

# Krüppel-like factor 6 interferes with cellular transformation induced by the H-ras oncogene

Lucas Daniel Trucco, Verónica Andreoli, Nicolás Gonzalo Núñez,<sup>1</sup> Mariana Maccioni, and José Luis Bocco<sup>2</sup>

Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

**ABSTRACT** KLF6 is a member of the Krüppel-like factor family of transcription factors, with diverse roles in the regulation of cell physiology, including proliferation, signal transduction, and apoptosis. Mutations or down-regulation of *KLF6* have been described in several human cancers. In this work, we found that KLF6-knockdown resulted in the formation of transformed foci and allowed the spontaneous conversion of NIH3T3 cells to a tumorigenic state. We further assessed the role of KLF6 in the context of oncogenic Ras. We showed that KLF6 was up-regulated by H-Ras<sup>G12V</sup> expression in a Jun N-terminal kinase (JNK)-dependent manner, correlated with enhanced *klf6* promoter activity. We found that ectopic KLF6 expression induced a G1-phase cell cycle arrest, thereby decreasing the cell proliferation rate. In addition, constitutive KLF6 expression impaired H-Ras<sup>G12V</sup>-mediated loss of density-dependent growth inhibition and anchorage-independent growth. Moreover, growth of H-Ras<sup>G12V</sup>-driven tumors was reduced in mice challenged with cells stably expressing KLF6. KLF6 expression correlated with the up-regulation of p21, whereas neither p53 induction nor apoptotic cell death was detected. Further, p21 knockdown impaired KLF6-induced cell cycle arrest. These findings provide novel evidence highlighting KLF6 function in response to malignant transformation, suggesting the relevance of KLF6 in controlling cell proliferation and hindering tumorigenesis. Trucco, L. D., Andreoli, V., Núñez, N. G., Maccioni, M., Bocco, J. L. Krüppel-like factor 6 interferes with cellular transformation induced by the H-ras oncogene. *FASEB J.* 28, 000–000 (2014). [www.fasebj.org](http://www.fasebj.org)

*Key Words:* tumor suppressor • c-Jun N-terminal kinase • p21

KRÜPPEL-LIKE FACTOR 6 (KLF6) is classified as part of the KLF family of transcription factors comprising 17 members characterized by a highly conserved Cys2-His2 zinc finger DNA-binding domain (DBD) located in the C-terminal region. A growing body of evidence indicates that these proteins have regulatory functions in diverse physiological processes, including cell proliferation, differentiation, development, and programmed cell death (1). KLFs are also involved in tumor biology, reprogramming somatic cells into inducible pluripotent stem cells, and maintaining the pluripotent state of embryonic stem cells (1).

KLF6 was identified on the basis of its properties as a regulator of the transcription of genes encoding the pregnancy-specific glycoproteins (2). The *klf6* gene is ubiquitously expressed and displays a differential expression pattern, being primarily enriched in placental cells (2, 3). In addition, *klf6* knockout in mice causes embryonic lethality associated with markedly reduced yolk sac hematopoiesis and a poorly defined liver (4). Regarding the role of KLF6 in carcinogenesis, studies over the past years have reported that KLF6 is functionally inactivated in different human tumors by loss of heterozygosity, somatic mutation, decreased expression, and dysregulated alternative splicing, supporting a tumor-suppressor activity (5). Human malignancies associated with alterations in the *KLF6* gene include colorectal cancer; gastric carcinoma; hepatocellular carcinoma; and prostate, ovarian, breast, and non-small-cell lung cancers (6). Moreover, ectopically expressed KLF6 suppresses glioblastoma multiforme tumorigenicity (7).

Abbreviations: CCND1, cyclin D1; CDK, cyclin-dependent kinase; DBD, DNA-binding domain; DDR, DNA-damage response; DMEM, Dulbecco's modified Eagle medium; ERK, extracellular-signal regulated kinase; H&E, hematoxylin-eosin; JNK, c-Jun N-terminal kinase; KLF6, Krüppel-like factor 6; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MKK, MAPK kinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SCR, scrambled; shRNA, short hairpin RNA; siRNA, small interfering RNA

<sup>1</sup> Current address: Section Recherche INSERM U932, Institut Curie, Paris, France.

<sup>2</sup> Correspondence: Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, CIBICI-CONICET, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina. E-mail: [jbocco@fcq.unc.edu.ar](mailto:jbocco@fcq.unc.edu.ar)

doi: 10.1096/fj.14-251884

This article includes supplemental data. Please visit <http://www.fasebj.org> to obtain this information.

A proposed function of KLF6 is the direct transactivation of the p21<sup>CIP1/WAF1</sup> promoter in a p53-independent manner, leading to inhibition of the cell cycle (8). In addition, KLF6 inhibits cyclin D1 (CCND1)/cyclin-dependent kinase 4 (Cdk4) activity *via* its interaction with CCND1, causing reduced Rb phosphorylation and growth suppression (9). Another mechanism whereby KLF6 regulates cell proliferation is through inhibition of the c-Jun proto-oncoprotein function, involving a physical interaction between c-Jun and KLF6, down-regulation of c-Jun transcription, and proteasome-dependent degradation of c-Jun (10). In relation to the transcriptional program of KLF6 in response to cell-death-inducing agents, several independent reports have described apoptotic and antiapoptotic functions by modulating Bcl-2 family proteins, as well as ATF-3 and p53 levels (5, 11); hence, the tumor-suppressor activity of KLF6 is relative and depends on cellular context and specific external or endogenous stimuli (11).

Furthermore, KLF6 expression is responsive to external stimulation that affects cell proliferation, such as growth factors, phorbol esters, and UV radiation (10, 12). However, how the cell cues and the particular signaling pathways regulate the gene expression, phosphorylation status, degradation, and subcellular localization of KLF6 remains unclear. Particularly, the ability of KLF6 to decrease cellular transformation induced by oncogenes, including K-Ras<sup>G12V</sup> and H-Ras<sup>G12R</sup>, remains under discussion (7, 13, 14).

In mammals, the Ras family includes 3 closely related members, H-*ras*, K-*ras* and N-*ras*, whose oncogenic variants generated by gain-of-function missense mutations are found in more than the 30% of all human tumors (15). These proteins operate as molecular switches that convey signals from surface receptors to the interior of cells, thereby regulating essential processes through the interaction with downstream effectors including the mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and Ral-GEFs (16). The wide proliferative signals generated by activated Ras culminate with the up-regulation of several transcription factors required for cell cycle entry and progression. Dysregulation of the Ras pathway is associated with increased KLF6 alternative splicing in human hepatocellular carcinoma, yielding the expression of the dominant negative KLF6-SV1 variant (17). Despite several lines of evidence obtained in cultured cells suggesting a potential interplay between KLF6 and Ras signaling, the underlying mechanism regarding KLF6 function in Ras-induced tumorigenesis has not been explored.

In this study, we investigated the ability of KLF6 to regulate the oncogenic activation triggered by the Ras pathway, focusing on the signal transduction cascades involved in the regulation of KLF6 expression. Herein, we demonstrate that KLF6 behaves as a tumor suppressor, restraining the spontaneous onset of the transformed phenotype and reducing the cell proliferation rate and tumor growth driven by mutated H-Ras. The

Jun N-terminal kinases (JNKs), which are downstream targets of activated Ras, are necessary for sustained KLF6 expression, suggesting KLF6 as part of a cell defense mechanism against *ras* oncogene induction.

## MATERIALS AND METHODS

### Plasmids, antibodies, and reagents

Lentiviral short hairpin RNA (shRNA) vectors were generated by ligating synthetic oligonucleotides (Sigma-Aldrich, St. Louis, MO, USA) into the *Age*I and *Eco*RI sites of pLKO.1-puro (18). The 21-mer target sequences were scrambled (SCR), 5'-GTAACTGCGTACCTTGAGTA; shKLF6-A, 5'-ACAGGGAATCTTCTCAACTAT; and shKLF6-B, 5'-GATCCC-ATTGGTGAAGTCTTA. Expression plasmids for KLF6 and its derivative mutants have already been described (10). The packaging plasmids pCMV-dR8.2-dvpr, pCMV-VSV-G, and pLenti-CMV/TO/RasV12-Puro, used to express H-Ras<sup>G12V</sup>, were obtained from Addgene.org (8455, 8454, and 22262; Addgene, Cambridge, MA, USA; <https://addgene.org>). The pLenti3.3/TR and pLenti6.3/TO/V5-GW/lacZ vectors containing the genes encoding the Tet repressor and  $\beta$ -galactosidase, respectively, were purchased from Invitrogen (Carlsbad, CA, USA). cDNA encoding mouse *klf6* was cloned into the pLenti6.3/TO/V5-DEST expression plasmid by the Gateway System (Invitrogen). The expression vectors encoding constitutively active mutants of MAPK kinase 6 (MKK6), MKK7, and MEK1 kinases and p21 shRNA constructs have been described elsewhere (19–21). All transfection experiments were performed with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

Primary antibodies used for Western blot analysis, immunofluorescence, and immunohistochemistry analysis were the following: anti-KLF6 (R-173), anti-H-Ras (C-20), and c-Jun (H79) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-c-Jun (Ser63; 9261), p44/42 MAPK [extracellular-signal regulated kinase 1/2 (Erk1/2); 137F5], phospho-p44/42 MAPK (Thr202/Tyr204; D13.14.4E), JNK (56G8), phospho-JNK (Thr183/Tyr185; 9251), Akt (pan) (C67E7), and phospho-Akt (Ser473; D9E) (Cell Signaling Technology, Danvers, MA, USA); Ki67 and CD34 (Cell Marque, Rocklin, CA, USA); p21 (556431; BD Pharmingen, San Diego, CA, USA); and  $\alpha$ -tubulin (T9026; Sigma-Aldrich). Peroxidase-conjugated secondary antibodies were purchased from Zymed (South San Francisco, CA, USA), and fluorescent dye-conjugated (Alexa Fluor 488) from Invitrogen.

JNK inhibitor (SP600125) and p38 inhibitor (SB203580) were obtained from Calbiochem (San Diego, CA, USA). MEK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) were from Cell Signaling Technology.

SCR and JNK1/2 small interfering RNA (siRNA; Signal-Silence SAPK/JNK siRNA I; Cell Signaling Technology) were transfected by using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions.

### Cell culture

All cell lines were from the American Type Culture Collection (ATCC, Manassas, VA, USA). NIH3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% newborn calf serum (Gibco) and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin; Gibco). MCF10A cells were cultured in DMEM/F12 medium supplemented with

10% newborn calf serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, and antibiotics. MDA-MB-231, H1299, A549, HCT116, and CCRF-CEM cells were grown in DMEM plus 10% heat-inactivated fetal bovine serum (PAA Laboratories, Pasching, Austria). The mutation data was obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC), Wellcome Trust Sanger Institute (Hinxton, UK) database; [http://cancer.sanger.ac.uk/cancergenome/projects/cell\\_lines/](http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/).

### Establishment of stable NIH3T3 cells

Lentiviruses were generated by cotransfecting HEK293T cells with pCMV-dR8.2-dvpr and pCMV-VSV-G helper plasmids and the appropriate transfer expression vector (pLKO.1-SCR, pLKO.1-shKLF6-A, pLKO.1-shKLF6-B, pLenti3.3/TR, pLentiCMV/TO/RasV12-Puro, pLenti6.3/TO/RasV12, pLenti6.3/TO/KLF6, or pLenti6.3/TO/V5-GW/lacZ). Viral supernatants were collected 48 h after transfection, supplemented with 6 µg/ml polybrene (Sigma-Aldrich), and used for transduction of NIH3T3 cells (multiplicity of infection, 0.5). After 48 h, the cells were resuspended in fresh medium containing puromycin (2 µg/ml; Sigma-Aldrich) and geneticin (500 µg/ml; Gibco) or blasticidin (5 µg/ml; Gibco) and selected during 3 wk. Resistant NIH3T3-TR-Ras<sup>G12V</sup> cells were maintained in DMEM containing 10% Tet system approved fetal bovine serum (Clontech, Mountain View, CA, USA).

### Western blot analysis

Western blot analysis was performed as described previously (22).

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA extraction, single-strand cDNA synthesis, and real-time PCR were performed as described elsewhere (22). The sequences of primers used are the following: KLF6, forward 5'-GTGACAAGGGTAATGGCGAC-3' and reverse 5'-ATGAGCATCTGTAAGGCTTTTCT-3'; p21, forward 5'-CTCTGGTGTCTGAGCGCCTGAAG-3' and reverse 5'-CTCTTGCAGAAGACC-AATCTGCGC-3'; TP53, forward 5'-TGGAAGACTCCAG-TGGGAAC-3' and reverse 5'-GCACAAACACGAACCTCAA-3'; and GAPDH, forward 5'-GGGGCTGGCATTGCTCT-3' and reverse 5'-TTGCTCAGTGTCTTGTCTGG-3'. Primers used for human KLF6 and cyclophilin A are described in Racca *et al.* (22).

### Cell proliferation

Growth rate was examined with the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison WI, USA). Briefly, NIH3T3 cells were plated in triplicate wells of a 96-well plate at a density of  $3 \times 10^3$  cells/well. The cells were incubated for 2 d at 37°C, and the medium was changed every 24 h. Cell proliferation was measured after incubation for 2 h with a combined MTS/PMS solution by reading the plates at 490 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Proliferation was calculated from the absorbance ratio between values obtained at the end point with respect to those measured in cells before treatment. All assays were repeated 3 times.

### Cell cycle analysis

Cells were fixed with ice-cold ethanol, and samples were resuspended in phosphate-buffered saline (PBS) containing

RNase I (50 µg/ml; Sigma-Aldrich) and propidium iodide (25 mg/ml; Sigma-Aldrich). The stained samples were subjected to fluorescence-activated cell sorting (FACSCanto II; Becton Dickinson, San Jose, CA, USA), and populations were analyzed with ModFitLT software (Verity Software House, Topsham, ME, USA).

### Immunofluorescence

Cells seeded onto glass coverslips were fixed in 3% paraformaldehyde, permeabilized in 0.01% Triton X-100, blocked in 0.4% fish skin gelatin and 0.2% Tween-20, and incubated with anti-KLF6 in PBS-2.5% goat serum. Secondary staining proceeded with the anti-rabbit Alexa Fluor 488-conjugated antibody in PBS-2.5% goat serum. Nuclei were counterstained with DAPI. Coverslips were mounted with FluorSave Reagent (Calbiochem), and images were captured with Fluoview 1000 (Olympus, Tokyo, Japan).

### Anchorage-independent cell transformation assay

Cells were plated in 0.3% agarose/growth medium onto 60 mm dishes with a 0.5% agarose underlayer. The cultures were maintained at 37°C in a moist atmosphere of 5% CO<sub>2</sub> in air, and fresh medium was added weekly. Colonies containing more than 100 cells in triplicate platings were counted under a microscope after 4 wk.

### Mice and tumor xenografts

N:NIH(S)-nu/nu athymic mice were purchased from Universidad Nacional de La Plata (Buenos Aires, Argentina). Animals were maintained at the Animal Resource Facility of the Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) [Animal Welfare Assurance no. A5802-01, U.S. National Institutes of Health (NIH), Bethesda, MD, USA], and experiments were performed in accordance with experimental ethics committee guidelines.

Tumors were established by s.c. injection of  $1 \times 10^6$  cells in 250 µl of sterile saline into the right flank of 6-wk-old male nude mice. Tumor measurements were taken by calipers, and tumor volume was calculated as width squared  $\times$  length  $\times$  0.5. The mice were euthanized when the tumors reached a volume of  $>4$  cm<sup>3</sup>. The tumors were excised, weighed, and formalin fixed for histologic and immunohistochemical analysis. Sections were embedded in paraffin and stained with hematoxylin and eosin (H&E), according to standard methods, and with antibodies to KLF6, Ki-67, and CD34 (23).

### Viability assay

NIH3T3-TR-Ras<sup>G12V</sup> cells were plated into 96-well plates ( $1 \times 10^4$  cells/well) and further exposed to doxorubicin (Sigma-Aldrich), *cis*-diammineplatinum (II) (Sigma-Aldrich), or methotrexate (Sigma-Aldrich). After 24 or 48 h, the cells were incubated with 120 nmol of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) for 3 h at 37°C. Then, the medium was removed, formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm on a microplate reader (Bio-Rad).

### Statistical analysis

Statistical significance was determined with 1- or 2-way analysis of variance (ANOVA), with Dunnett's multiple comparison *post hoc* analysis (Prism 5; GraphPad, San Diego, CA,



USA). Comparisons between 2 groups were made by Student's *t* test. Unless specified otherwise, all data are presented as means  $\pm$  SEM, with data considered significant at  $P < 0.05$ .

## RESULTS

### KLF6 knockdown increases spontaneous cellular transformation

Since KLF6 inactivation was observed in several tumors, we asked whether KLF6 down-regulation can lead to malignant transformation. We used an RNA interference approach to mimic KLF6 loss. Two independent shRNAs targeted against KLF6 (shKLF6-A and -B) were used to ablate KLF6 expression by stable transduction of the NIH3T3 cell line. A construct expressing SCR shRNA was used as the control. Analysis of KLF6 mRNA and protein expression demonstrated a knockdown efficiency  $>80\%$  (Fig. 1A, B). We determined whether KLF6 depletion affects *in vitro* growth capacity. Although the cells proliferate at similar rates when cultured in complete medium, the shKLF6 cells grew under serum-starved conditions, bypassing the arrest observed in the SCR cells, which remain sensitive to serum withdrawal (Fig. 1C). It has been shown that KLF6 contributes to cell growth suppression through p21 activation, in both cultured cells and in a transgenic mouse model (8, 24). We therefore evaluated p21 expression in KLF6-knockdown cells and observed in both shKLF6 cell lines a significant decrease in the p21 levels (Fig. 1A, B).

Thereafter, we tested the responsiveness of the shKLF6-expressing cells in the induction of morphologically transformed foci when grown at high cellular densities. Foci formation became evident in cultures of KLF6 knockdown cells, whereas the SCR controls formed a monolayer of contact-inhibited cells (Fig. 1D, E). Subsequently, we investigated the *in vivo* relevance of our *in vitro* observations. We performed xenograft experiments in which cells expressing shKLF6 were implanted by s.c. injection into nude mice and monitored for tumor growth. Whereas cells expressing SCR shRNA completely failed to generate any visible or palpable tumor, even 4 wk after inoculation, KLF6-knockdown cells produced a high incidence of tumors (11/12 mice). The latency periods before the appearance of readily visible tumors ranged from 14 to 20 d (Fig. 1F), and all developing tumors grew progressively until they were surgically excised (Fig. 1G). These results demonstrate that KLF6 knockdown enhances susceptibility to spontaneous cellular transformation, indicating that KLF6 loss may be an important event at early stages of tumorigenesis.

### Constitutively active H-Ras induces KLF6 expression in a JNK-dependent manner

Activation of Ras oncogenes has an initiating role in tumor development, attributed principally to its capac-

ity to endow incipient cancer cells with a sufficiency of growth signals (16). To investigate the potential role of KLF6 in controlling the oncogenic cascade triggered by mutated Ras, we took advantage of a Ras-inducible model. We stably transduced NIH3T3 fibroblasts to express a constitutively active form of Ras, H-Ras<sup>G12V</sup>, under the control of a tetracycline-inducible promoter (NIH3T3-TR-Ras<sup>G12V</sup> cells). Induction of H-Ras<sup>G12V</sup> expression resulted in an increase in the endogenous KLF6 protein in a time-dependent manner, reaching maximum levels at 12 h, with elevation persisting until at least 48 h (Fig. 2A). Tetracycline treatment, *per se*, did not produce a significant change in the expression of KLF6, as determined in cells transduced with the empty vector (NIH3T3-TR-Puro). Furthermore, the KLF6 mRNA level was up-regulated 5- and 8-fold after 24 and 48 h of H-Ras<sup>G12V</sup> expression, respectively (Fig. 2C), in correlation with induction of the *klf6* promoter activity (Fig. 3A).

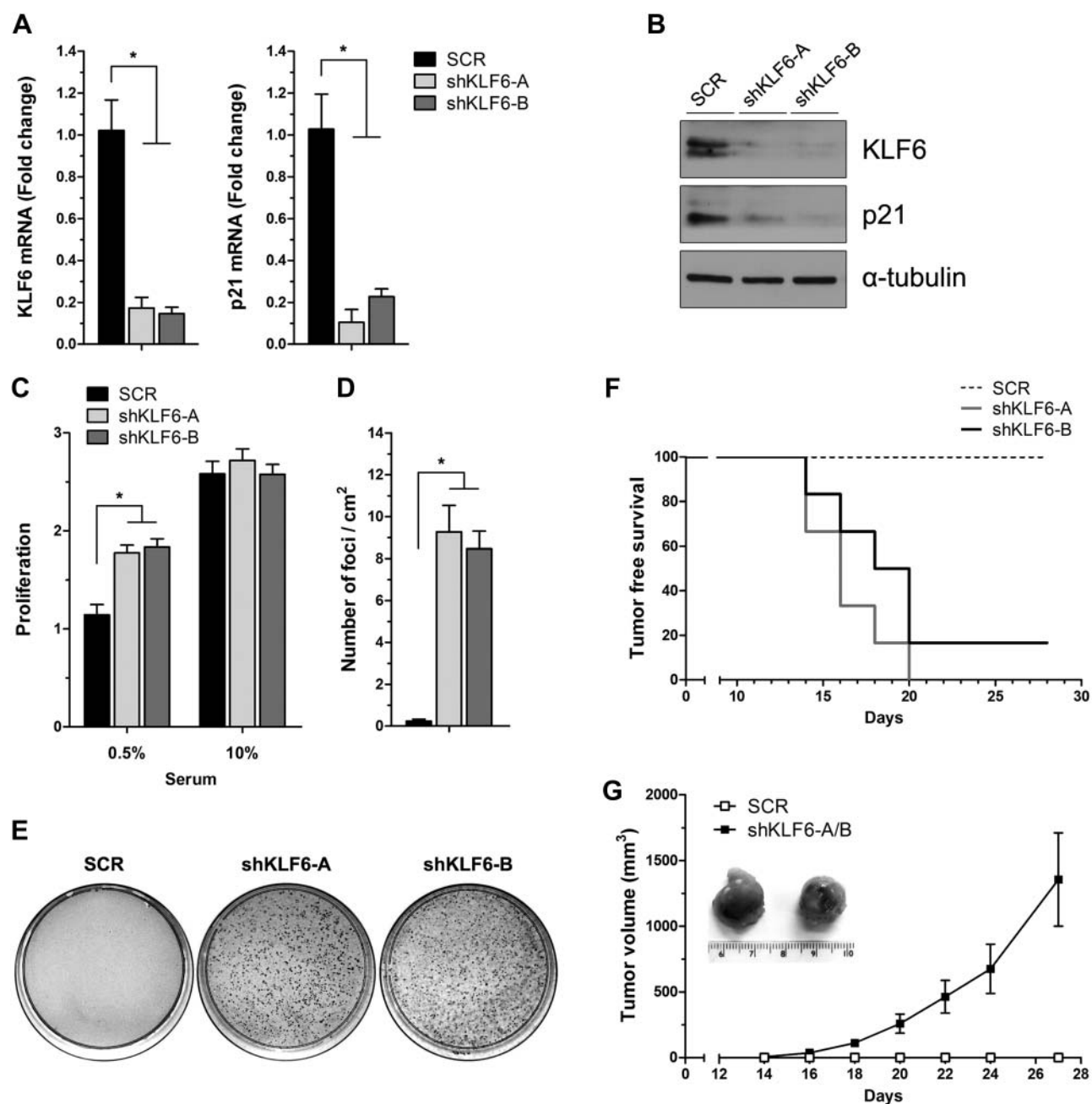
Ras activates several signaling pathways that promote cell survival and proliferation. Although the Raf/MEK/ERK pathway is the best characterized, it is now well established that Ras also activates the JNK and p38 MAPKs and PI3K/AKT, contributing overall to the establishment of the transformed phenotype (25–27). We observed that H-Ras<sup>G12V</sup> expression activated ERK, JNK, and AKT signaling (Fig. 2A). Hence, we tested the signaling pathways mediating the H-Ras<sup>G12V</sup>-induced KLF6 expression. To this end, cells were treated with selective pharmacological inhibitors of MEK, JNK, p38, and PI3K. We observed that the increase in KLF6 obtained after the addition of tetracycline was impaired by the JNK inhibitor SP600125, at both the protein and transcript levels, indicating that the expression of KLF6 induced by H-Ras<sup>G12V</sup> is dependent on JNK activity (Fig. 2B, C and Supplemental Fig. S1A). The JNKs are encoded by 3 genes: *jnk1* and *jnk2*, ubiquitously expressed, and *jnk3*, primarily restricted to the nervous system (28). We observed that JNK1- and JNK2-deficient mouse embryonic fibroblasts (MEFs) expressed lower KLF6 levels compared with parental wild-type cells, showing both isoforms to have redundant activities in the regulation of KLF6 in response to H-Ras<sup>G12V</sup> expression (Supplemental Fig. S1B). In addition, we observed a decreased KLF6 protein expression on siRNA-mediated silencing of JNKs in the MEFs constitutively expressing H-Ras<sup>G12V</sup> (Fig. 2D), indicating that JNK is essential for the induction of KLF6 in response to Ras activation.

Conversely, neither MEK inhibition with U0126, which prevents ERK phosphorylation, nor SB203580-mediated p38 inhibition significantly altered the KLF6 levels in response to H-Ras<sup>G12V</sup> expression. Nevertheless, the p38 inhibitor enhanced Ras-mediated c-Jun phosphorylation (Fig. 2C), in agreement with the previously described antagonism between p38 and JNK (29). Similarly, inhibition of PI3K with LY294002, which in turn reduced AKT phosphorylation, had no significant effect on KLF6 protein expression in NIH3T3-TR-Ras<sup>G12V</sup> cells (Fig. 2C). We also examined

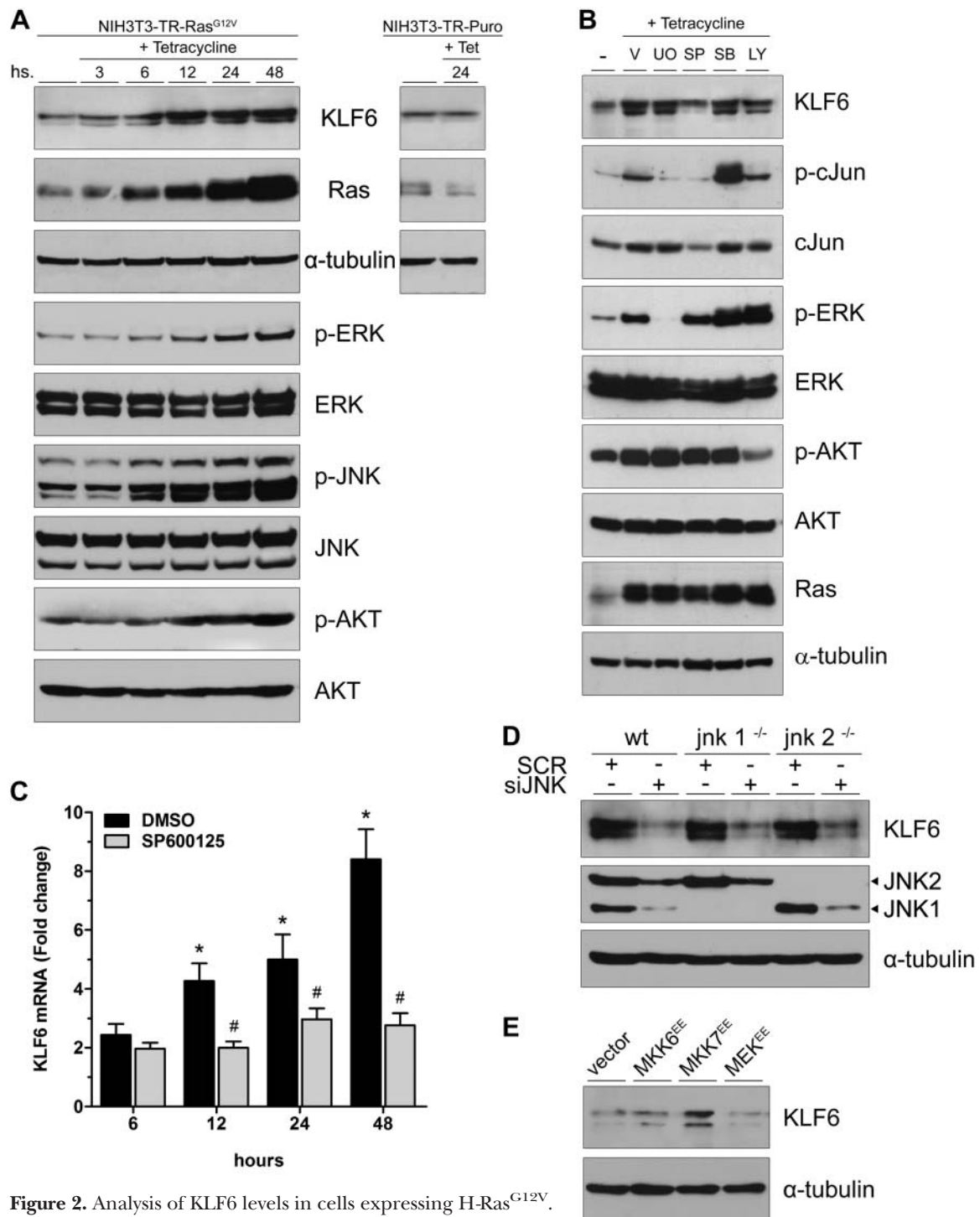
the effect of MKK activation on KLF6 induction. A constitutively active mutant of the JNK upstream activator MKK7, but not the activated forms of MKK6 or MEK (activators of p38 and ERK, respectively), increased KLF6 protein expression (Fig. 2E), supporting a role for the JNK signaling pathway in the regulation of KLF6 expression.

To further explore whether this mechanism operates in other cellular backgrounds, we transduced

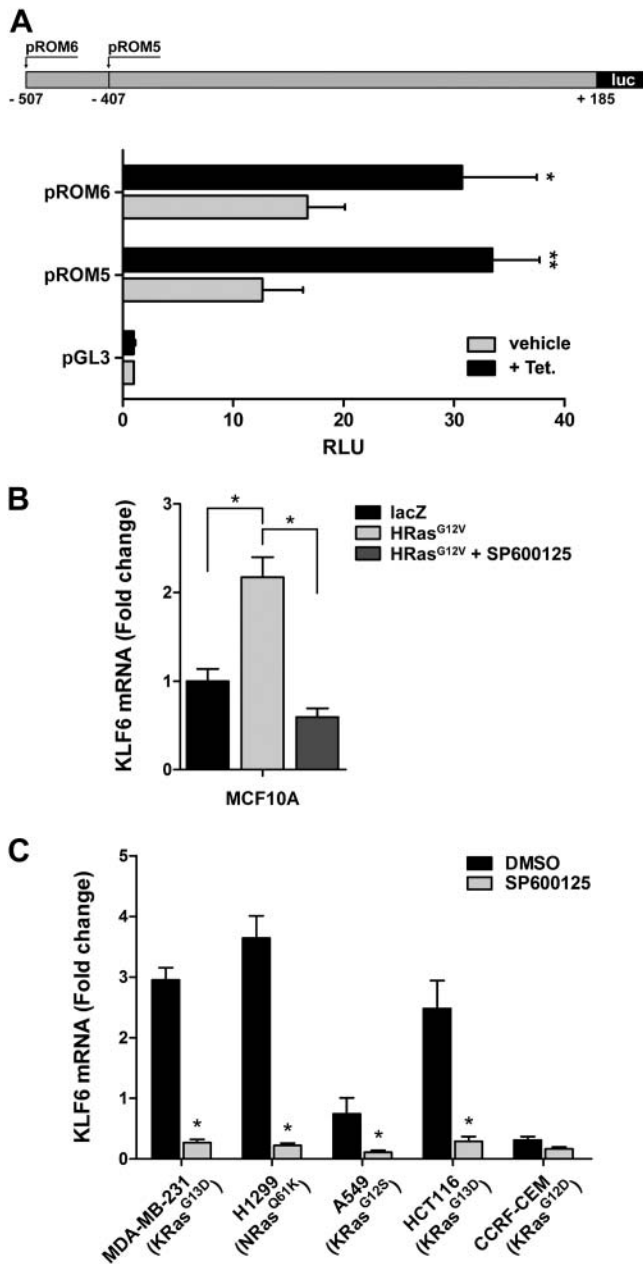
nontumorigenic human breast epithelial MCF10A cells with lentiviral vectors encoding either H-Ras<sup>G12V</sup> or lacZ (control). We found that KLF6 expression was increased in MCF10A cells transduced with H-Ras<sup>G12V</sup>, and this increment was reduced by the JNK inhibitor SP600125 (Fig. 3B). In complementary experiments, we evaluated KLF6 levels in human cancer-derived cells harboring activating mutations in Ras oncogenes. Blockage of JNK signaling reduced



**Figure 1.** shRNA-mediated KLF6 knockdown promotes cellular transformation in NIH3T3 cells. Experiments were performed in NIH3T3 cells expressing either SCR or KLF6-specific shRNA. *A*) qRT-PCR analysis of KLF6 and p21 mRNA expression. Data are expressed as fold increase  $\pm$  SD after normalization with GAPDH mRNA; \* $P < 0.01$ . *B*) Immunoblot analysis showing KLF6 and p21 protein expression.  $\alpha$ -Tubulin was used as the loading control. *C*) Proliferation rates of cells cultured at the indicated serum concentrations for 48 h. \* $P < 0.01$ . *D*) Number of transformed foci formed after 20 d of culture, through loss of contact inhibition of growth. *E*) Representative dishes showing focus formation. *F*) Tumor-free survival of xenografted athymic nude mice presented by Kaplan-Meier plot ( $n=6$ /group). *G*) Growth curves of generated tumors. Inset: tumor size at the end of the experimental period (left, shKLF6-A; right, shKLF6-B).



**Figure 2.** Analysis of KLF6 levels in cells expressing H-Ras<sup>G12V</sup>. *A*) Ras and KLF6 protein expression determined in NIH3T3-TR-Ras<sup>G12V</sup> and NIH3T3-TR-Puro cells at the indicated time points after addition of tetracycline (1 μg/ml). Total and phosphorylated forms of ERK, JNK, and AKT and α-tubulin (loading control) are shown. *B*) Immunoblot analysis of cell lysates obtained from NIH3T3-TR-Ras<sup>G12V</sup> cells treated with tetracycline for 18 h in the presence of the indicated small molecule kinase inhibitors. -, control without tetracycline; V, vehicle control (DMSO); UO, U0126 (MEK 1/2 inhibitor); SP, SP600125 (JNK inhibitor); SB, SB203580 (p38 inhibitor); LY, LY294002 (PI3K inhibitor). *C*) KLF6 mRNA expression in NIH3T3-TR-Ras<sup>G12V</sup> cells after addition of tetracycline, measured by qRT-PCR. Fold induction ± SD was calculated after normalization with GAPDH mRNA. \**P* < 0.05 vs. noninduced controls; #*P* < 0.05 vs. vehicle-treated cells. *D*) Western blot analysis of KLF6 and JNK in H-Ras<sup>G12V</sup>-transduced wild-type (wt) and JNK1- and JNK2-knockout MEFs (*jnk1*<sup>-/-</sup> and *jnk2*<sup>-/-</sup>, respectively) transfected with JNK-specific siRNA (siJNK) or nontargeting SCR siRNA. *E*) Immunoblot analysis assessing KLF6 expression in NIH3T3 cells transiently transfected with constitutively active forms of MKK6, MKK7, and MEK1. Mock transfection with empty vector served as the control.



**Figure 3.** Regulation of KLF6 expression by Ras oncogenes. *A*) KLF6 promoter activity was induced after H-Ras<sup>G12V</sup> expression. Reporter plasmids containing DNA fragments of different lengths, encompassing the 5'-flanking region of the *KLF6* gene governing luciferase gene expression, were transiently cotransfected into NIH3T3-TR-Ras<sup>G12V</sup> cells, along with the normalizing vector coding for *Renilla*. Luciferase activity was measured in cell extracts obtained from cells treated or not with tetracycline. Transcriptional activity is expressed as the fold increase, with pGL3-basic vector activity arbitrarily set at 1. \**P* < 0.05; \*\**P* < 0.001 *vs.* vehicle-treated cells. *B*) KLF6 mRNA levels measured by qRT-PCR in human mammary epithelial MCF10A cells transfected to express H-Ras<sup>G12V</sup> and treated with either vehicle or the JNK inhibitor SP600125 for 18 h. Values were normalized to cyclophilin A amplification and expressed as the fold change  $\pm$  SD with respect to lacZ-transfected control cells. \**P* < 0.01. *C*) Analysis of KLF6 mRNA levels in human cancer cell lines harboring the indicated activating mutations in endogenous Ras isoforms (COSMIC) after treatment with vehicle (DMSO) or SP600125. Values were calculated as in *B*. \**P* < 0.05 *vs.* vehicle-treated cells.

KLF6 mRNA expression in mammary adenocarcinoma (MDA-MB-231), colorectal carcinoma (HCT116), and lung carcinoma (H1299 and A549) cell lines (Fig. 3C). KLF6 was marginally expressed in lymphoblastic leukemia cells (CCRF-CEM). Altogether, our data support the notion that JNK activity mediates KLF6 expression in response to Ras oncogene expression.

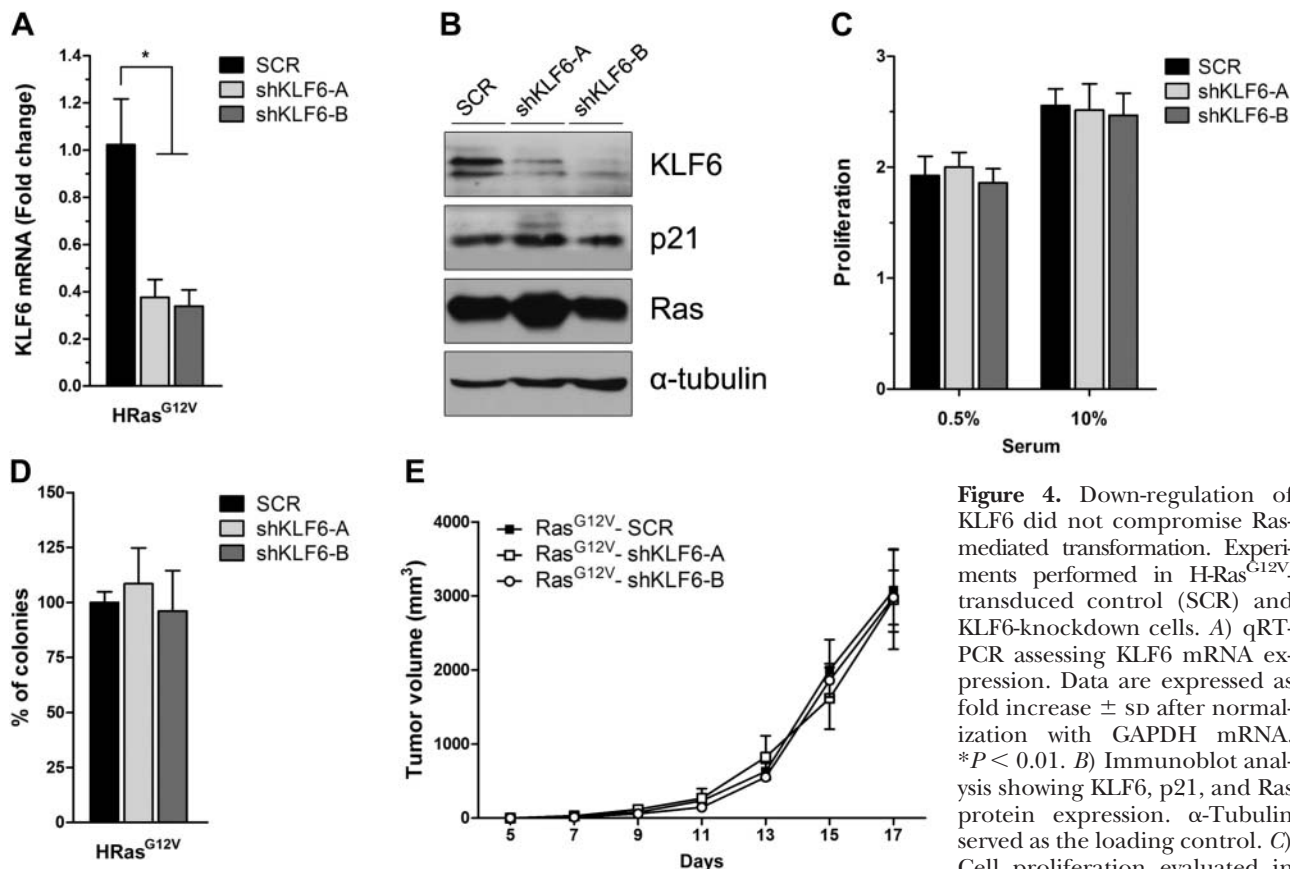
### KLF6 is dispensable for transformation induced by oncogenic Ras

As other members of the KLFs family have been described to have diverse functions in Ras-mediated cell growth, including both suppressive and proliferation-promoting activity (30), we asked whether KLF6 induction is needed for Ras-mediated cell transformation. To address this question, we transfected H-Ras<sup>G12V</sup> into the KLF6 knockdown NIH3T3 cells and performed proliferation assays. Immunoblot analysis showed comparable Ras protein levels between the 3 cell lines and corroborated reduced KLF6 mRNA and protein expression in the shKLF6-A and -B cells, when compared to the SCR cells (Fig. 4A, B). Similar proliferation rates were observed in activated Ras-expressing cells when cultured in media supplemented with 0.5 or 10% serum (Fig. 4C), in correlation with equivalent p21 expression levels (Fig. 4B). Hence, KLF6-knockdown cells expressing H-Ras<sup>G12V</sup> were assayed for anchorage-independent growth potential. Regardless of the presence of KLF6, H-Ras<sup>G12V</sup>-expressing cells formed colonies to the same extent when cultured in soft agar (Fig. 4D and Supplemental Fig. S2A). We also tested whether KLF6 is necessary for Ras-promoted tumor formation *in vivo*. Consistent with results of growth and transformation assays, depletion of KLF6 had no effect on Ras-induced tumor growth, since both groups of nude mice grafted with either shKLF6-A or shKLF6-B cells developed tumors as efficiently as those injected with the SCR cells (Fig. 4E and Supplemental Fig. S2B, C). Thus, we conclude that KLF6 expression is not necessary to confer a Ras-dependent transformed phenotype on NIH3T3 cells.

### Ectopic KLF6 expression inhibits H-Ras<sup>G12V</sup>-mediated cell proliferation and promotes cell cycle arrest

To further analyze the role of KLF6 in H-Ras<sup>G12V</sup> growth potential, we evaluated the effect of KLF6 expression on the cell proliferation rate and serum growth factor dependence of subconfluent cell cultures. We transfected NIH3T3-TR-Ras<sup>G12V</sup> fibroblasts with a KLF6 encoding vector and quantified the proliferation rate in the presence or absence of tetracycline in media containing different concentrations of serum. As observed in Fig. 5A, H-Ras<sup>G12V</sup>-promoted cell proliferation of serum-starved NIH3T3 cells was impaired by KLF6 ectopic expression. Interestingly, overexpression of KLF6 also caused marked inhibition of serum-in-





**Figure 4.** Down-regulation of KLF6 did not compromise Ras-mediated transformation. Experiments performed in H-Ras<sup>G12V</sup>-transduced control (SCR) and KLF6-knockdown cells. **A)** qRT-PCR assessing KLF6 mRNA expression. Data are expressed as fold increase  $\pm$  sd after normalization with GAPDH mRNA.  $*P < 0.01$ . **B)** Immunoblot analysis showing KLF6, p21, and Ras protein expression.  $\alpha$ -Tubulin served as the loading control. **C)** Cell proliferation evaluated in

cells in 0.5 or 10% serum for 48 h. **D)** Soft agar colony formation assay. Number of colonies was scored and is expressed as a percentage of values obtained in SCR cells. **E)** Tumor growth in xenografted athymic nude mice. Results are shown as means  $\pm$  SEM ( $n=6$ /group).

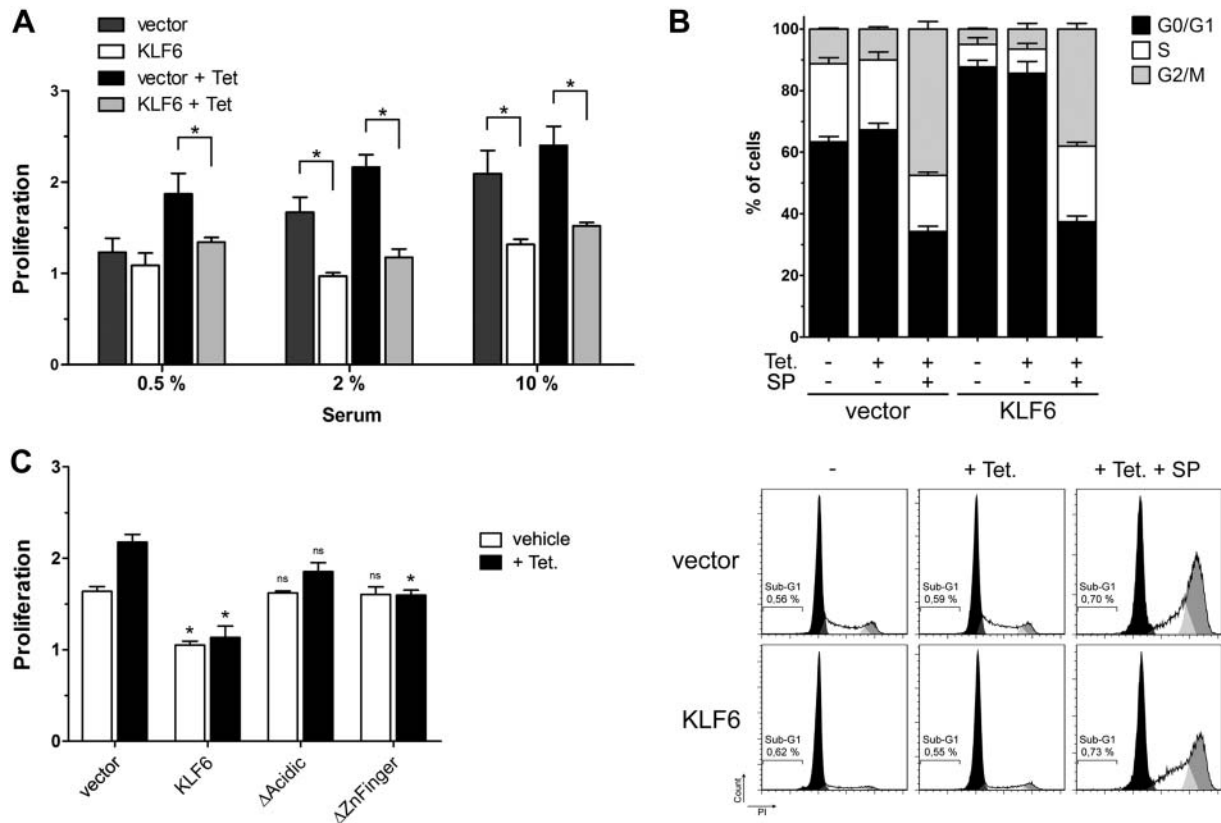
duced cellular proliferation relative to the empty vector as control.

Subsequently, we studied the effect of KLF6 expression on cell cycle distribution by propidium iodide staining. No significant differences were observed in the distribution of cells in the different phases of the cell cycle between noninduced and H-Ras<sup>G12V</sup>-expressing cells (Fig. 5B), as reported before for exponentially growing cultures (31). However, KLF6 expression led to an increase in the proportion of cells in the G<sub>1</sub> phase of the cell cycle in noninduced cells (63.4–87.7% for cells transfected with the empty or KLF6 expression vector, respectively; Fig. 5B). More significantly, the KLF6-mediated cell cycle arrest at the G<sub>1</sub> phase was not resumed by induction of oncogenic Ras (85.7–87.7% for cells treated or not with tetracycline). The accumulation of cells at the G<sub>1</sub> phase was concomitant with a reduced number of cells in both the S and G<sub>2</sub>/M phases upon KLF6 expression. Furthermore, the cell cycle arrest mediated by KLF6 overexpression did not affect cell death, since no significant differences were observed on the hypodiploid cell population (Fig. 5B) or in the activity of the final executioner caspases-3/7 (Supplemental Fig. S3), thus excluding changes in basal apoptosis rates as a cause of the observed growth differences. The previous findings identifying the role of Ras/JNK pathway for KLF6 expression prompted us to test the effect of JNK inhibition on the cell cycle.

Treatment of H-Ras<sup>G12V</sup>-expressing cells with the JNK inhibitor SP600125 caused a large increase in the percentage of cells in the G<sub>2</sub>/M phase (Fig. 5B). Even though blockade of JNK delayed the progression of mitosis, it also prevented KLF6-induced G<sub>1</sub> arrest, indicating that KLF6 function in cell cycle regulation depends on JNK activity.

KLF6 influences cellular processes mainly by activating or, in some cases, repressing the expression of its targets genes (11). Besides the conserved zinc-finger DBD present in the C-terminal region of the KLF family members, the acidic amino-terminal portion of KLF6 is also required for transcriptional activity. Then, we tested the ability of KLF6 deletion mutants lacking the 3 zinc-finger DBDs ( $\Delta$ ZnFinger) or the acidic transactivation domain ( $\Delta$ Acidic) to modulate cell proliferation. Only the construct encoding the full-length KLF6 sequence impaired cell proliferation (Fig. 5C), suggesting that growth inhibition mediated by KLF6 is dependent on its transcriptional function, whereas no significant effects were detected for the  $\Delta$ Acidic and DNA-binding deletion mutants. Although cells expressing the  $\Delta$ ZnFinger exhibited a slight reduction in the growth rate compared with those cells transfected with the empty vector during Ras induction, the decrease was less than half of that produced by wild-type KLF6.





**Figure 5.** KLF6 mediates growth inhibition. *A*) Cell proliferation of NIH3T3-TR-Ras<sup>G12V</sup> cells. Cells were transfected with control or KLF6 expression vector. Next, cells were incubated with 0.5, 2, or 10% serum for 48 h. Proliferation rate was quantified in cells stimulated or not with tetracycline for 24 h. \**P* < 0.05). *B*) Cell cycle distribution of NIH3T3-TR-Ras<sup>G12V</sup> cells. After 24 h of transfection with either an empty vector or the KLF6 expression plasmid, cells were treated as indicated. Percentages of cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases (top) and cell cycle profiles (bottom) are shown. SP, SP600125. *C*) Cell proliferation evaluated in NIH3T3-TR-Ras<sup>G12V</sup> cells transiently transfected with expression vectors encoding KLF6 or deletion derivative mutants lacking the transactivation domain (ΔAcidic) or the DBD (ΔZnFinger) in 2% serum for 48 h. ns, not significant. \**P* < 0.05 vs. empty-vector cells.

### Constitutive expression of KLF6 suppresses transformed phenotype induced by activated Ras

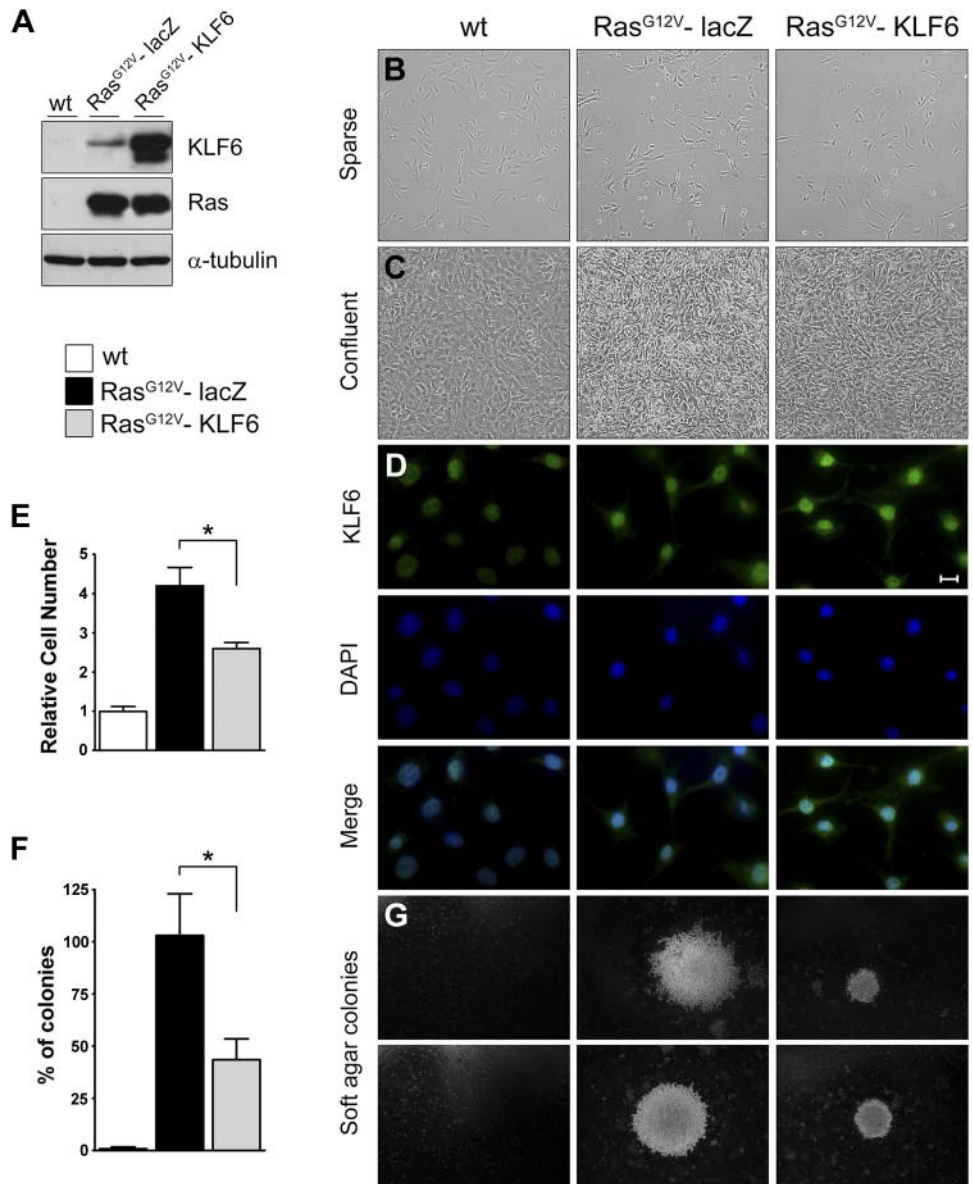
We next investigated the effect of KLF6 expression on Ras-induced transformation using *in vitro* studies based on the ability of activated Ras to promote alterations in cell morphology, loss of density-dependent growth inhibition, and anchorage-independent growth capability. We established NIH3T3 cells stably expressing H-Ras<sup>G12V</sup> and KLF6 (Ras<sup>G12V</sup>-KLF6) or β-galactosidase (Ras<sup>G12V</sup>-lacZ, control cells) and verified the expression of these proteins by immunoblot analysis (Fig. 6A). In morphologic examination by phase-contrast microscopy, we observed that cells expressing H-Ras<sup>G12V</sup>, along with lacZ, displayed the characteristic spindle-shaped, highly refractile appearance of transformed cells, whereas these features were abrogated in Ras<sup>G12V</sup>-KLF6 cells, which showed a flattened morphology similar to wild-type cells (Fig. 6B, C). Then, we examined the KLF6 subcellular distribution by immunofluorescence microscopy, showing that KLF6 has a predominant nuclear localization in NIH3T3 fibroblasts, although cytoplasmic staining became evident in Ras-expressing cells (Fig. 6D).

We also assessed the cell density at saturation of the culture and observed a significant reduction in the cell number at confluence of Ras<sup>G12V</sup>-KLF6 cells compared with Ras<sup>G12V</sup>-lacZ cells (Fig. 6E); hence, KLF6 may aid in preventing loss of contact growth inhibition. Finally, we evaluated the cell growth in soft agar (Fig. 6F). As expected, NIH3T3 cells failed to grow in soft agar unless oncogenic Ras was expressed. We found that expression of KLF6 in H-Ras<sup>G12V</sup>-transformed cells led to a significant reduction of anchorage-independent growth when compared with the control cells (Fig. 6F). Besides, KLF6-expressing cells developed smaller colonies compared with the cells expressing β-galactosidase along with activated Ras (Fig. 6G), demonstrating that KLF6 expression attenuates Ras-induced transformation *in vitro*.

### KLF6 hinders oncogenic Ras in promoting tumorigenicity *in vivo*

To investigate whether KLF6 is able to interfere with the oncogenic pathways triggered by Ras to regulate tumorigenesis *in vivo*, Ras<sup>G12V</sup>-lacZ and Ras<sup>G12V</sup>-KLF6 cells were s.c. injected into athymic nude mice and

**Figure 6.** Effect of KLF6 on Ras-transformed NIH3T3 fibroblast phenotype. Experiments performed in wild-type (wt) and H-Ras<sup>G12V</sup> expressing NIH3T3 cells stably transduced with lacZ (control) or KLF6 vectors. *A*) Western blot analysis of KLF6, Ras, and  $\alpha$ -tubulin (loading control) expression. *B, C*) Representative phase-contrast microscopy images showing cells growing in sparse (*B*) or confluent (*C*) culture conditions ( $\times 100$ ). *D*) Immunofluorescence assays showing KLF6 subcellular distribution. KLF6 signal appears in green; nuclei were counterstained with DAPI (blue). Merged images of both panels are also shown. Scale bar = 10  $\mu$ m. *E*) Cell density at saturation. Cells were cultured for 14 d in complete medium, and the number of cells was determined with a hemocytometer. Data represent values relative to those of the wild-type cells.  $*P < 0.05$ . *F*) Soft agar colony formation assay. Number of colonies was counted and expressed as a percentage of those obtained in NIH3T3-Ras<sup>G12V</sup>-lacZ cells.  $*P < 0.05$ . *G*) Images of representative colonies formed in soft agar.



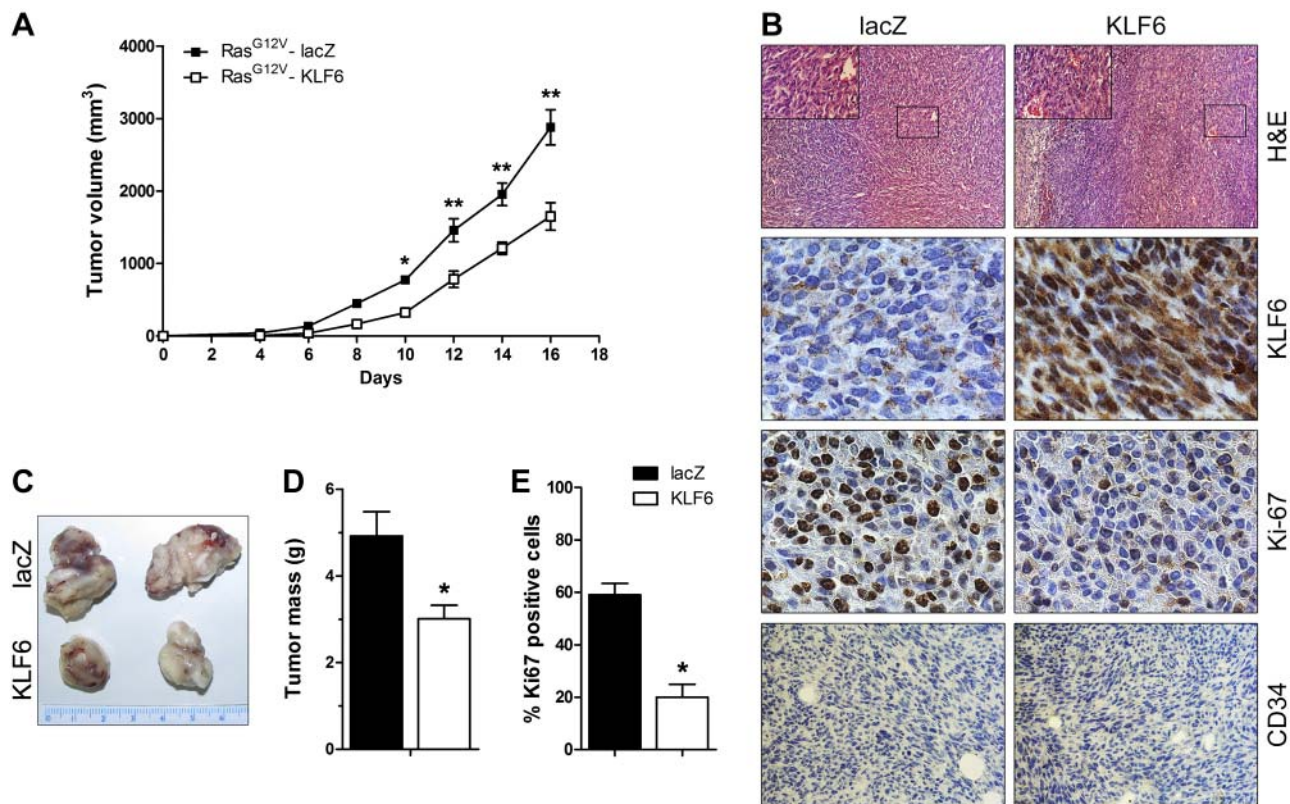
monitored for tumor growth. Whereas Ras<sup>G12V</sup>-lacZ cells formed rapidly growing tumors, the development of tumors was significantly reduced in cells expressing Ras<sup>G12V</sup> in combination with KLF6 (Fig. 7A). A marked reduction in the final size and weight was observed in the tumors developed from Ras<sup>G12V</sup>-KLF6 fibroblasts compared with Ras<sup>G12V</sup>-lacZ cells (Fig. 7C, D).

Histologic examination confirmed that inoculated cells allowed the development of fibrosarcoma-like tumors composed of pleomorphic spindle cells (Fig. 7B). Of note, expression of ectopic KLF6 remained stable *in vivo* at the time of surgical excision, as revealed by immunohistochemical analysis (Fig. 7B). Further studies of biopsies revealed a lower proliferative index of the tumors obtained from Ras<sup>G12V</sup>-KLF6-injected mice according to the staining for the Ki-67 marker compared with the control tumors derived from cells expressing lacZ (Fig. 7E). In addition, staining of the vascular endothelial cell marker CD34 indicated no marked differences in angiogenesis (Fig. 7B). Collec-

tively, *in vitro* and tumor xenografts experiments using transformed cells support a tumor-suppressor function for KLF6, with the ability to counterbalance the tumorigenesis triggered by activated H-Ras.

#### Inhibition of H-Ras<sup>G12V</sup>-mediated oncogenesis by KLF6 is associated with enhanced p21<sup>CIP1/WAF1</sup> gene expression

To further understand the mechanism involved in KLF6-mediated growth arrest, we analyzed the expression of cell cycle regulators subsequent to transfection with the expression vector encoding KLF6 after H-Ras<sup>G12V</sup> induction with tetracycline in NIH3T3-TR-Ras<sup>G12V</sup> cells. As observed by immunoblot analysis, H-Ras<sup>G12V</sup> caused a marked increase of KLF6 protein levels (Fig. 2A). Moreover, KLF6 protein overexpression obtained after transfection was further increased by H-Ras<sup>G12V</sup> induction (Fig. 8A). Since KLF6 protein stability was not modified by activated Ras expression



**Figure 7.** Effects of stably expressed KLF6 on tumor burden. *A*) Growth curves of tumors formed by NIH3T3 cells expressing H-Ras<sup>G12V</sup>, along with either lacZ or KLF6, injected subcutaneously into nude mice ( $n=8/\text{group}$ ).  $*P < 0.05$ ,  $**P < 0.001$ . *B*) Representative micrographs of H&E-stained tissue sections. Immunohistochemical analysis of tumor sections showing the expression of KLF6, the cell proliferation marker Ki-67 ( $\times 400$ ), and the endothelial cell protein CD34 ( $\times 200$ ). *C*) Representative photographs of the excised tumors induced by H-Ras<sup>G12V</sup>-transduced cells expressing  $\beta$ -galactosidase (top images) or KLF6 (bottom images) at 16 d after inoculation. *D*) Mass of tumors obtained from xenografted nude mice.  $*P < 0.05$ . *E*) Ki-67 labeling index. Representative fields of Ki-67-stained tumor sections were quantified by counting positive and negative nuclei. Data represent percentage of positive cells  $\pm$  SD,  $*P < 0.01$ .

(Supplemental Fig. S4), we presume that this finding may be a consequence of increased KLF6 mRNA half-life or increased *klf6* transcription autoregulated by its own gene product. In addition, inhibition of JNK with SP600125, besides repressing c-Jun phosphorylation, impaired the H-Ras<sup>G12V</sup>-induced KLF6 protein up-regulation (Fig. 8B).

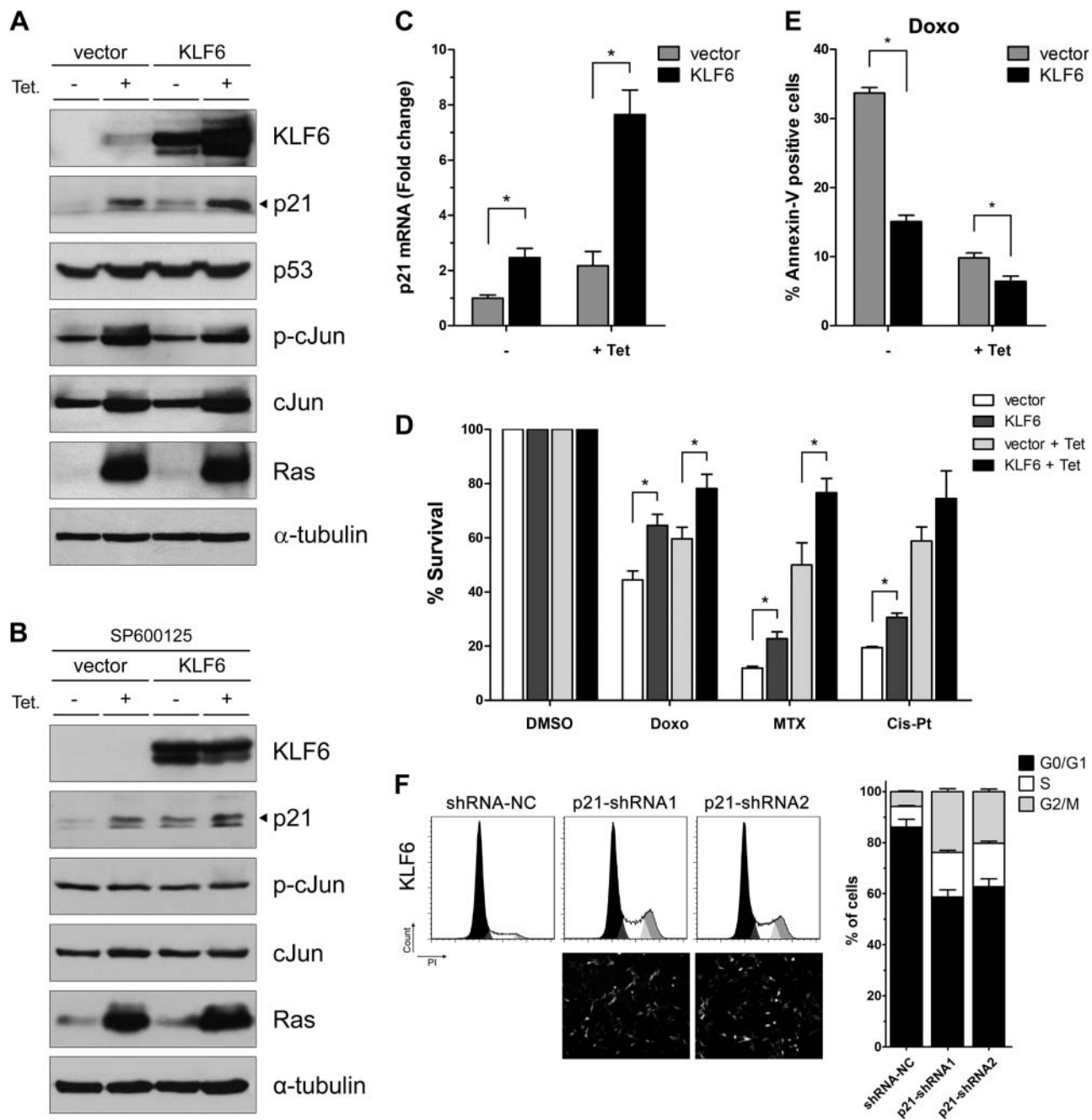
As mentioned before, KLF6-mediated growth suppression has been linked to repression of CCND1 function and induction of the CDK inhibitor p21 (9, 24). In this study, we observed that KLF6-transfected cells expressed higher levels of p21 compared with empty vector-transfected cells (Fig. 8A). Likewise, KLF6 expression correlated with higher amounts of p21 mRNA (Fig. 8C). No significant changes were observed on p53 protein (Fig. 8A) or mRNA expression (Supplemental Fig. S5), indicating that KLF6 mediates p21 up-regulation in a p53-independent fashion after expression of the Ras mutant. In addition, we observed lower levels of p21 in cells expressing H-Ras<sup>G12V</sup> when treated with SP600125 (Fig. 8B), correlating with the abrogation of KLF6-induced cell cycle arrest caused by JNK inhibition and with previous studies demonstrating that JNK mediates increased p21 protein stability by phosphorylation (32). We further evaluated the effect

of p21 knockdown in the KLF6-mediated cell cycle control. We observed that KLF6-induced cell cycle arrest was overridden by p21 knockdown, supporting the contribution of this CDK inhibitor in the antiproliferative response (Fig. 8F). Of interest, NIH3T3 cells overexpressing KLF6 showed a relative resistance to cell death caused by treatment with the chemotherapeutic drugs doxorubicin, cisplatin, and methotrexate, which trigger DNA damage, leading to apoptosis (Fig. 8D, E). This effect is consistent with the reduced fraction of cells reaching the S phase as a consequence of the G<sub>1</sub>-cell cycle arrest mediated by KLF6. Therefore, besides the cytostatic action, high KLF6 expression provides a selective survival advantage to transformed cells after treatment with DNA-damaging drugs.

## DISCUSSION

Oncogenesis is a stepwise process whereby normal cells are transformed into cancer cells. It is characterized by various genetic and epigenetic changes that uncouple the molecular network connecting proliferation and tumor suppression, thus reprogramming cells to undergo uncontrolled cell division. Ras mutations have





**Figure 8.** KLF6 increases p21 expression. *A*) Immunoblot analysis of whole-cell lysates from NIH3T3-TR-Ras<sup>G12V</sup> cells transfected with empty or KLF6 expression vectors, incubated in the presence or absence of tetracycline. *B*) Same experiments were also performed in cells treated with the JNK inhibitor SP600125. *C*) Levels of p21 mRNA quantified by qRT-PCR. Values were normalized with GAPDH expression. Data are expressed as fold increase  $\pm$  SD, with p21 expression in vector transfected cells arbitrarily set as 1. \* $P < 0.05$ . *D*) Cell viability of NIH3T3-TR-Ras<sup>G12V</sup> cells treated with DNA-damaging drugs. Cells transiently expressing KLF6 cultured in the presence or absence of tetracycline for 24 h were exposed to cisplatin (cis-Pt; 20  $\mu$ g/ml) or methotrexate (MTX; 100  $\mu$ g/ml) for 24 h or to doxorubicin (doxo; 1  $\mu$ M) for 48 h. Survival of cells treated with vehicle (DMSO) was established as 100%. \* $P < 0.05$ . *E*) Percentages of annexin-V-positive cells after 48 h of exposure to doxorubicin (1  $\mu$ M), determined by FACS analysis. \* $P < 0.05$ . *F*) Cell cycle distribution of NIH3T3-TR-Ras<sup>G12V</sup> cells transiently transfected with KLF6 in combination with control shRNA (shRNA-NC) or 2 different shRNA constructs targeting p21 (p21-shRNA1 and p21-shRNA2) coexpressing enhanced green fluorescent protein (eGFP). Cell cycle analysis was performed in cells gated on eGFP-positive populations. Representative cell cycle profiles (top panels), fluorescence microscopy images (bottom panels), and percentages of cells in each cell cycle phase (right panel) are shown.

been identified as one of the most prevalent genetic alterations in human malignancies, constituting a driver oncogenic event in most pancreatic, colorectal, and lung cancers, generally associated with limited

therapeutic options and poor survival (33). Although Ras isoforms have similar structural and biochemical properties, with all of them being capable of transforming cells, there is evidence indicating that may have

different functions or activities related to their preferential expression in different compartments. Indeed, gene inactivation studies in mice have revealed tissue specificity for the expression of different Ras isoforms throughout development, whereas H-Ras<sup>G12V</sup> shows the highest transformation potential in NIH3T3 fibroblasts and rat epithelial RIE-1 cells (16, 34). In the current study, we showed that KLF6 down-regulation predisposes to spontaneous cellular transformation and increases the tumorigenic potential of NIH3T3 cells, associated with a reduced expression of the CDK inhibitor p21. Using constitutive and tetracycline-inducible expression of the H-Ras<sup>G12V</sup> oncogene, we demonstrated that Ras activation leads to a progressive increase in endogenous KLF6 expression, dependent on JNK activity. In addition, ectopic expression of KLF6 following Ras activation induced G1 cell cycle arrest and decreased cell proliferation in correlation with increased p21 levels. We also showed that constitutive KLF6 expression reverses some transformed phenotypes typically induced by activated Ras, such as loss of density-dependent growth inhibition and colony formation in soft agar, and reduces the ability to form tumors when xenografted into immunodeficient mice.

Depending on the strength of the signal, the context in which the signaling occurs, and differences in cell type or tissue origin, oncogenic Ras signaling is capable of eliciting different biological outcomes. Ras proteins couple mitogenic stimuli to intracellular effectors; therefore, constitutive activation of Ras enhances the proliferative capacity of cells by up-regulation of several mitogenic transcription factors (35). These regulators increase the levels of CCND1 and suppress the CDK inhibitor p27, thereby promoting phosphorylation of Rb and leading to transition through the G1/S cell cycle checkpoint (35). However, although active Ras mutants can transform nearly all immortalized cell lines, the usual outcome of normal primary cells is death rather than survival, except in the presence of a cooperating oncogene such as *c-myc* or in association with the loss of certain tumor-suppressor genes such as p53 (36). Previous studies have indicated that expression of Ras oncogenes leads primary fibroblasts to premature senescence due to oncogenic stress associated with genome instability and activation of a robust DNA-damage response (DDR; ref. 37). Indeed, KLF6-induced cell cycle arrest may be part of its ability to disrupt the CCND1/CDK4 complex and to counteract c-Jun function, the major component of the AP-1 transcription factor, an important cooperating partner of oncogenic Ras in cell transformation (5, 38). Further, the phenotypic changes induced in KLF6-expressing NIH3T3 cells also superficially resembled those of senescent cells, which adopted a flat, enlarged morphology and ceased proliferation at subconfluent densities, despite the presence of serum. However, senescence-associated  $\beta$ -galactosidase activity was not detected (data not shown). Our results reveal insights into

novel functions of KLF6, including cell cycle arrest in the absence of cell death. Meanwhile, KLF6 interferes with oncogenic signaling triggered by activated Ras that is detrimental for tumor growth *in vivo*.

A complex interplay between different and divergent signaling pathways determines the capacity of the cancer cells to proliferate, survive, and invade. KLFs have been linked to carcinogenesis, counteracting, or cooperating with the process, acting both upstream and downstream of Ras signaling (30). For instance, elevated oncogenic K- or H-Ras expression correlates with increased *klf6* alternative splicing mediated by the PI3K/Akt pathway (17) and generates the isoform KLF6-SV1 which is associated with a dominant negative activity against wild-type KLF6 protein and a shorter metastasis-free survival correlating with elevated expression of epithelial-to-mesenchymal transition markers in breast cancer patients (17, 39). These findings, together with the results presented here, support an enhanced transcription of the KLF6 gene under H-Ras<sup>G12V</sup> expression, thus providing the basis for further investigation to elucidate the transcription factors that are involved in this regulatory circuitry.

Several studies have uncovered the key role of JNK in tumor development. JNK has been reported to act either as a tumor promoter or tumor suppressor in different types of cancer. Activated JNK is present in non-small-cell lung cancer and promotes oncogenesis in the bronchial epithelium (40). In line with that finding, JNK deficiency suppresses Ras-induced lung tumor formation (26). Further, JNK2-dependent signaling, conjointly with ERK, is essential for amplifying the gene expression that supports Ras transformation (41). In sharp contrast, lack of JNK activity promotes the growth of carcinogen-induced tumors in hepatic parenchymal cells, progression to prostatic adenocarcinoma in the context of PTEN-loss, and development of Ras-driven breast cancer in p53-null mice (42–44). In addition, JNK1-knockout mice are hypersensitive to chemical-induced skin papilloma and carcinoma formation (45). Moreover, a potent tumor suppressor function for the MKK7/JNK signaling axis was described in K-Ras<sup>G12D</sup>-induced lung cancer (46). Our observations associate the activation of the Ras/JNK pathway with the function of KLF6 to regulate cell cycle progression and carcinogenesis. Indeed, phosphorylation of p21 by JNK increases p21 protein stability (32), which would explain the requirement for JNK activation for KLF6-induced growth arrest. In addition, similar impairment in the G<sub>2</sub>/M transition on JNK inhibition in Ras-expressing cells has been described in several cancer cell lines, such as breast cancer (47), multiple myeloma (48), epithelial carcinoma (49), erythroleukemia (50), and human leukemia (51), consistent with the functional role of JNK during the G<sub>2</sub> phase and early mitosis (52).

Susceptibility to Ras-mediated transformation seems to rely on the requirement to bypass the INK4A–Rb and ARF–p53–p21 tumor-suppressor pathways (16). Since the NIH3T3 cell line harbors a deletion of the Ink4a/

Arf locus, which encodes the inhibitors of CDK4/6 p16 and p19 (53), KLF6-elicited p21 up-regulation emerges as an alternative mechanism to overcome the growth-promoting activity triggered by activated Ras. Thus, although transcriptional activation of p21 in response to a variety of stimuli, including DNA damage, is dependent on p53, Ras-mediated p21 induction may be p53-independent (54). KLF6 may contribute to p21 expression, directly recognizing the consensus Sp1-binding sites present in the proximal region of the p21 promoter and interacting with the Sp1 factor enhancing gene transcription, as previously reported (55). Nonetheless, given that KLF6 knockdown neither affected the acquired capabilities, nor p21 levels of Ras-transformed cells, we cannot formally exclude that other compensatory mechanisms may be involved. Cell cycle inhibition involving the association of p21 with cyclin-CDK complexes and proliferating cell nuclear antigen, matches with protection from apoptosis, implying that increased p21 expression maintains the transformed state providing an explanation for the low frequency of p21 loss in human tumors (32). In accordance with this view, H-Ras<sup>G12V</sup>-transformed cells expressing KLF6 showed increased survival upon treatment with DNA-damaging agents. Thus, although KLF6 expression inhibited tumors growth *in vivo*, our data suggest that high levels of KLF6 may also confer resistance to therapy based on the activation of the DDR.

In summary, in the current study, we further identified KLF6 function as a *bona fide* tumor suppressor, considering that its expression is responsive to oncogenic activation mediated by Ras and has the ability to prevent tumorigenesis. Our data also provide evidence of the involvement of KLF6 as a component of a tumor-surveillance system activated by oncogenic stress that plays an important role in tumor suppression. Further determination of the mechanisms modulating KLF6 expression and regulating its activity to control proper growing capacity of cells will help in identifying novel selective therapeutic approaches for cancer treatment. F

The authors thank Drs. R. Weinberg, S. Temple, and O. Coso for their kind gifts of plasmids; the entire Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI; Universidad Nacional de Córdoba, Córdoba, Argentina) team for their encouragement and support—particularly, Dr. J. P. Nicola for critically reading the manuscript; G. Panzetta-Dutari and L. Patrino for stimulating discussions; and P. Abadie, M. P. Crespo and F. Navarro for excellent technical assistance. This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica [Proyecto de Investigación en Ciencia y Tecnología (PICT) 2007-01630 and PICT 2011-0435]; Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba and Instituto Nacional del Cáncer-Argentina. L.D.T., V.A., and N.G.N. were supported by a CONICET fellowship. M.M. and J.L.B. are Investigator members of CONICET. The authors declare no conflicts of interest.

## REFERENCES

1. McConnell, B. B., and Yang, V. W. (2010) Mammalian Krüppel-like factors in health and diseases. *Physiol. Rev.* **90**, 1337–1381
2. Koritschoner, N. P., Bocco, J. L., Panzetta-Dutari, G. M., Dumur, C. I., Flury, A., and Patrino, L. C. (1997) A novel human zinc finger protein that interacts with the core promoter element of a TATA box-less gene. *J. Biol. Chem.* **272**, 9573–9580
3. Slavin, D., Sapin, V., López-Díaz, F., Jacquemin, P., Koritschoner, N., Dastugue, B., Davidson, I., Chatton, B., and Bocco, J. L. (1999) The Krüppel-like core promoter binding protein gene is primarily expressed in placenta during mouse development. *Biol. Reprod.* **61**, 1586–1591
4. Matsumoto, N., Kubo, A., Liu, H., Akita, K., Laub, F., Ramirez, F., Keller, G., and Friedman, S. L. (2006) Developmental regulation of yolk sac hematopoiesis by Kruppel-like factor 6. *Blood* **107**, 1357–1365
5. DiFeo, A., Martignetti, J. A., and Narla, G. (2009) The role of KLF6 and its splice variants in cancer therapy. *Drug Resist. Update* **12**, 1–7
6. Bureau, C., Hanoun, N., Torrisani, J., Vinel, J.-P., Buscail, L., and Cordelier, P. (2009) Expression and function of Kruppel like-factors (KLF) in carcinogenesis. *Curr. Genomics* **10**, 353–360
7. Kimmelman, A. C., Qiao, R. F., Narla, G., Banno, A., Lau, N., Bos, P. D., Nuñez Rodriguez, N., Liang, B. C., Guha, A., Martignetti, J. A., Friedman, S. L., and Chan, A. M. (2004) Suppression of glioblastoma tumorigenicity by the Kruppel-like transcription factor KLF6. *Oncogene* **23**, 5077–5083
8. Narla, G., Heath, K. E., Reeves, H. L., Li, D., Giono, L. E., Kimmelman, A. C., Glucksman, M. J., Narla, J., Eng, F. J., Chan, A. M., Ferrari, A. C., Martignetti, J. A., and Friedman, S. L. (2001) KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science* **294**, 2563–2566
9. Benzeno, S., Narla, G., Allina, J., Cheng, G. Z., Reeves, H. L., Banck, M. S., Odin, J. A., Diehl, J. A., Germain, D., and Friedman, S. L. (2004) Cyclin-dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1. *Cancer Res.* **64**, 3885–3891
10. Slavin, D. A., Koritschoner, N. P., Prieto, C. C., López-Díaz, F. J., Chatton, B., and Bocco, J. L. (2004) A new role for the Kruppel-like transcription factor KLF6 as an inhibitor of c-Jun proto-oncoprotein function. *Oncogene* **23**, 8196–8205
11. Andreoli, V., Gehrau, R. C., and Bocco, J. L. (2010) Biology of Krüppel-like factor 6 transcriptional regulator in cell life and death. *IUBMB Life* **62**, 896–905
12. Tahara, E., Kadara, H., Lacroix, L., Lotan, D., and Lotan, R. (2009) Activation of protein kinase C by phorbol 12-myristate 13-acetate suppresses the growth of lung cancer cells through KLF6 induction. *Cancer Biol. Ther.* **8**, 801–807
13. Fernandez-Zapico, M. E., Lomber, G. A., Tsuji, S., DeMars, C. J., Bardsley, M. R., Lin, Y.-H., Almada, L. L., Han, J.-J., Mukhopadhyay, D., Ordog, T., Buttar, N. S., and Urrutia, R. (2011) A functional family-wide screening of SP/KLF proteins identifies a subset of suppressors of KRAS-mediated cell growth. *Biochem. J.* **435**, 529–537
14. Sangodkar, J., Dhawan, N. S., Melville, H., Singh, V. J., Yuan, E., Rana, H., Izadmehr, S., Farrington, C., Mazhar, S., Katz, S., Albano, T., Arnovitz, P., Okrent, R., Ohlmeyer, M., Galsky, M., Burstein, D., Zhang, D., Politi, K., Difeo, A., and Narla, G. (2012) Targeting the FOXO1/KLF6 axis regulates EGFR signaling and treatment response. *J. Clin. Invest.* **122**, 2637–2651
15. Solomon, H., Brosh, R., Buginim, Y., and Rotter, V. (2010) Inactivation of the p53 tumor suppressor gene and activation of the Ras oncogene: cooperative events in tumorigenesis. *Discov. Med.* **9**, 448–454
16. Karnoub, A. E., and Weinberg, R. A. (2008) Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.* **9**, 517–531
17. Yea, S., Narla, G., Zhao, X., Garg, R., Tal-Kremer, S., Hod, E., Villanueva, A., Loke, J., Tarocchi, M., Akita, K., Shirasawa, S., Sasazuki, T., Martignetti, J. A., Llovet, J. M., and Friedman, S. L. (2008) Ras promotes growth by alternative splicing-mediated inactivation of the KLF6 tumor suppressor in hepatocellular carcinoma. *Gastroenterology* **134**, 1521–1531
18. Stewart, S. A., Dykxhoorn, D. M., Palliser, D., Mizuno, H., Yu, E. Y., An, D. S., Sabatini, D. M., Chen, I. S. Y., Hahn, W. C., Sharp, P. A., Weinberg, R. A., and Novina, C. D. (2003)



- Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* **9**, 493–501
19. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1999) The MKK7 gene encodes a group of c-Jun NH2-terminal kinase kinases. *Mol. Cell. Biol.* **19**, 1569–1581
  20. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gukkind, J. S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137–1146
  21. Fasano, C. A., Dimos, J. T., Ivanova, N. B., Lowry, N., Lemischka, I. R., and Temple, S. (2007) shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. *Cell Stem Cell* **1**, 87–99
  22. Racca, A. C., Camolotto, S. A., Ridano, M. E., Bocco, J. L., Genti-Raimondi, S., and Panzetta-Dutari, G. M. (2011) Krüppel-like factor 6 expression changes during trophoblast syncytialization and transactivates  $\beta$ hCG and PSG placental genes. *PLoS One* **6**, e22438
  23. Gehrau, R. C., D'Astolfo, D. S., Dumur, C. I., Bocco, J. L., and Koritschoner, N. P. (2010) Nuclear expression of KLF6 tumor suppressor factor is highly associated with overexpression of ERBB2 oncoprotein in ductal breast carcinomas. *PLoS One* **5**, e8929
  24. Narla, G., Kremer-Tal, S., Matsumoto, N., Zhao, X., Yao, S., Kelley, K., Tarocchi, M., and Friedman, S. L. (2007) In vivo regulation of p21 by the Kruppel-like factor 6 tumor-suppressor gene in mouse liver and human hepatocellular carcinoma. *Oncogene* **26**, 4428–4434
  25. Pruitt, K., Pruitt, W. M., Bilter, G. K., Westwick, J. K., and Der, C. J. (2002) Raf-independent deregulation of p38 and JNK mitogen-activated protein kinases are critical for Ras transformation. *J. Biol. Chem.* **277**, 31808–31817
  26. Cellurale, C., Sabio, G., Kennedy, N. J., Das, M., Barlow, M., Sandy, P., Jacks, T., and Davis, R. J. (2011) Requirement of c-Jun NH(2)-terminal kinase for Ras-initiated tumor formation. *Mol. Cell. Biol.* **31**, 1565–1576
  27. Der, C. J., and Van Dyke, T. (2007) Stopping ras in its tracks. *Cell* **129**, 855–857
  28. Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239–252
  29. Wagner, E. F., and Nebreda, A. R. (2009) Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat. Rev. Cancer* **9**, 537–549
  30. Tetreault, M.-P., Yang, Y., and Katz, J. P. (2013) Krüppel-like factors in cancer. *Nat. Rev. Cancer* **13**, 701–713
  31. Jacobsen, K., Groth, A., and Willumsen, B. M. (2002) Ras-inducible immortalized fibroblasts: focus formation without cell cycle deregulation. *Oncogene* **21**, 3058–3067
  32. Jung, Y.-S., Qian, Y., and Chen, X. (2010) Examination of the expanding pathways for the regulation of p21 expression and activity. *Cell. Signal.* **22**, 1003–1012
  33. Cox, A. D., and Der, C. J. (2010) Ras history: the saga continues. *Small GTPases* **1**, 2–27
  34. Li, W., Zhu, T., and Guan, K.-L. (2004) Transformation potential of Ras isoforms correlates with activation of phosphatidylinositol 3-kinase but not ERK. *J. Biol. Chem.* **279**, 37398–37406
  35. Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2011) RAS oncogenes: weaving a tumorigenic web. *Nat. Rev. Cancer* **11**, 761–774
  36. Frame, S., and Balmain, A. (2000) Integration of positive and negative growth signals during ras pathway activation in vivo. *Curr. Opin. Genet. Dev.* **10**, 106–113
  37. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A., Maestro, R., Pelicci, P. G., and d'Adda di Fagnana, F. (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444**, 638–642
  38. Johnson, R., Spiegelman, B., Hanahan, D., and Wisdom, R. (1996) Cellular transformation and malignancy induced by ras require c-jun. *Mol. Cell. Biol.* **16**, 4504–4511
  39. Hatami, R., Sieuwerts, A. M., Izadmehr, S., Yao, Z., Qiao, R. F., Papa, L., Look, M. P., Smid, M., Ohlssen, J., Levine, A. C., Germain, D., Burstein, D., Kirschenbaum, A., DiFeo, A., Foekens, J. A., and Narla, G. (2013) KLF6-SV1 drives breast cancer metastasis and is associated with poor survival. *Sci. Transl. Med.* **5**, 169ra12
  40. Khatlani, T. S., Wislez, M., Sun, M., Srinivas, H., Iwanaga, K., Ma, L., Hanna, A. E., Liu, D., Girard, L., Kim, Y. H., Pollack, J. R., Minna, J. D., Wistuba, I. I., and Kurie, J. M. (2007) c-Jun N-terminal kinase is activated in non-small-cell lung cancer and promotes neoplastic transformation in human bronchial epithelial cells. *Oncogene* **26**, 2658–2666
  41. Mathiasen, D. P., Egebjerg, C., Andersen, S. H., Rafn, B., Puustinen, P., Khanna, A., Daugaard, M., Valo, E., Tuomela, S., Bøttzauw, T., Nielsen, C. F., Willumsen, B. M., Hautaniemi, S., Laheesmaa, R., Westermarck, J., Jäättelä, M., and Kallunki, T. (2012) Identification of a c-Jun N-terminal kinase-2-dependent signal amplification cascade that regulates c-Myc levels in ras transformation. *Oncogene* **31**, 390–401
  42. Das, M., Garlick, D. S., Greiner, D. L., and Davis, R. J. (2011) The role of JNK in the development of hepatocellular carcinoma. *Genes Dev.* **25**, 634–645
  43. Hübner, A., Mulholland, D. J., Standen, C. L., Karasarides, M., Cavanagh-Kyros, J., Barrett, T., Chi, H., Greiner, D. L., Tournier, C., Sawyers, C. L., Flavell, R. A., Wu, H., and Davis, R. J. (2012) JNK and PTEN cooperatively control the development of invasive adenocarcinoma of the prostate. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 12046–12051
  44. Cellurale, C., Girmius, N., Jiang, F., Cavanagh-Kyros, J., Lu, S., Garlick, D. S., Mercurio, A. M., and Davis, R. J. (2012) Role of JNK in mammary gland development and breast cancer. *Cancer Res.* **72**, 472–481
  45. She, Q., Chen, N., Bode, A. M., Flavell, R. A., and Dong, Z. (2002) Deficiency of c-Jun-NH(2)-terminal kinase-1 in mice enhances skin tumor development by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.* **62**, 1343–1348
  46. Schramek, D., Kotsinas, A., Meixner, A., Wada, T., Elling, U., Pospisilik, J. A., Neely, G. G., Zwick, R.-H., Sigl, V., Forni, G., Serrano, M., Gorgoulis, V. G., and Penninger, J. M. (2011) The stress kinase MKK7 couples oncogenic stress to p53 stability and tumor suppression. *Nat. Genet.* **43**, 212–219
  47. Mingo-Sion, A. M., Marietta, P. M., Koller, E., Wolf, D. M., and Van Den Berg, C. L. (2004) Inhibition of JNK reduces G2/M transit independent of p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells. *Oncogene* **23**, 596–604
  48. Hideshima, T., Hayashi, T., Chauhan, D., Akiyama, M., Richardson, P., and Anderson, K. (2003) Biologic sequelae of c-Jun NH(2)-terminal kinase (JNK) activation in multiple myeloma cell lines. *Oncogene* **22**, 8797–8801
  49. Du, L., Lyle, C. S., Obey, T. B., Gaarde, W. A., Muir, J. A., Bennett, B. L., and Chambers, T. C. (2004) Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: evidence that mitotic Bcl-2 phosphorylation is JNK-independent. *J. Biol. Chem.* **279**, 11957–11966
  50. Nishigaki, K., Hanson, C., Thompson, D., Yugawa, T., and Ruscetti, S. (2005) Activation of the Jun N-terminal kinase pathway by friend spleen focus-forming virus and its role in the growth and survival of friend virus-induced erythroleukemia cells. *J. Virol.* **79**, 12752–12762
  51. Moon, D. O., Kim, M. O., Kang, C. H., Lee, J. D., Choi, Y. H., and Kim, G. Y. (2009) JNK inhibitor SP600125 promotes the formation of polymerized tubulin, leading to G2/M phase arrest, endoreduplication, and delayed apoptosis. *Exp. Mol. Med.* **41**, 665–677
  52. Gutierrez, G. J., Tsuji, T., Cross, J. V., Davis, R. J., Templeton, D. J., Jiang, W., and Ronai, Z. A. (2010) JNK-mediated phosphorylation of Cdc25C regulates cell cycle entry and G(2)/M DNA damage checkpoint. *J. Biol. Chem.* **285**, 14217–14228
  53. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995) Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83**, 993–1000
  54. Abbas, T., and Dutta, A. (2009) p21 in cancer: intricate networks and multiple activities. *Nat. Rev. Cancer* **9**, 400–414
  55. Kivinen, L., Tsubari, M., Haapajarvi, T., Datto, M. B., Wang, X. F., and Laiho, M. (1999) Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1-binding sites. *Oncogene* **18**, 6252–6261

Received for publication February 14, 2014.

Accepted for publication August 26, 2014.