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Oncogenic *KRAS* and *BRAF* Differentially Regulate Hypoxia-Inducible Factor-1α and -2α in Colon Cancer

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Abstract

***KRAS* and *BRAF* mutations are frequently observed in human colon cancers. These mutations occur in a mutually exclusive manner, and each is associated with distinctive biological features. We showed previously that K-ras can interact with hypoxia to activate multiple signaling pathways. Many hypoxic responses are mediated by hypoxia-inducible factor (HIF)-1α and HIF-2α, and we sought to define the roles of mutant *KRAS* and *BRAF* in the induction of HIF-1α and HIF-2α in colon cancer cells. Ectopic expression of mutant K-ras in Caco2 cells enhanced the hypoxic induction of only HIF-1α, whereas mutant BRAF enhanced both HIF-1α and HIF-2α. Knockout or knockdown of mutant *KRAS* in DLD-1 and HCT116 cells impaired the hypoxic induction of only HIF-1α. HIF-1α mRNA levels were comparable in cells with and without a *KRAS* mutation. However, the rate of HIF-1α protein synthesis was higher in cells with a *KRAS* mutation, and this was suppressed by the phosphoinositide 3-kinase inhibitor LY294002. In contrast, knockdown of mutant BRAF in HT29 cells suppressed both HIF-1α and HIF-2α. Although BRAF regulated mRNA levels of both HIF-1α and HIF-2α, knockdown of BRAF or treatment with the MEK inhibitor PD98059 impaired the translation of only HIF-2α. Our data reveal that oncogenic *KRAS* and *BRAF* mutations differentially regulate the hypoxic induction of HIF-1α and HIF-2α in colon cancer, and this may potentially contribute to the phenotypic differences of *KRAS* and *BRAF* mutations in colon tumors.** [Cancer Res 2009;69(21):8499–506]

Introduction

Activating mutations of *KRAS* are found in ~45% of colorectal cancers (1, 2). Oncogenic activation of *KRAS* can influence several cellular processes that regulate morphology, proliferation, and motility, and *KRAS* mutations are clinically associated with a poor prognosis (3, 4). K-ras interacts with several effector proteins, including Raf kinases and phosphoinositide 3-kinases (PI3K). *BRAF*, one of the Raf kinases, is also mutated in ~15% of colorectal cancers (5–7). Interestingly, *KRAS* and *BRAF* mutations are mutually exclusive (5–7), suggesting that they may have similar functions. However, the biological properties of tumors with *KRAS* or *BRAF* mutations are quite distinctive. For example, *BRAF* mutant tumors are more likely to exhibit microsatellite instability, poor histologic grade, mucinous histology, and a better prognosis (7, 8). However, it remains unclear how these oncogenes that signal through the same pathways can give rise to such distinctive tumor phenotypes.

As tumor cells proliferate, their oxygen and nutrient demands increase, and cells adapt to this hypoxic environment through a switch to anaerobic glycolysis and induction of survival factors and angiogenic growth factors such as vascular endothelial growth factor (9). Hypoxia-inducible factors (HIF) are thought to play a major role in controlling the transcriptional responses to hypoxia (10, 11). HIF-1 was the first HIF isoform to be recognized, and it is composed of two subunits, HIF-1α and HIF-1β. HIF-2α and HIF-3α were identified by homology searches or screens for interaction partners with HIF-1β (12). Whereas HIF-3α is the most distantly related isoform, HIF-1α and HIF-2α are closely related and both activate hypoxia-responsive element–dependent gene expression. Although the two isoforms are frequently expressed in human cancers, their functions vary. In colon cancer, we have reported previously that HIF-1α may promote the growth of cancer cells both *in vitro* and *in vivo*. However, HIF-2α appears to restrain tumor growth, and loss of expression of HIF-2α but not HIF-1α in human colon cancer tissues is strongly correlated with advanced tumor stage (13). The mechanisms that regulate the expression of HIF-1α versus HIF-2α in colon cancer are unknown.

Under normoxic conditions, HIF-1α and HIF-2α are hydroxylated by O_2-dependent prolyl hydroxylases within the oxygen-dependent degradation domain and targeted by the von Hippel-Lindau protein (pVHL) E3 ubiquitin ligase complex, leading to proteasomal degradation. Under hypoxic conditions, prolyl hydroxylase activity is inhibited, thereby allowing stabilization and accumulation of HIF-1α and HIF-2α protein (12). In addition to hypoxia, HIF-1α expression can be regulated by certain oncogenic pathways. For instance, insulin, insulin-like growth factor, epidermal growth factor, and fibroblast growth factor can induce expression of HIF-1α protein in normoxia via mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways (14–16). Transformation by H-ras or mutant K-ras has also been shown to enhance the levels of HIF-1α protein (17, 18). In melanoma cells, mutant *BRAF* (V600E) can enhance HIF-1α expression in normoxia (19). However, the relevance of these mechanisms in hypoxia as well as the effects on the HIF-2α isoform are unclear. We therefore sought to identify the roles of mutant *KRAS* and *BRAF* on the hypoxic induction of HIF-1α and HIF-2α in colon cancer.

Materials and Methods

Cell culture. DLD-1, HCT116, HT29, Caco2, DKs-5, DKO-3, HK2-10, and HKe-3 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Hypoxic conditions were achieved by culturing cell lines in a sealed hypoxia chamber (Billups-Rothenberg) after flushing with a mixture of 1% or 5% O_2 and 5% CO_2 balanced with N_2 as described previously (20).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Plasmid construction and establishment of stable cells. Lentivirus vector pHR-SIN-CSGW GFP-LC3 was kindly provided by Dr. Ramnik Xavier and the GFP-LC3 fragment was excised with *Bam*HI and *Not*I (21). Human K-ras and BRAF cDNA were amplified by reverse transcription-PCR (RT-PCR) using RNA from Caco2 cells for wild-type K-ras, SW480 cells for mutant K-ras V12, DLD-1 cells for wild-type BRAF, and HT29 cells for mutant BRAF V600E. The PCR primers used were 5′-AAGGAAGGATCCAGGCCTGCTGAAAATGACTG-3′ and 5′-AACCAAGCGGCCGCAAGGCATCATCAACACCCAG-3′ for K-ras and 5′-AAGGAAAGATCTTCTCGGTTATAAGATGGCGG-3′ and 5′-AACCAAGCGGCCGCTCTCCTGAACTCTCTCACTC-3′ for BRAF. The PCR-amplified products were digested with *Bam*HI and *Not*I for K-ras and *Bgl*II and *Not*I for BRAF and subcloned into the pCSGW vector.

pCSGW K-ras wild-type, K-ras V12, BRAF wild-type, BRAF V600E, or empty vector were introduced along with packaging plasmid pCMV-dR8.91 and envelope plasmid pMD2.G into HEK293T cells by transfection with the Fugene 6 regent (Roche). The culture medium containing lentivirus was harvested and filtered, and polybrene was added at 8 μg/mL. Caco2 cells were infected with the virus for 48 h.

pSicoR shBRAF plasmids were kindly provided by Dr. Kevin M. Haigis. pSicoR shBRAF or empty vector was introduced into HEK293T cells and lentivirus was harvested as described above. HT29, DLD-1, and Caco2 cells were infected with the virus and then selected by puromycin resistance.

RNA interference. Cells were transfected with 20 nmol/L small interfering RNA (siRNA) duplex oligos using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Nucleotide sequences of siRNAs were as follows: 5′-r(GGUGACUUAGGUUCUAGAU)d(TT)-3′ for K-ras #1, 5′-r(GGAAGCAAGUAGUAAUUGA)d(TT)-3′ for K-ras #2, 5′-r(GGCCCUAUUGGACAAAUUU)d(TT)-3′ for BRAF #1, 5′-r(GGAGGUGUGGAAUAUCAAA)d(TT)-3′ for BRAF #2, and 5′-r(GCGCGCUUUGUAGGAUUCG)d(TT)-3′ for control.

Quantitative PCR analysis. Total RNA was isolated from cultured cells using the Isogen kit (Wako) and reverse transcription with random hexanucleotide primers and SuperScript reverse transcriptase III (Invitrogen) was done. The resulting cDNA was amplified by real-time PCR using the iQ5 Real-time PCR Detection System (Bio-Rad) and Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences were 5′-GGCGCGAACGACAAGAAAAAG-3′ and 5′-CCTTATCAAGATGCGAACTCACA-3′ for HIF-1α, 5′-GGAGGTGTTCTATGAGCTGG-3′ and 5′-GACAGAAAGATCATGTCGCCA-3′ for HIF-2α, 5′-AGGCCAGCACATAGGAGAGA-3′ and 5′-TTTCTTGCGCTTTCGTTTTT-3′ for vascular endothelial growth factor, and 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′ for 18S rRNA. Transcript levels of HIF-1α, HIF-2α, and vascular endothelial growth factor were normalized to 18S rRNA.

Western blotting. Cells were lysed in chilled lysis buffer supplemented with the Complete protease inhibitor cocktail (Roche). Protein extracts (50 μg) were resolved on a NuPAGE 3% to 8% Tris-acetate or 10% Bis-Tris SDS-polyacrylamide gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Millipore). The blots were probed with mouse monoclonal anti–HIF-1α (BD Transduction Laboratories; 1:500), rabbit polyclonal anti–HIF-2α (Novus; 1:500), mouse monoclonal anti–K-ras (Santa Cruz Biotechnology; 1:2,000), mouse monoclonal anti-BRAF (Santa Cruz Biotechnology; 1:10,000), rabbit polyclonal anti–phospho-p44/42 kinase (Cell Signaling; 1:2,000), rabbit polyclonal anti-p44/42 kinase (Cell Signaling; 1:2,000), rabbit polyclonal anti–phospho-Akt (Cell Signaling; 1:1,000), rabbit polyclonal anti-Akt (Cell Signaling; 1:1,000), mouse monoclonal anti-pVHL (BD Pharmingen; 1:500), and anti-β-actin (Sigma; 1:500,000) antibodies. Immunoreactive proteins were visualized using the Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences).

MG132 and cycloheximide treatment. Cells were treated with the proteasome inhibitor MG132 (Calbiochem; 10 μmol/L) for the indicated times. HIF-1α and HIF-2α proteins were measured by Western blotting. Band densities were quantified using ImageJ image analysis software and HIF protein level was normalized to β-actin at each time point. Translation of HIF was measured by its accumulation during MG132 treatment. Cells were cultured in hypoxia for 6 h and the medium was then switched to cycloheximide-containing medium under normoxia for the indicated times. HIF-1α and HIF-2α proteins were measured by Western blotting, and protein stability of HIF was measured by its degradation during cycloheximide treatment.

Statistical analysis. Statistical differences were analyzed by the Student's *t* test, and *P* values < 0.05 were considered statistically significant.

Results

Mutant *KRAS* enhances the hypoxic induction of HIF-1α, but mutant *BRAF* induces both HIF-1α and HIF-2α. To determine whether activating mutations of *KRAS* or *BRAF* regulate the hypoxic induction of HIF-1α and HIF-2α, lentiviral vectors expressing wild-type K-ras, mutant K-ras (V12), wild-type BRAF, or mutant BRAF (V600E) were transduced into Caco2 cells that do not carry mutations in either *KRAS* or *BRAF* gene (Fig. 1*A*). Stably transfected

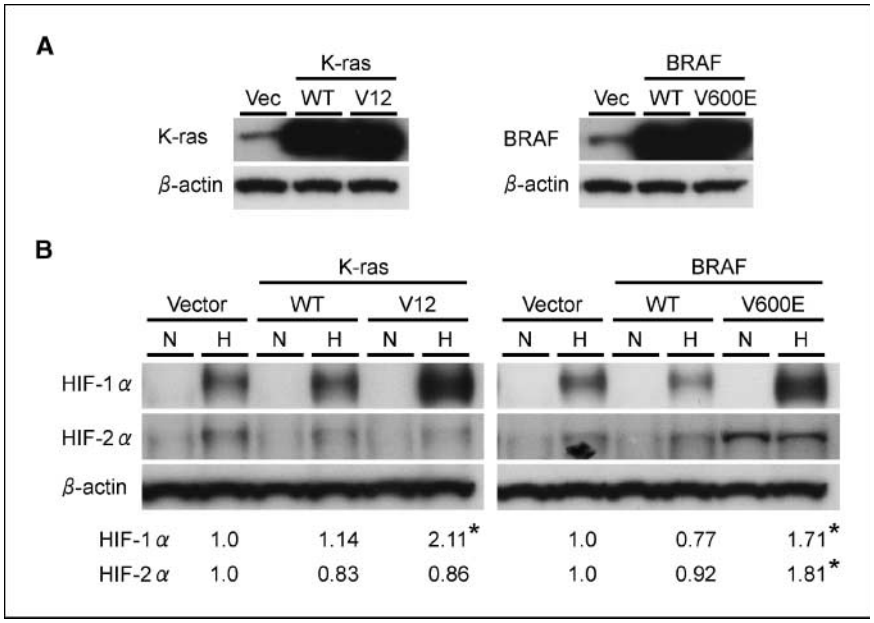

Figure 1. Overexpression of K-ras or BRAF and hypoxic induction of HIF proteins. Wild-type (*WT*) K-ras, mutant (V12) K-ras, wild-type BRAF, mutant (V600E) BRAF, or empty vector (*Vec*) were lentivirally transduced into Caco2 cells. *A,* K-ras and BRAF expression was confirmed by Western blotting. *B,* cells were incubated in either normoxia (*N*) or hypoxia (*H*) for 12 h and HIF-1α and HIF-2α protein induction was analyzed by Western blotting. Band densities were quantified and HIF protein levels were normalized to β-actin. *Numbers at the bottom,* mean fold change relative to vector control in hypoxia for two independent experiments. *, $P < 0.05$.

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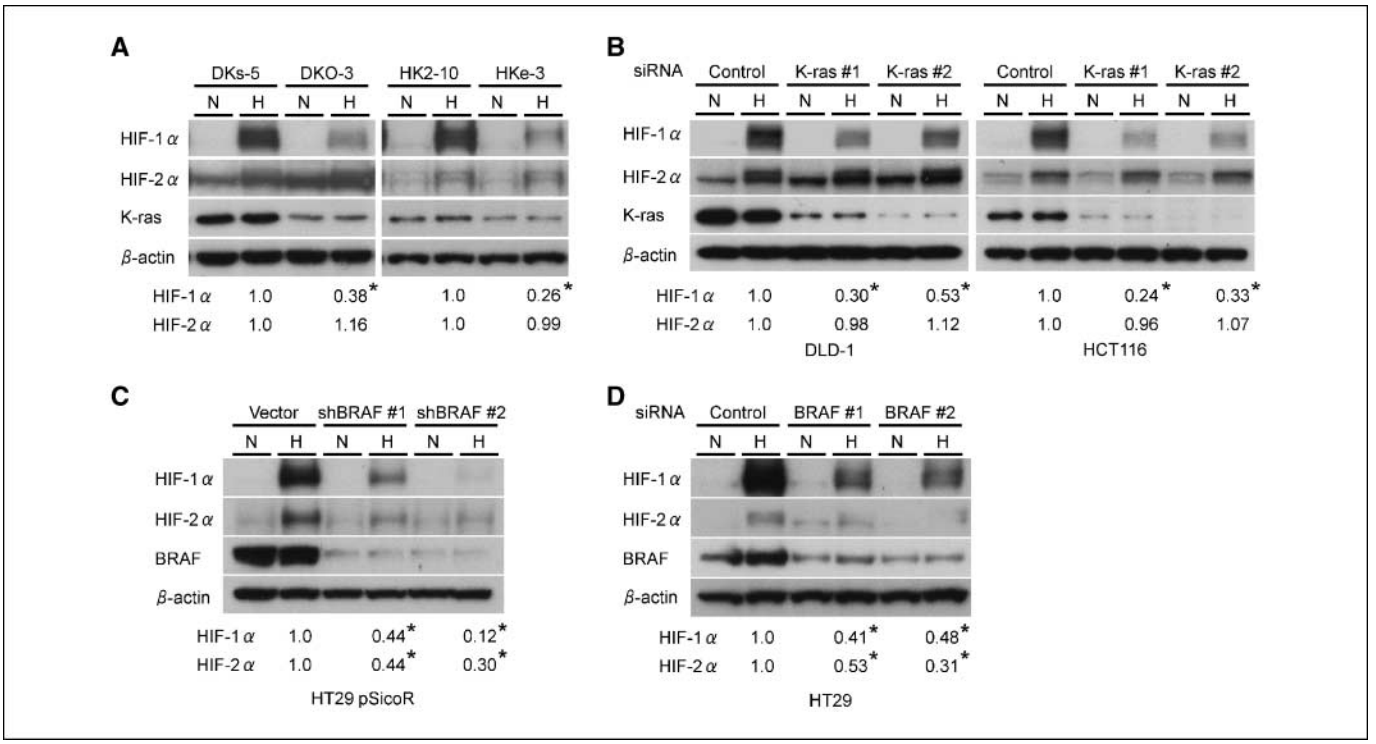

Figure 2. Depletion of mutant *KRAS* and *BRAF* oncogenes and hypoxic induction of HIF proteins. Hypoxic induction of HIF-1α and HIF-2α proteins was analyzed by Western blotting. *A,* DKs-5 and DKO-3 and HK2-10 and HKe-3 cells were incubated in either normoxia or hypoxia for 12 h. The weak K-ras bands seen in the DKO-3 and Hke-3 cells correspond to the remaining wild-type *KRAS* allele. *B,* DLD-1 and HCT116 cells were treated with siRNA targeting K-ras (K-ras #1 and #2) or control siRNA. Thirty-six hours after transfection, cells were subjected to either normoxia or hypoxia for 12 h. *C,* short hairpin RNA against BRAF (shBRAF #1 and #2) or empty vector was lentivirally transduced into HT29 cells. Stable cells (HT29 pSicoR) were incubated in either normoxia or hypoxia. *D,* HT29 cells were treated with siRNA targeting BRAF (BRAF #1 and #2) or control siRNA, and cells were subjected to either normoxia or hypoxia. Band densities were quantified and HIF protein levels were normalized to β-actin. *Numbers at the bottom,* mean fold change in hypoxia relative to control for two independent experiments. *, $P < 0.05$.

cells were cultured in either normoxia or hypoxia for 12 h, and hypoxic induction of HIF proteins was analyzed by Western blotting. The induction of HIF-1α was enhanced by ectopic expression of K-ras (V12) or BRAF (V600E; 2.11- and 1.71-fold, respectively; both $P < 0.05$) in hypoxic conditions (Fig. 1*B*). However, overexpression of wild-type K-ras or wild-type BRAF did not increase levels of either HIF isoform. Interestingly, BRAF (V600E) enhanced HIF-2α expression in hypoxia (1.81-fold; $P < 0.05$) but also induced expression in normoxic conditions. K-ras (V12) did not upregulate HIF-2α in either condition (Fig. 1*B*).

We then evaluated whether the hypoxic induction of HIF-1α and HIF-2α was regulated by endogenous *KRAS* mutations in colon cancer cells. DKO-3 and HKe-3 cells are derived from DLD-1 and HCT116 cells, respectively, in which the endogenous mutant *KRAS* allele was disrupted by homologous recombination, and control DKs-5 and HK2-10 cells have a mutant *KRAS* allele (22). These cells were incubated in either normoxia or hypoxia. HIF-1α induction was impaired by genetic disruption of mutant *KRAS* in both DKO-3 and HKe-3 cells (62% and 74% reduction, respectively; both $P < 0.05$), whereas the induction of HIF-2α was unaffected (Fig. 2*A*). To verify this observation, DLD-1 and HCT116 cells were transfected with two independent siRNA oligos targeting K-ras and then cultured in either normoxia or hypoxia. The hypoxic induction of HIF-1α was suppressed by knockdown of K-ras by 47% to 76% ($P < 0.05$), whereas HIF-2α was unaffected (Fig. 2*B*). Interestingly, knockdown of wild-type K-ras only slightly suppressed HIF-1α expression by 15% to 26% (P = nonsignificant) in Caco2 and HT29 cells that do not carry a *KRAS* mutation (Supplementary Fig. S1*A*). These data suggest that activating mutations in the *KRAS* gene play an important role in enhancing the hypoxic induction of HIF-1α but not HIF-2α.

To address the role of endogenous mutant *BRAF* (V600E) in HT29 cells, we established stable knockdown cells using the pSicoR lentivirus expressing BRAF short hairpin RNA. The stable cells (HT29 pSicoR) were incubated in either normoxia or hypoxia. Hypoxic induction of both HIF-1α and HIF-2α was impaired by the stable knockdown of BRAF by 56% to 88% and 56% to 70% (both $P < 0.05$), respectively (Fig. 2*C*). This suppression of HIF-1α and HIF-2α was also observed when BRAF was transiently knocked down by siRNA in HT29 cells (52-59% and 47-69% reductions, respectively; both $P < 0.05$; Fig. 2*D*). Interestingly, stable knockdown of wild-type BRAF in DLD-1 and Caco2 cells that do not carry a *BRAF* mutation minimally suppressed HIF-1α expression by 21% and HIF-2α expression by 20% to 29% (Supplementary Fig. S1*B*). These data indicate that activating mutations in *BRAF* play an important role in enhancing the hypoxic induction of both HIF-1α and HIF-2α.

Mutant *KRAS* does not regulate HIF-1α mRNA expression in either normoxia or hypoxia. We next addressed the mechanisms by which mutant *KRAS* enhances the hypoxic induction of HIF-1α. DKs-5, DKO-3, HK2-10, and HKe-3 cells were cultured in normoxia and relative levels of HIF-1α mRNA were measured by quantitative PCR. HIF-1α mRNA levels were comparable between DKs-5 and DKO-3 cells as well as between HK2-10 and HKe-3 cells (Fig. 3*A*). DLD-1 and HCT116 cells were treated with siRNA oligos against K-ras, and HIF-1α mRNA levels were measured. HIF-1α mRNA expression was not affected by knockdown of K-ras in either cell line

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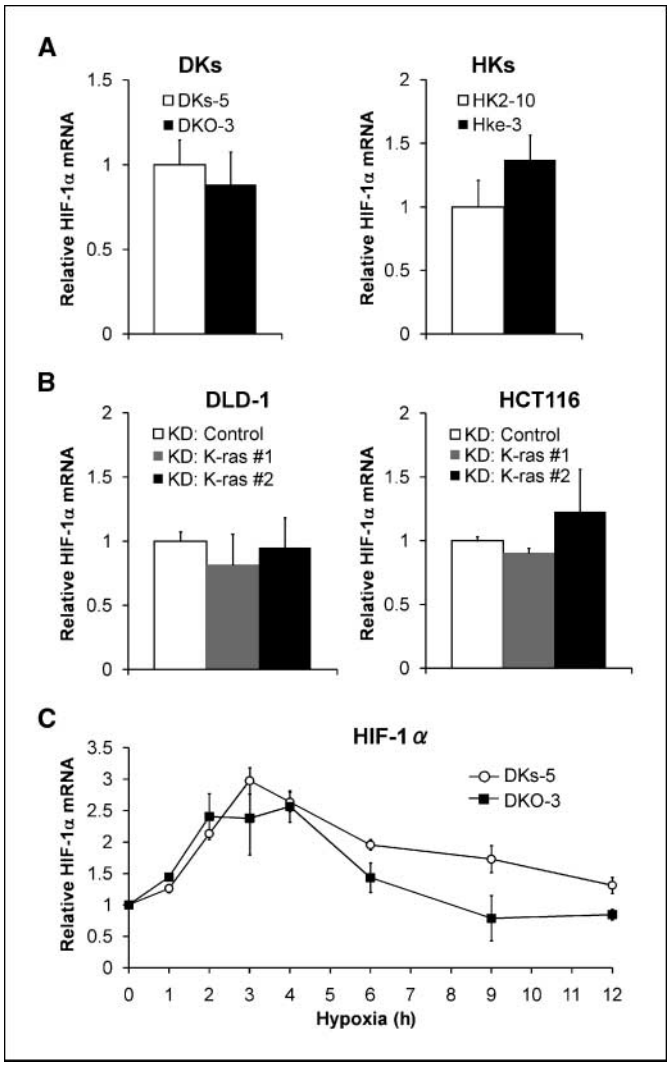

Figure 3. Depletion of mutant K-ras and mRNA expression of HIF-1α. Total RNA was extracted from DKs-5 and DKO-3 cells (*DKs*) or HK2-10 and HKe-3 (*HKs*) cultured in normoxia (*A*) or DLD-1 and HCT116 cells treated with control siRNA or K-ras siRNA (*B*). Relative levels of HIF-1α mRNA were determined by quantitative reverse transcription-PCR. Data were normalized to the levels of 18S rRNA. All reactions were done in triplicate. Mean ± SD for three different cDNA samples. *C,* DKs-5 and DKO-3 cells were incubated in hypoxia for the indicated times. Relative levels of HIF-1α mRNA were determined by quantitative reverse transcription-PCR. Data were normalized to the levels of 18S rRNA at each time point. Mean ± SD relative to time 0 in each cell line for two different cDNA samples.

(Fig. 3*B*). It has been shown previously that HIF-1α mRNA can be induced early in the response to hypoxia (0.5-3 h) in pulmonary artery smooth muscle cells (23). To investigate whether the hypoxic regulation of HIF-1α mRNA in colon cancer may also be time-dependent and whether this can be affected by mutant *KRAS*, DKs-5 and DKO-3 cells were cultured in hypoxia for 1 to 12 h. HIF-1α mRNA was induced ~3-fold at 3 h after exposure to hypoxia in both DKs-5 and DKO-3 cells, and there were no significant differences in mRNA levels at any of the time points analyzed (P = nonsignificant; Fig. 3*C*). These data suggest that mutant *KRAS* does not regulate HIF-1α mRNA expression in either normoxia or hypoxia.

Mutant *KRAS* regulates the translation of HIF-1α through the PI3K pathway. Although the levels of mRNA were similar, HIF-1α protein was induced by hypoxia to a greater extent in DKs-5 compared with DKO-3 cells (Supplementary Fig. S2*A*). This resulted in higher induction of vascular endothelial growth factor mRNA (Supplementary Fig. S2*B*). These data suggest that mutant *KRAS* may not play a key role in the hypoxic induction of HIF-1α mRNA but may regulate the accumulation of HIF-1α protein at the translational level. To further address this hypothesis, DKs-5 and DKO-3 cells were treated with MG132 for 0, 10, 20, and 30 min to block proteasomal degradation of HIF-1α protein. The rate of translation of HIF-1α in normoxia was measured by its accumulation during MG132 treatment. The rate of HIF-1α protein synthesis was significantly lower in DKO-3 cells (45% and 39% reductions at 10 and 20 min, respectively; both $P < 0.05$) compared with DKs-5 cells (Fig. 4*A*). DLD-1 cells were then treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY294002 for 9 h followed by MG132 treatment. HIF-1α protein synthesis was inhibited by LY294002 (63% and 55% reductions at 20 and 30 min, respectively; both $P < 0.05$) but not PD98059 (Fig. 4*B*). DLD-1 cells were treated with PD98059 or LY294002 and then incubated in hypoxia for 6 h. Hypoxic induction of HIF-1α protein was reduced by 61% ($P < 0.05$) with LY294002 but not PD98059 despite the activation of extracellular signal-regulated kinase (ERK) in hypoxia (Fig. 4*C*). These data suggest that mutant *KRAS* may regulate the translation of HIF-1α through the PI3K pathway.

HIF-1α protein is unstable in normoxic conditions, and the half-life of HIF-1α protein is estimated at 2 to 5 min. To examine whether mutant *KRAS* affects the stability of HIF-1α protein, DKs-5 and DKO-3 cells were incubated in hypoxia for 6 h and then cultured in the presence of the translational inhibitor cycloheximide. The half-life of HIF-1α protein was comparable between DKs-5 and DKO-3 cells (Supplementary Fig. S3*A*). Furthermore, pVHL protein levels were not affected by either knockout of mutant *KRAS* or knockdown of K-ras in DLD-1 and HCT116 cells (Supplementary Fig. S3*B*). These data indicate that mutant *KRAS* does not regulate HIF-1α protein stability.

Mutant *BRAF* enhances mRNA expression of HIF-1α and HIF-2α in normoxia. We next addressed the mechanisms by which mutant *BRAF* enhances the hypoxic induction of both HIF-1α and HIF-2α. HT29 pSicoR cells were cultured in normoxia and HIF mRNA levels were measured. The mRNA levels of both HIF-1α and HIF-2α were significantly reduced in BRAF knockdown cells (57-72% reduction in HIF-1α ($P < 0.05$) and 80-89% reduction in HIF-2α ($P < 0.005$) compared with control cells (Fig. 5*A*). BRAF signals through MAPK, and we tested the significance of this pathway with the MEK inhibitor PD98059. The mRNA levels of both HIF-1α and HIF-2α in HT29 cells were suppressed by PD98059 [33% reduction in HIF-1α ($P < 0.05$) and 43% reduction in HIF-2α ($P < 0.005$); Fig. 5*B*]. To investigate whether the early induction of HIF mRNA is enhanced by mutant BRAF, HT29 pSicoR cells were cultured in hypoxia for 1 to 12 h. HIF-1α and HIF-2α mRNA were induced 2- and 2.5-fold, respectively, at 3 h after exposure to hypoxia, but these changes were not affected by knockdown of mutant BRAF (Fig. 5*C*). These data suggest that although mutant *BRAF* can enhance HIF-1α and HIF-2α mRNA expression in basal normoxic conditions, it does not enhance the induction of HIF mRNA in hypoxia.

Mutant *BRAF* enhances the translation of HIF-2α through the MAPK pathway. Although there were no changes in the hypoxic induction of HIF mRNAs, mutant *BRAF* did regulate the hypoxic induction of HIF-1α and HIF-2α protein (Supplementary Fig. S2*C*), which was accompanied by an increase in the levels of vascular endothelial growth factor mRNA (Supplementary

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Fig. S2*D*). To examine whether mutant *BRAF* controlled the translation of HIF protein, HT29 pSicoR cells were treated with MG132 for 0, 10, 20, and 30 min. Interestingly, the rate of HIF-1α protein synthesis was not affected, but the rate of HIF-2α protein synthesis was reduced by the knockdown of BRAF at 20 min ($P < 0.05$; Fig. 6*A*). The rate of HIF-2α protein synthesis was also suppressed at 10 and 20 min when HT29 cells were treated with PD98059 (both $P < 0.05$; Fig. 6*B*). Interestingly, PD98059 treatment suppressed the hypoxic induction of HIF-1α protein by 68% and HIF-2α by 58% in HT29 cells (both $P < 0.05$; Fig. 6*C*). These data suggest that mutant *BRAF* regulates the translation of HIF-2α through MAPK pathways.

To examine whether mutant *BRAF* affects the stability of HIF proteins, HT29 pSicoR cells were incubated in hypoxia for 6 h and then cultured in the presence of cycloheximide. The half-life of HIF-1α or HIF-2α protein was not altered by stable knockdown of BRAF (Supplementary Fig. S3*C*). Furthermore, pVHL expression was not affected by either stable or transient knockdown of BRAF in HT29 cells (Supplementary Fig. S3*D*). These data indicate that mutant *BRAF* does not affect the stability of either HIF-1α or HIF-2α protein.

Discussion

Although HIF-1α and HIF-2α are both induced by hypoxia in colon cancer cells, they have distinctive biological functions (13). We sought to determine whether the induction of these factors may be influenced by the underlying tumor genotype. In particular, *KRAS* and *BRAF* are among the most frequently mutated oncogenes in colon cancer, and tumors with these mutations are associated with distinct clinical phenotypes (1, 2, 5–8). Our previous studies have shown a unique interaction between K-ras and hypoxia (20, 24). We now show that oncogenic *KRAS* functions primarily to induce HIF-1α at the level of translation. In contrast, oncogenic *BRAF* can enhance the mRNA expression of both HIF-1α and HIF-2α, but at the translational level, only HIF2α is induced by *BRAF*.

Transformation with H-ras or mutant K-ras in fibroblasts enhances protein levels of HIF-1α (17, 18), which is consistent with our results in Caco2 cells. However, ectopic overexpression of mutant K-ras can produce misleading results (25, 26). Thus, it is essential to evaluate the oncogenic regulation of HIF-1α at physiologic levels of mutant K-ras. In the present study, we used

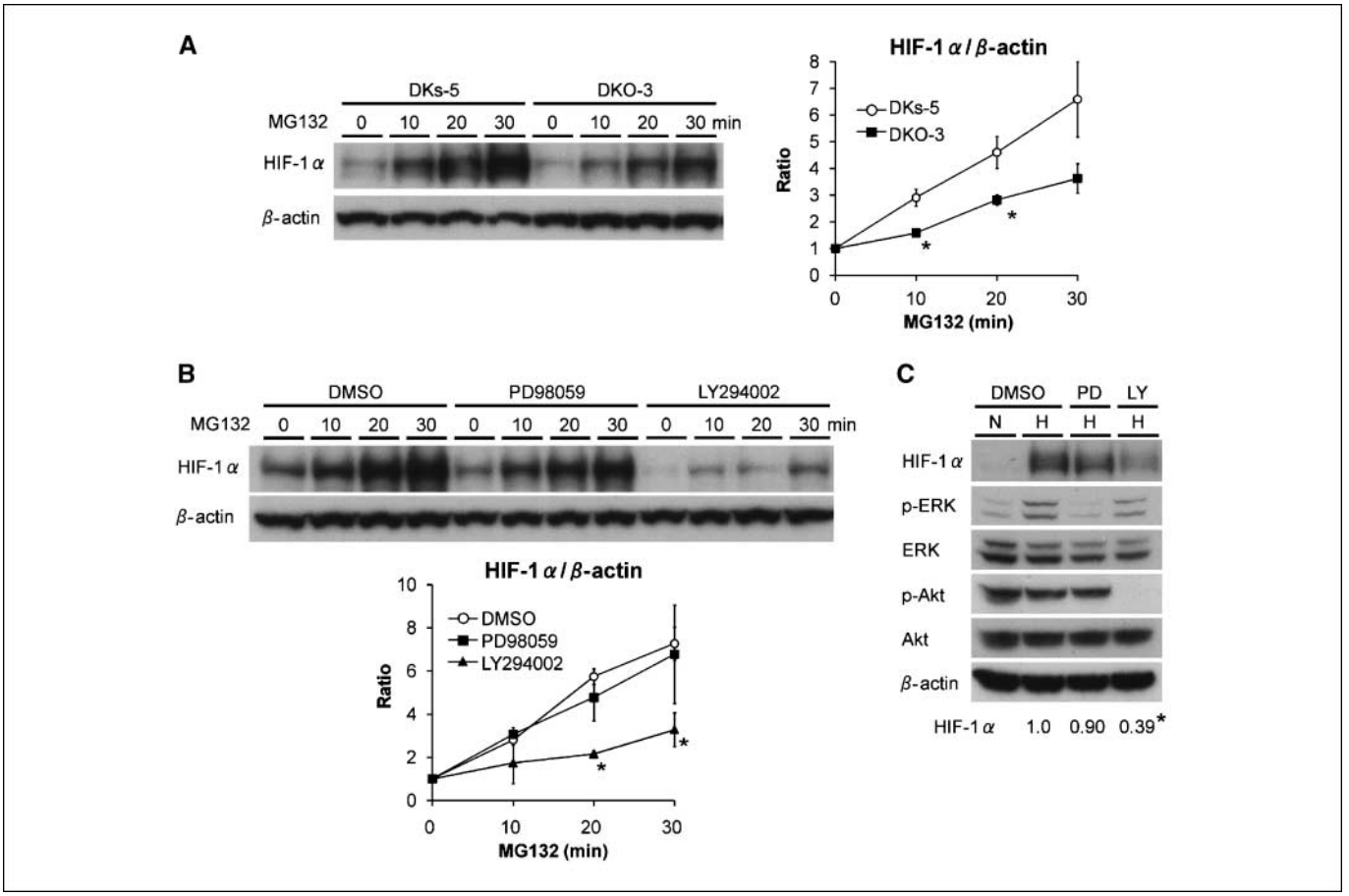

Figure 4. Knockout of mutant *KRAS* and translation of HIF-1α. *A,* DKs-5 and DKO-3 cells were cultured in normoxia and treated with MG132 for the indicated times. Protein levels of HIF-1α were analyzed by Western blotting (*left*). Band densities were quantified and HIF-1α protein level was normalized to β-actin at each time point. Protein synthesis of HIF-1α was measured by its accumulation during MG132 treatment. Mean ± SD for two independent experiments (*right*). *, $P < 0.05$. *B,* inhibition of MAPK and PI3K and translation of HIF-1α. DLD-1 cells were cultured in normoxia and treated with DMSO, PD98059 (*PD*), or LY294002 (*LY*) and then treated with MG132 for the indicated times. Protein levels of HIF-1α were analyzed by Western blotting (*top*). Protein synthesis of HIF-1α was measured as indicated above. Mean ± SD for two independent experiments (*bottom*). *, $P < 0.05$. *C,* inhibition of MAPK and PI3K and hypoxic induction of HIF-1α. DLD-1 cells were treated with DMSO, PD98059, or LY294002 for 6 h and then cultured in normoxia or hypoxia for 6 h. Hypoxic induction of HIF-1α and HIF-2α proteins and phosphorylation of p42/p44 (ERK) and Akt were analyzed by Western blotting. Band densities were quantified and HIF-1α protein levels were normalized to β-actin. *Numbers at the bottom,* mean fold change relative to DMSO control for two independent experiments. *, $P < 0.05$.

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mutant *KRAS* knockout cells or transiently knocked down endogenous K-ras in DLD-1 and HCT116 cells. The effects of endogenous mutant *KRAS* on the hypoxic induction of HIFs were consistent. Importantly, these effects on HIF appear to be specific to mutant *KRAS*, as no such regulation of HIF was seen with the wild-type *KRAS* gene.

A previous report suggested that mutant *BRAF* may regulate HIF-1α expression in melanoma cells, but its role in colon cancer was unknown (19). In our study, stable knockdown of BRAF in HT29 cells suppressed mRNA expression of HIF-1α but did not regulate its translation, which is consistent with the previous report (19). However, HIF-1α protein stability or pVHL protein expression was not affected by knockdown of BRAF in HT29 cells, whereas stable knockdown of BRAF in WM793 melanoma cells increased pVHL protein, resulting in lower stability of HIF-1α protein (19). These findings suggest that the effects of mutant *BRAF* on HIF-1α protein stability and pVHL protein expression are cell-specific.

In our study, mutant *KRAS* and *BRAF* appear to regulate HIF protein primarily at the level of translation. Whereas *KRAS* primarily regulates the translation of HIF-1α, *BRAF* may selectively regulate the translation of HIF-2α. Global protein translation is generally suppressed under hypoxic conditions (27), and in our study, p70 S6 kinase, which regulates global translation and can be phosphorylated by mammalian target of rapamycin, was inactivated in hypoxia (data not shown). Recent reports have shown that the translation of HIF-1α may also be regulated by mammalian target of rapamycin–independent pathways (28, 29). In addition, hypoxia increases HIF-2α translation by disrupting the iron-regulatory protein 1-HIF-2α iron-responsive element interaction (30). This may explain how HIF-2α protein can accumulate during hypoxia while global translation is suppressed. However, it is unknown whether oncogenic signaling regulates the binding activity of iron-regulatory protein 1.

Although HIF-1α and HIF-2α are similarly induced by hypoxia and both can bind hypoxia-responsive elements at target gene loci, several reports have shown that they have distinct expression patterns and functions (31–41). Our previous study suggested that HIF-1α may promote the growth of colon cancer cells, whereas HIF-2α may restrain growth. Expression of HIF-2α in human colon cancer tissues was inversely correlated with tumor stage (13). In the present study, we show that mutant *BRAF* but not *KRAS* upregulates HIF-2α in hypoxia. These differential effects of oncogenic *KRAS* and *BRAF* on the hypoxic induction of HIF-2α may potentially contribute to the more favorable clinical behavior associated with mutant *BRAF* tumors (7). It is important to recognize that *BRAF* mutations typically occur in the setting of high levels of MSI, but there are likely to be other genetic alterations associated with MSI as well as additional targets of *BRAF* that may contribute to the unique clinical phenotype. Of note, our studies of *BRAF* were done in HT29 and Caco2 cells, neither of which exhibits MSI, suggesting that the observed relationship between *BRAF* and HIF isoforms does not depend on the presence of MSI.

In conclusion, we have shown that oncogenic *KRAS* and *BRAF* mutations differentially regulate the hypoxic induction of HIF-1α and HIF-2α in colon cancer. Mutant *KRAS* enhances the hypoxic induction of HIF-1α by regulating its translation through PI3K pathways. Mutant *BRAF* enhances HIF-1α and HIF-2α mRNA expression but, more importantly, regulates HIF-2α translation. These differential effects on HIFs highlight the unique interaction

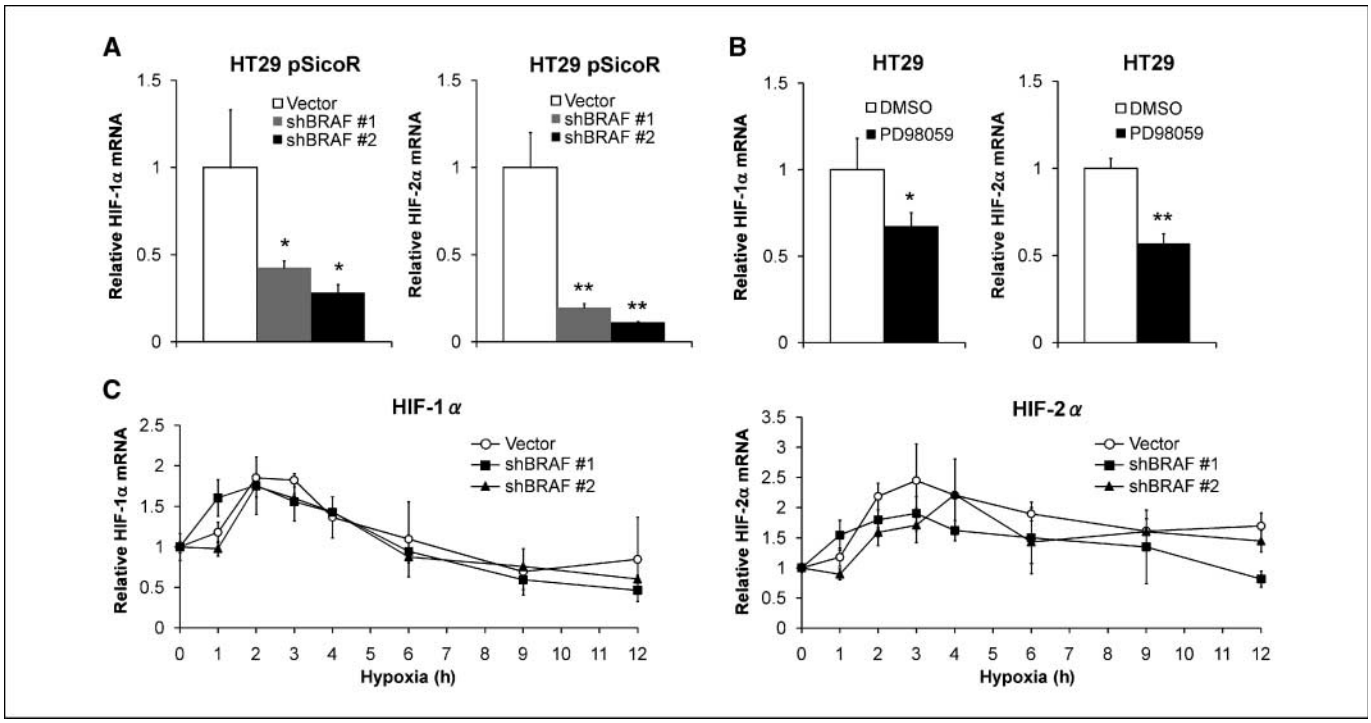

Figure 5. Knockdown of BRAF and mRNA expression of HIF-1α and HIF-2α. Total RNA was extracted from HT29 pSicoR (vector, shBRAF #1, and shBRAF #2) cells cultured in normoxia (*A*) or HT29 cells treated with DMSO or PD98059 (*B*). Relative levels of HIF-1α and HIF-2α mRNA were determined by quantitative reverse transcription-PCR and normalized to the levels of 18S rRNA. All reactions were done in triplicate. Mean ± SD for three different cDNA samples. *, $P < 0.05$; **, $P < 0.005$. *C,* HT29 pSicoR cells were incubated in hypoxia for the indicated times. Relative levels of HIF-1α and HIF-2α mRNA were determined by quantitative reverse transcription-PCR and normalized to the levels of 18S rRNA at each time point. Mean ± SD relative to time 0 in each cell line for two different cDNA samples.

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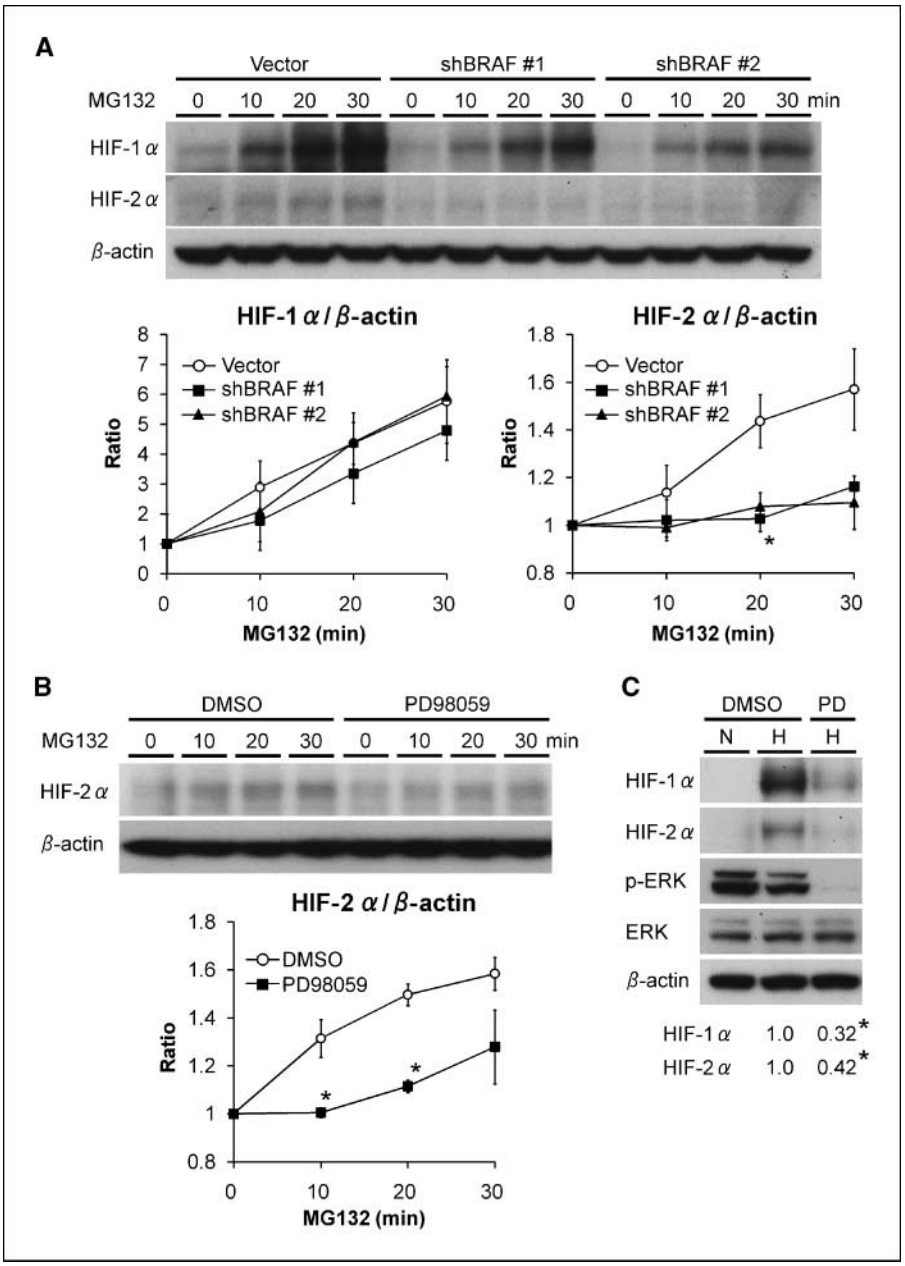

Figure 6. Knockdown of BRAF and translation of HIF-1α and HIF-2α. *A,* HT29 pSicoR cells were cultured in normoxia and treated with the proteasome inhibitor MG132 for the indicated times. Protein levels of HIF-1α, HIF-2α, and β-actin were analyzed by Western blotting (*top*). Band densities were quantified and HIF-1α and HIF-2α protein levels were normalized to β-actin at each time point. Protein synthesis of HIFs was measured by its accumulation during MG132 treatment. Mean ± SD for two independent experiments (*bottom*). *, $P < 0.05$. *B,* inhibition of MAPK and translation of HIF-1α and HIF-2α. HT29 cells were treated with DMSO or PD98059 in normoxia and then treated with MG132 for the indicated times. Protein levels of HIF-1α and HIF-2α were analyzed by Western blotting (*top*). Protein synthesis of HIFs was measured as indicated above. Mean ± SD for two independent experiments (*bottom*). *, $P < 0.05$. *C,* inhibition of MAPK and hypoxic induction of HIF-1α and HIF-2α. HT29 pSicoR cells were treated with PD98059 for 6 h and then incubated in normoxia or hypoxia for 6 h. Hypoxic induction of HIF-1α and HIF-2α proteins and phosphorylation of p42/p44 (ERK) were analyzed by Western blotting. Band densities were quantified and HIF protein levels were normalized to β-actin. *Numbers at the bottom,* mean fold change relative to DMSO control for two independent experiments. *, $P < 0.05$.

between oncogenes and the tumor microenvironment and may potentially contribute to the some of the phenotypic differences in mutant *KRAS* and *BRAF* colon tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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