observed using a construct under a different FOXO-responsive promoter (i.e., MnSOD) and a synthetic promoter encompassing six optimal FOXO DBEs (6× DBE; Supplementary Fig. S10), suggesting that the cooperative induction indeed reflects increased FOXO activity.

As HA-FOXO4-4A did not induce senescence in Colo829 cells, whereas HA-FOXO4-4E did, we also determined the ability of these mutants to induce $p21^{cip1}$ transcription. In line with the lack of senescence induction, HA-FOXO4-4A, but not HA-FOXO4-4E, was significantly less capable of driving $p21^{cip1}$ transcription (Fig. 4C). These data indicate that $BRAF^{V600E}$ activates FOXO4 through JNK-mediated phosphorylation to promote $p21^{cip1}$ transcription, which in Colo829 cells correlates with the induction of senescence.

To address to what extent $p21^{cip1}$ is required for the FOXO4-induced cell cycle arrest and senescence in response to $BRAF^{V600E}$ signaling, we used short hairpin RNA (shRNA)–mediated knockdown of $p21^{cip1}$. This impaired $p21^{cip1}$

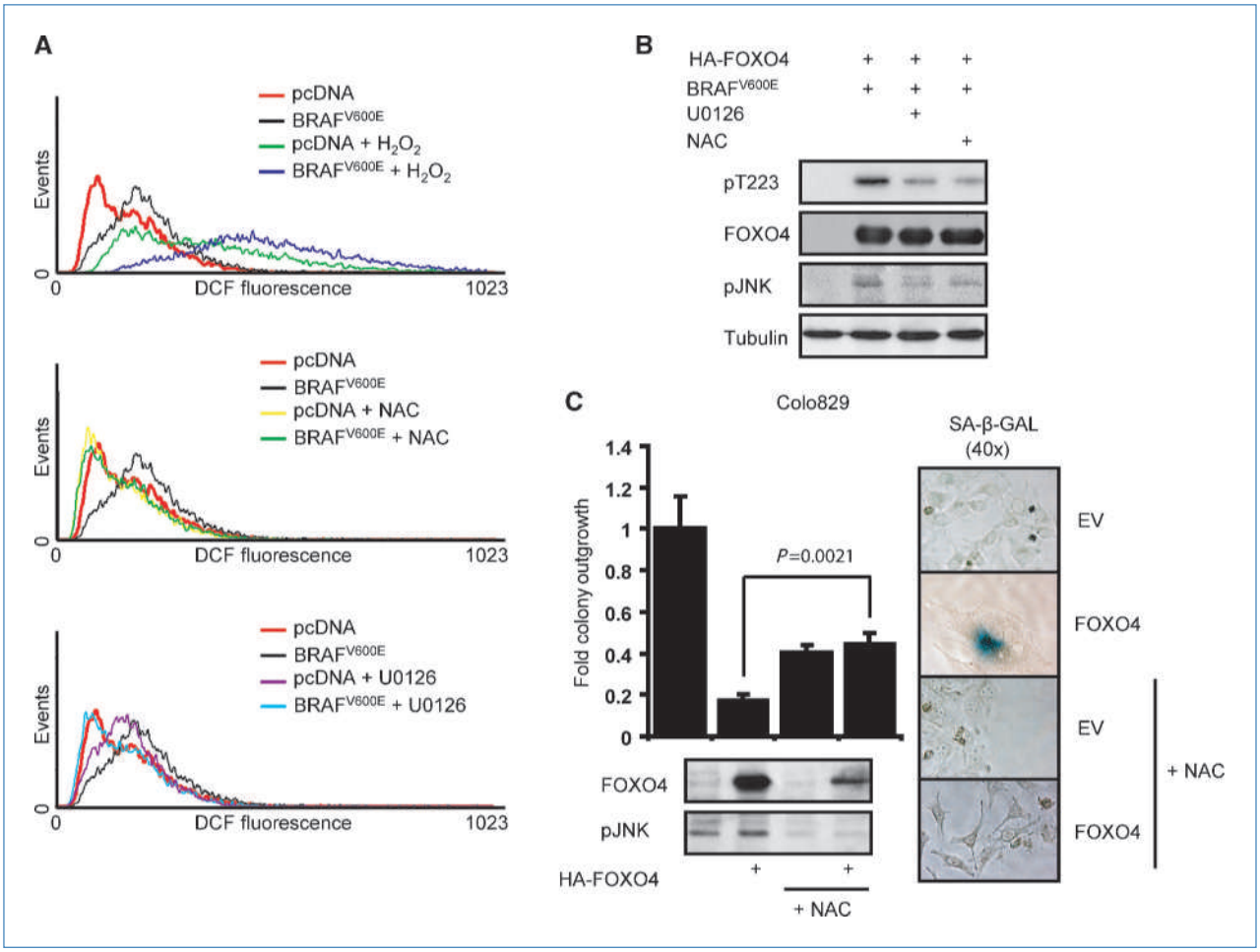

Figure 3. MEK-dependent BRAF^{V600E} signaling elevates cellular ROS levels, which stimulate Thr^{223} phosphorylation of FOXO4 by JNK. A, BRAF^{V600E} expression increases cellular ROS. BRAF^{V600E}-expressing HEK293T cells were treated 24 h with 4 mmol/L NAC and 20 μmol/L U0126 or 45 min with 200 μmol/L H_2O_2 and analyzed for DCF fluorescence. B, reduced MEK activity or cellular ROS inhibits BRAF^{V600E}-induced FOXO4 phosphorylation by JNK. Experiment as in Fig. 2C, but on pretreatment of 20 μmol/L U0126 or 4 mmol/L NAC for 24 h. C, interference with cellular ROS levels inhibits FOXO4-induced senescence. Colo829 cells were transfected as in Fig. 1B and treated at days 1.5 and 5.5 posttransfection with 1 mmol/L NAC and analyzed for colony formation and SA-β-GAL positivity.

expression induced by BRAF^{V600E} FOXO4 coexpression (Supplementary Fig. S11). Whereas BRAF^{V600E} and FOXO4 together induced a strong G_1 arrest as determined by FACS analysis, this effect was abolished on knockdown of p21^{cip1} (Fig. 4D). Because p21^{cip1} expression is elevated in FOXO4-induced senescence in Colo829 cells, we also addressed the effect of p21^{cip1} knockdown on the induction of senescence. Strikingly, FOXO4 expression did not induce SA-β-GAL staining in Colo829 cells on p21^{cip1} knockdown (Fig. 4D), indicating that p21^{cip1} is required in FOXO4-induced senescence in these cells. Altogether, these data show that FOXO4 is a downstream target of BRAF^{V600E} that can facilitate a cell cycle arrest and OIS through regulation of p21^{cip1}.

BRAF^{V600E} regulates p21^{cip1} expression through MEK and ROS-dependent phosphorylation of FOXOs

Following our observations that suggest BRAF^{V600E}-mediated JNK/FOXO4 activation runs through MEK-ROS signaling, we addressed the involvement of MEK and ROS in the regulation of p21^{cip1} and cell cycle arrest by BRAF^{V600E} and FOXO4. Pretreatment of cells with either NAC, to reduce ROS (Fig. 5A), or U0126, to inhibit MEK (Fig. 5B), repressed JNK activation by BRAF^{V600E}, phosphorylation of FOXO4 on the JNK target site Thr^{223}, and the cooperative induction of p21^{cip1}. Furthermore, whereas ectopic expression of FOXO4 in Colo829 cells significantly enhanced p21^{cip1} promoter activity, pretreatment of these cells with U0126 or NAC reduced this effect (Fig. 5C). This shows that JNK-mediated phosphorylation of FOXO4 and the concomitant activation of p21^{cip1} transcription are dependent on MEK activity and elevations in cellular ROS.

Next, we investigated the role of endogenous FOXOs in signaling from BRAF^{V600E} toward p21^{cip1} transcription. High ectopic expression of BRAF^{V600E} strongly induced p21^{cip1} promoter activity (ref. 8; Fig. 5D). This induction was abrogated on shRNA-mediated simultaneous depletion of endogenous FOXO1, 3a, and 4, whereas add-back of a FOXO4

mutant insensitive to shRNA-mediated knockdown (FOXO4-SM) was sufficient to rescue BRAF^{V600E}-induced transactivation of the p21^{cip1} promoter (Fig. 5D; Supplementary Fig. S12 and S13). Thus, endogenous FOXOs are essential for ectopic BRAF^{V600E} to induce p21^{cip1} transcription.

Endogenous BRAF^{V600E} regulates FOXO4 phosphorylation and p21^{cip1} expression in cultured melanoma cells and *in vivo*

To further investigate the endogenous regulation of FOXO4 by oncogenic BRAF, we used a distinct human melanoma-derived cell line WM266.4 (BRAF^{V600D}; Fig. 6A). WM266.4 cells are tumorigenic yet express very high levels of p21^{cip1}. This, we reasoned, made them suitable to investigate the entire endogenous signaling cascade from oncogenic BRAF toward p21^{cip1}. Like Colo829 and in agreement with hyperactive BRAF signaling, WM266.4 cells expressed a significant amount of active ERK and JNK. As for Colo829 cells, expression of p16^{ink4a} was not detectable in this cell line (Fig. 6A; ref. 38). Small interfering RNA (siRNA)–mediated knockdown of BRAF in WM226.4 cells reduced ERK and JNK activity and, importantly, resulted in diminished p21^{cip1} expression (Supplementary Fig. S14), arguing that the high p21^{cip1} level in WM266.4 is indeed driven by the oncogenic BRAF. Treatment of WM266.4 cells with U0126 inhibited MEK activity and subsequent JNK activation, indicating that, indeed also in these cells, MEK signaling is essential for JNK activation by oncogenic BRAF (Fig. 6B). Interestingly, next to impaired p21^{cip1} expression, the U0126-mediated repression of JNK reduced phosphorylation of endogenous FOXO4 on the JNK sites Thr^{223} + Ser^{226} and also siRNA-mediated knockdown of endogenous FOXOs reduced the p21^{cip1} expression (Fig. 6B). U0126 further enhanced this reduction, probably reflecting incomplete knockdown of FOXOs by these siRNAs. Together, these experiments indicate that oncogenic BRAF can regulate p21^{cip1} expression through phosphorylation of endogenous FOXOs by JNK, confirming the results we obtained in our overexpression studies.

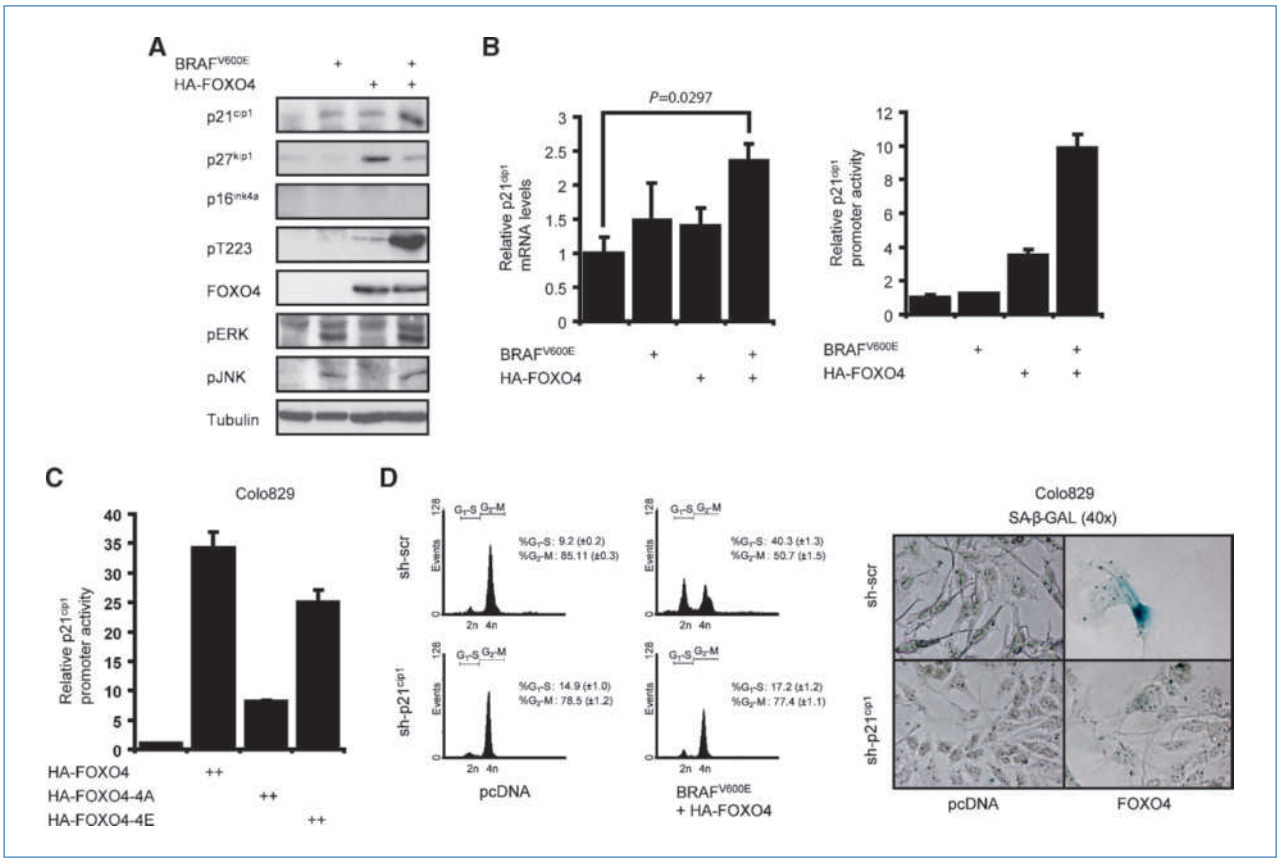

Figure 4. BRAF^{V600E}-FOXO4 signaling induces transcription of p21^{cip1}, not p27^{kip1} or p16^{ink4a}. A, BRAF^{V600E} and HA-FOXO4 coexpression results in increased p21^{cip1}. Total lysates of puromycin selected HEK293T cells expressing HA-FOXO4 and BRAF^{V600E} were analyzed by immunoblotting. B, BRAF^{V600E} and FOXO4 cooperatively promote p21^{cip1} transcription. Quantitative real-time PCR for p21^{cip1} mRNA in HEK293T (left) and p21^{cip1}-luciferase assay on A14 cell lysates (right), which transiently expressed HA-FOXO4 and BRAF^{V600E}. C, mutation of the JNK sites in FOXO4 affects the ability to transactivate p21^{cip1} transcription. p21^{cip1}-luciferase assay in Colo829 cells, using wild-type FOXO4, HA-FOXO4-4A, and HA-FOXO4-4E. D, p21^{cip1} is required for FOXO-mediated G$_1$ arrest and senescence response in a background of BRAF^{V600E} signaling. Left, U2OS cells (optimal for FOXO-mediated G$_1$ arrest; ref. 19) were transfected with BRAF^{V600E} and HA-FOXO4 in combination with a plasmid encoding a short hairpin against p21^{cip1} or a scrambled control. Right, SA-β-GAL staining after expression of HA-FOXO4 in combination with a plasmid encoding a scrambled or p21^{cip1} short hairpin in Colo829.

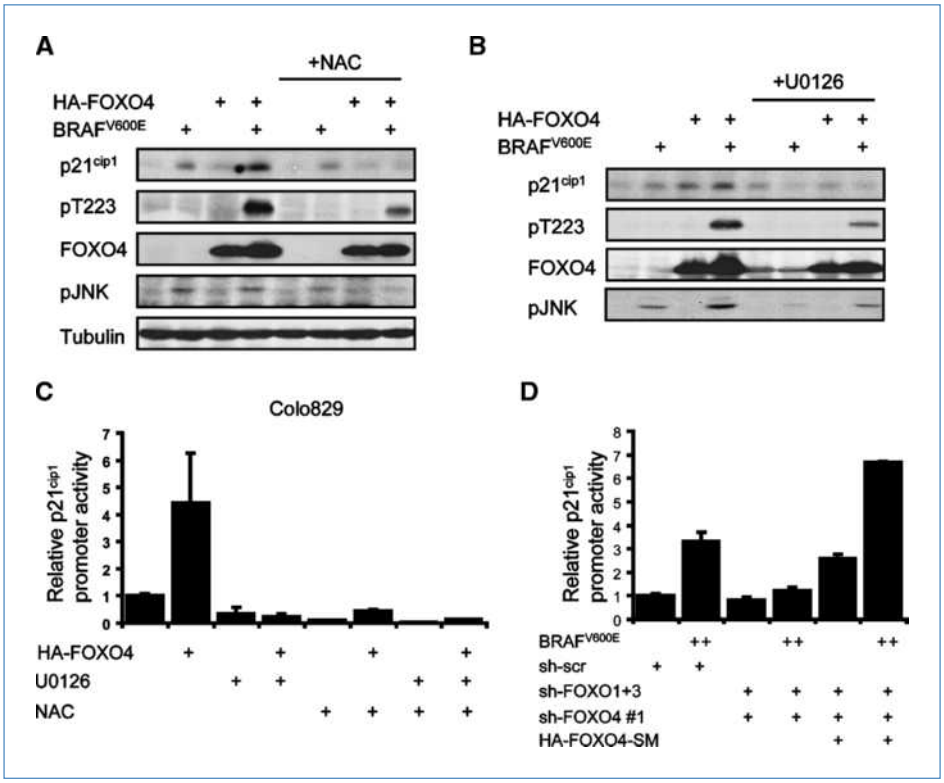

Figure 5. BRAF^{V600E} regulates p21^{cip1} expression through MEK-ROS-JNK signaling toward endogenous FOXO4. A, scavenging of cellular ROS represses JNK activation, Thr^{223}-FOXO4 phosphorylation, and subsequent p21^{cip1} expression. Lysates of puromycin selected, untreated, or NAC-treated (4 mmol/L, 24 h) HEK293T cells were analyzed by immunoblotting. Cells were transfected and treated as in Fig. 3A. B, interference with MEK signaling represses JNK activation, Thr^{223}-FOXO4 phosphorylation and subsequent p21^{cip1} expression. Experiment as in A, except with pretreatment for 24 h with the MEK inhibitor U0126 (20 μmol/L). C, FOXO4-induced p21^{cip1} transcription in Colo829 cells requires MEK activity and cellular ROS. p21^{cip1}-luciferase assay from lysates of Colo829 cells expressing HA-FOXO4 following 24 h pretreatment with 10 μmol/L U0126 or 4 mmol/L NAC. D, endogenous FOXOs mediate BRAF^{V600E}-induced p21^{cip1} transcription. A14 cells expressing BRAF^{V600E}, short hairpins against FOXO1 + 3a and FOXO4 or a scrambled sequence and a FOXO4 mutant, insensitive to its corresponding short hairpin (HA-FOXO4-SM), were subjected to a p21^{cip1}-luciferase assay. High levels of BRAF^{V600E} were transfected [2 μg (++) compared with 200 ng otherwise used throughout the study to force higher p21^{cip1} transcription].

Ultimately, to study the biological relevance of our observations *in vivo*, we used a Braf$^{+/LSL\text{-}V600E}$; Tyr::CreERT2$^{+/o}$ mouse model, which expresses BRAF^{V600E} in melanocytes off the endogenous *Braf* gene in a tamoxifen-inducible manner (39). As reported before, activation of BRAF^{V600E} signaling induced melanocytic nevi within the dermis, composed of nests of pigmented epitheloid cells intermingled with whorls of lightly pigmented and amelanotic spindle cells (Supplementary Fig. S15). At the periphery of these melanocytic nevi, we observed multiple patches of darkly pigmented, large polygonal cells, interpreted as neoplastic melanocytes. p21^{cip1} expression was significantly expressed within these neoplastic melanocytes at the periphery of the BRAF^{V600E}-induced nevi (Fig. 6C), and minor p21^{cip1} expression was detected in the less pigmented regions of the nevi and within epidermal layers. To investigate endogenous FOXO4 expression in the mouse skin, we developed novel monoclonal antisera. The antisera could immunostain ectopically expressed mouse HA-FOXO4 in Colo829 cells (Supplementary Fig. S16). When applied to the mouse skin sections, the antisera showed expression of endogenous FOXO4 in the mouse skin (Fig. 6C). To determine the phosphorylation status of FOXO4 on the JNK target sites in the BRAF^{V600E}-expressing skin samples, we used pT^{223}/S^{226} antisera. Detection with the pT^{223}/S^{226} antisera showed nuclear staining in unstimulated cells, including Colo829 (Supplementary Fig. S17; data not shown). Knockdown of endogenous FOXO4 reduced, although not abolished, the signal, showing the extent of specificity of this antisera for endogenous FOXO4. Importantly, endogenous Thr^{223}/Ser^{226} phosphorylation of FOXO4 was specifically enriched in the areas of the nevi that also showed p21^{cip1} staining (Fig. 6C). Thus, in line with the cell culture data, *in vivo* activation of oncogenic BRAF promotes nevi formation, i.e., senescence *in vivo*, which harbor phosphorylation of FOXO4 on the JNK target sites Thr^{223}/Ser^{226} and elevated p21^{cip1} expression within similar compartments.

Discussion

Here, we describe a role for FOXO4 in BRAF^{V600E}-induced senescence. BRAF^{V600E} activates FOXO4 through a MEK-ROS-JNK signaling cascade to induce p21^{cip1} expression

and senescence (Fig. 6D). Senescence represents a barrier for tumor formation, and consequently, the melanoma-derived cells we have used *de facto* have bypassed this barrier. Irrespectively, in cell culture active FOXO reimposes this barrier, suggesting that FOXO inactivation is one of the requirements for senescence bypass. This conclusion is supported by data showing that, in mice, loss of PTEN and consequently reduced FOXO activity synergize with $BRAF^{V600E}$ to induce melanoma (40). Despite limitations in studying senescence in melanoma cell lines in culture, our histochemical analysis of lesions from $BRAF^{V600E}$ mice clearly suggests that *in vivo* FOXO and $p21^{cip1}$ indeed function in the senescence response induced by $BRAF^{V600E}$.

Oncogenes induce senescence through various mechanisms. Although HRAS is an upstream regulator of RAF, $HRAS^{G12V}$ expression in primary melanocytes induces senescence through the ER-associated unfolded protein response, whereas oncogenic (B)RAF does not (32). This difference between RAS and RAF is also reflected in mice models in which $BRAF^{V600E}$ induces both melanocyte senescence and

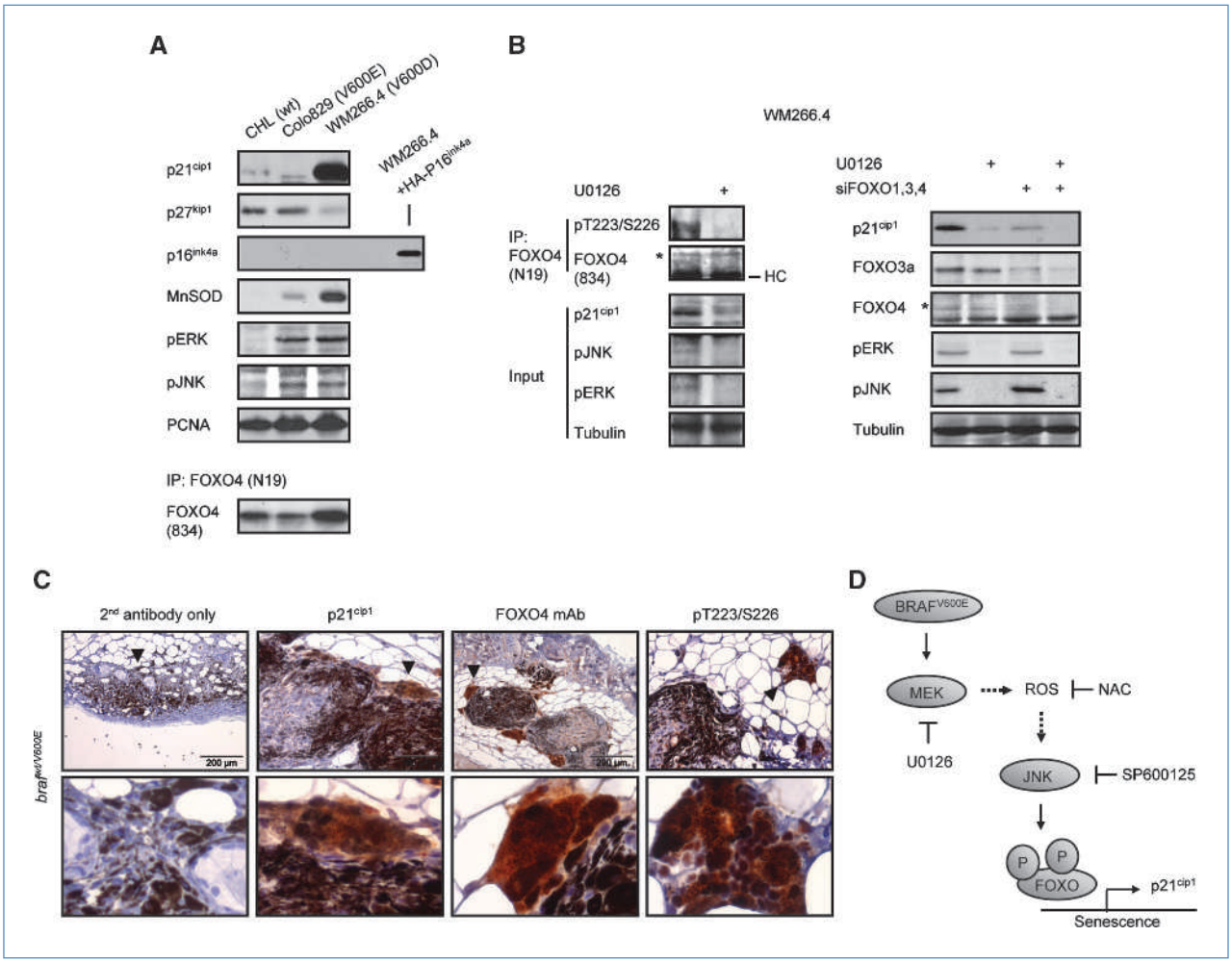

Figure 6. Endogenous $BRAF^{V600E}$ regulates $p21^{cip1}$ transcription through FOXO4 phosphorylation on the JNK target sites. A, characterization of WM266.4 ($BRAF^{V600D}$) cells. CHL (wt BRAF), Colo829 ($BRAF^{V600E}$), and WM266.4 ($BRAF^{V600D}$) cells were lysed and analyzed by immunoblotting. Endogenous FOXO4 expression was determined after immunoprecipitation. B, left, U0126 abrogates JNK signaling, endogenous phosphorylation of FOXO4 on Thr^{223} + Ser^{226} and $p21^{cip1}$ expression in WM266.4 cells. WM266.4 cells were untreated or treated for 24 h with 10 µmol/L U0126 and analyzed as in A). The phosphorylation status of endogenous FOXO4 was determined after immunoprecipitation. HC, heavy chain. Right, endogenous FOXOs regulate $p21^{cip1}$ expression in WM266.4 cells. Lysates of WM266.4 cells transfected with scrambled siRNA or siRNA against FOXO1,3a and 4 (siFOXO) and untreated or treated for 24 h with 20 µmol/L U0126 were analyzed by immunoblotting. C, expression of $p21^{cip1}$, total FOXO4, and Thr^{223}/Ser^{226}-phosphorylated FOXO4 is elevated in neoplastic regions of $BRAF^{V600E}$-induced nevi. Top, skin sections of tamoxifen-treated $Braf^{+/LSL\text{-}V600E}$; Tyr::$CreERT2^{+/o}$ mice were analyzed for background signal (second antibody only), $p21^{cip1}$ expression, total FOXO4, and Thr^{223}/Ser^{226}-phosphorylated FOXO4. Higher magnifications of the nevus (arrowheads) are shown in the bottom. The top right shows undifferentiated nevi. The bottom right represents a magnification of epidermal staining from the bottom left. Untreated tissue did not typically show positive staining. D, model on the regulation of FOXO4 by $BRAF^{V600E}$, resulting in $p21^{cip1}$-mediated senescence. $BRAF^{V600E}$ signaling activates MEK. This, in turn, induces elevations in cellular ROS levels, thereby promoting activation of JNK. JNK subsequently phosphorylates FOXO4 and thereby promotes specific transcription of $p21^{cip1}$, rather than $p27^{kip1}$ or $p16^{ink4a}$, and triggers a senescence response.

melanoma (39), whereas HRAS^{G12V}, but not NRAS^{Q61K}, induces senescence and only melanoma if combined with loss of tumor suppressors p16^{ink4a} or p19^{Arf} (41). Interestingly, senescence in general, including melanocyte senescence (33), frequently correlates with elevated levels of ROS and OIS can be bypassed by ROS scavenging compounds (34, 35). BRAF^{V600E} chronically increases cellular ROS, which, as we showed, is required for activation of FOXO4, p21^{cip1} transcription, and subsequent senescence. Together, these and our data suggest that, besides oncogene-specific pathways, increased ROS direct part of the senescence program, which may be more generic. Recently, RAS-induced senescence was shown to require a RAS-dependent negative feedback loop repressing PI3K-PKB/AKT activity (42). As ROS, reduced PKB/AKT activity also activates FOXO, suggesting that activation of FOXO is the general event in senescence rather than the ROS/JNK signaling mechanism. Interestingly, the idea that FOXO activation will be a general component of senescence onset is in agreement with the current notion that the reverse, i.e., FOXO inactivation, represents a general component of tumor onset (18).

Mechanisms of senescence induction also greatly differ between cell types. In cell culture, melanocyte senescence differs from fibroblast senescence (discussed in ref. 43). Human melanocytes deficient in INK4a show an impaired senescence response, but INK4a-deficient human fibroblasts senesce normally. Because a number of families with inherited predisposition to melanoma showed loss of p16^{ink4a} (44, 45), these and other data suggest that INK4a-dependent senescence is especially important in melanocytes. However, loss of p16^{ink4a} is not very common in early-stage melanomas (46), and in oncogenic BRAF-positive human and mouse nevi, examples of cellular senescence *in vivo* p16^{ink4a} expression are mosaic (47, 48). Also recently, we showed in the Braf$^{+/LSL\text{-}V600E}$; Tyr::CreERT2$^{+/o}$ mouse model that loss of p16^{ink4a} does not affect BRAF^{V600E}-induced nevus formation (39). Furthermore, in these mice, BRAF^{V600E}-induced melanoma showed nuclear p16^{ink4a} staining in agreement with clinical data showing significant nuclear p16^{ink4a} expression in primary melanoma (30%–85%) as well as metastatic melanoma (15%; ref. 48). Thus, although p16^{ink4a} fulfills an important role in the suppression of melanoma progression, it seems not to be essential for establishing senescence (see also ref. 4). Here, we show firstly that, in the absence of p16^{ink4a}, FOXO4 can induce senescence, and secondly, this requires p21^{cip1}. This confirms the earlier suggestion that p21^{cip1} may facilitate melanocyte senescence in the absence of p16^{ink4a} (5). Thus, in BRAF^{V600E} signaling, p21^{cip1} and p16^{ink4a} seem to regulate two independent cell cycle inhibitory responses that are functionally redundant to the induction of BRAF^{V600E}-induced senescence.

Besides INK4a, the requirement for FOXO further defines differences between fibroblasts and melanocytes in senescence induction. In contrast to our observations with respect to OIS in melanoma cells and melanocytes *in vivo*, in fibroblasts loss of FOXO3a rather than activation of FOXO has been implicated in replicative senescence (49). Recently, a differential requirement for FOXO has been suggested in tumor progression (50), and it will be of interest to see whether a similar differential requirement applies to the various stages at which cellular senescence can be induced.

FOXOs function as tumor suppressors (18), and senescence induction by FOXO as shown here provides one mechanism for this function of FOXO. Importantly, although a mechanism of tumor suppression, it is argued that cellular senescence is also causative to organismal aging (51, 52). OIS may therefore represent a trade-off between tumor suppression and life span. Interestingly, both lack of growth factor signaling and increased ROS result in FOXO activation. However, the absence of growth factor signaling can impose a reversible p27^{kip1}-mediated G_1 cell cycle arrest and/or quiescence, which may be used to repair, for example, cellular damage (24). In this manner FOXO may positively affect life span and importantly with little cost to the organism. However, in response to BRAF^{V600E}-induced ROS, FOXOs protect against tumorigenesis through induction of senescence, and unlike the former, this protection is not without cost. Our findings underline the pivotal role that FOXOs play in mediating the role of ROS in normal signaling as well as aging, and it will be of interest to see whether, for example, age in return affects the ability of FOXO to mediate senescence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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