# Aberrant Splicing of Cyclin-Dependent Kinase–Associated Protein Phosphatase KAP Increases Proliferation and Migration in Glioblastoma

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## Abstract

**The cyclin-dependent kinase (Cdk)–associated protein phosphatase KAP is a dual-specificity phosphatase of which the only known function is to dephosphorylate Cdk2 and inhibit cell cycle progression. Paradoxically, we find increased KAP mRNA expression in malignant astrocytomas, which correlates with increasing histologic grade and decreased patient survival. We have resolved this apparent paradox with the discovery of aberrant KAP splicing in malignant astrocytomas that leads to increased expression of KAP-related transcripts but decreased KAP protein expression. In addition, the aberrant splicing generates a dominant negative KAP variant that increases proliferation. We provide the first evidence that KAP not only regulates proliferation but also inhibits migration by decreasing *cdc2* mRNA and protein expression. The effect of KAP on cdc2 expression requires its phosphatase activity but does not involve direct dephosphorylation of cdc2. Thus, KAP regulates both cdc2-dependent migration and Cdk2-dependent proliferation, and its loss due to aberrant splicing increases malignancy in human gliomas.** [Cancer Res 2007;67(1):130–8]

## Introduction

Cyclin-dependent kinases (Cdk) and the cyclins with which they interact are major regulators of cell cycle progression. The overall activity of these proteins is dependent on a complex system of phosphorylation and dephosphorylation events. The $G_1$-S transition is controlled to a large extent by Cdk2 and cyclin A, which interact downstream with other proteins such as E2F and the retinoblastoma protein (RB1). Cdc2/Cdk1 also participates in cell cycle progression at this stage (1, 2). Numerous proteins involved in the regulation of this pathway have been implicated in tumorigenesis, including p53, p21/Cip1/Waf1 (a Cdk2 inhibitor), p27/Kip1, E2F, and RB1 (3).

The Cdk-associated protein phosphatase KAP (also known as Cdi1) is the product of the *CDKN3* gene and is a dual-specificity phosphatase that dephosphorylates Cdk2 on $Thr^{160}$ (4, 5). Phosphorylation of $Thr^{160}$ is necessary for full Cdk2 activity and cell cycle progression. KAP expression increases at the $G_1$-S transition, where it counteracts the stimulatory effect of Cdk-activating kinase on Cdk2 activity (6). The binding of cyclin A to Cdk2 inhibits the KAP-mediated dephosphorylation of Cdk2 (4–6). Recent evidence suggests that another protein, Htm4, participates in a protein complex with KAP and Cdk2 to promote KAP phosphatase activity and inhibit cell cycle (7, 8). Interestingly, KAP also binds to two other cell cycle regulators, cdc2 and Cdk3 (4, 5). However, direct evidence for regulation of these proteins by KAP is lacking.

The finding that KAP is a regulator of cell cycle progression raises the possibility that it may be involved in carcinogenesis. However, reports on the role of KAP in tumors have been somewhat contradictory. Aberrant KAP mRNA transcripts that code for proteins lacking Cdk2-dephosphorylating activity have been identified in hepatocellular carcinoma and in a hepatoblastoma cell line, although a clear role for these transcripts in tumorigenesis was not determined (9, 10). In contrast, KAP was reported to be overexpressed and to increase tumorigenicity in breast and prostate cancer (11). This latter result is difficult to explain, given the established role of KAP in cell cycle inhibition (4). Thus, the role of KAP in cancer remains unclear.

We show here that KAP inhibits not only proliferation but also migration of human malignant astrocytoma (glioblastoma) cells. The effect of KAP on migration is mediated via phosphatase-dependent inhibition of cdc2 mRNA and protein expression. We also show that KAP mRNA increases during astrocytoma progression as a result of the production of aberrant mRNA splice variants that generate nonfunctional KAP proteins. The resultant loss of full-length KAP promotes astrocytoma cell migration and proliferation through dysregulation of cdc2 and Cdk2. These findings establish a role for KAP as a suppressor of malignancy in cancer.

## Materials and Methods

**Tumor samples and cell lines.** All investigations described in this study were done after informed consent was obtained and in accordance with an Institutional Review Board (IRB) protocol approved by the Partners Human Research Committee at Brigham and Women's Hospital. Fresh frozen primary human tissue samples of 83 WHO grade 2, 3, and 4 astrocytomas and nontumor brain samples from surgical procedures for epilepsy were obtained from the Brain Tumor Tissue Bank at Brigham and Women's Hospital under the auspices of an IRB-approved human subjects study protocol. In some cases, DNA was extracted from paraffin-embedded tumor tissues obtained from the Department of Pathology at Brigham and Women's Hospital. Histology of glioblastoma (WHO grade 4 astrocytoma) samples was confirmed by H&E before inclusion in this study. Human glioblastoma cell lines were obtained from the American Type Culture Collection (U87 or U343; Manassas, VA) or were a gift (D566, D. Bigner, Duke University, Durham, NC). Human embryonic kidney (HEK) 293T cells were also obtained from the American Type Culture Collection. Glioblastoma cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a 5% $CO_2$ atmosphere.

**mRNA expression profiling.** Total RNA was isolated from fresh frozen human astrocytoma samples and from nontumor brain samples. The mRNA

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

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was reverse transcribed to generate cDNA, which was then biotinylated and hybridized to Affymetrix HG-U133A expression arrays. Statistical comparisons were done using the *t* test to identify differentially expressed genes. To account for multiple hypothesis testing, both *P* and *Q* values were calculated. For significant differential expression, we defined a *Q* value of ≤0.03, which corresponded to an unadjusted *P* value of 0.05.

**Survival analysis.** After informed consent was obtained, clinical data from hospital records and from the Brigham and Women's Hospital Brain Tumor Registry were reviewed under the auspices of an IRB protocol approved by the Partners Human Research Committee at Brigham and Women's Hospital. Patient survival was calculated from the time of tumor diagnosis to the time of death. Using Kaplan-Meier survival analysis, KAP mRNA expression was analyzed in a panel of 33 human malignant gliomas for which survival data and microarray expression data were both available (27 glioblastomas/WHO grade 4, and 6 anaplastic astrocytomas/WHO grade 3). The tumors were divided into two groups based on the median level of KAP expression: those with high KAP expression ($n$ = 18) and those with low expression ($n$ = 15). The mean age for the two groups was not statistically different [53 ± 16 versus 45 ± 15 years (mean ± SD); $P$ < 0.06, unpaired $t$ test]. Statistical significance with respect to survival was calculated using the Mantel-Cox log-rank test.

**RNA extraction, reverse transcription-PCR, and DNA sequencing.** Total RNA from cultured cells or primary human brain tissue samples was isolated using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). KAP-related cDNAs were obtained by reverse transcription-PCR (RT-PCR) using the SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) and the following primers: 5′-ACTGGTCTCGACGTGGGGCG-3′ (nucleotides 25–44, sense) and 5′-GTTGATAACACTGGTGGTTTC-3′ (nucleotides 780–760, antisense; IDT, Coralville, IA). The PCR products were resolved on agarose gels, cloned into a pGEM-T vector (Promega, Madison, WI), and sequenced using an ABI 3730XL DNA Sequencer. At least 10 clones for each tissue sample were sequenced.

**Real-time quantitative PCR.** Total RNA (1 μg) isolated from primary frozen tumor tissues or from frozen nontumor brain tissues was used for generation of cDNAs using SuperScript III (Invitrogen). ABI TaqMan reagents (Applied Biosystems, Foster City, CA) for KAP, cdc2, or GAPDH (as an endogenous control) and 5 ng of cDNA were then used for real-time quantitative PCR on an ABI 7300 Thermocycler Real Time PCR machine (Applied Biosystems). At least four wells were run for each sample. The results were indicated as a fold increase relative to one sample, which was chosen as a reference point.

**Reagents and recombinant plasmid construction.** pLenti-internal ribosome entry site (IRES)-enhanced green fluorescent protein (EGFP) was generated by inserting IRES-EGFP from pIRES2-EGFP (Clontech, Mountain View, CA) into pLenti6/V5 (Invitrogen) using the Directional TOPO Cloning kit according to the manufacturer's instructions. Full-length human KAP cDNA was cloned from human 293T cells by RT-PCR and validated by direct DNA sequencing. A myc-KAP fusion protein was created by cloning full-length KAP into an expression vector that resulted in adding a myc tag to the $NH_2$ terminus of the protein. The KAP phosphatase-dead mutant (KAPC140S) was generated by single amino acid substitution from $Cys^{140}$ to Ser and a hemagglutinin tag was added to its $NH_2$ terminus. Myc-KAP and KAPC140S were cloned into a pLenti-IRES-EGFP vector. The KAP splice variant d was cloned from primary glioblastoma samples by RT-PCR and validated by sequencing. The d variant of KAP was then tagged with hemagglutinin at its $NH_2$ terminus and cloned into the pcDNA3 plasmid. A KAP shRNA expression vector directed against KAP sequence ($^{534}$GCAA-TACAGACCATCAAGCAA$^{555}$) and a control shRNA vector (pSM2c) were purchased from Open Biosystems (Huntsville, AL). *Cdc2* cDNA was cloned from human *cdc2* cDNA in pOTB7 (Open Biosystems) by PCR, and a *cdc2* kinase-dead mutant (cdc2T161A) was generated by single amino acid substitution from $Thr^{161}$ to Ala.

**Transfection and lentiviral transduction methods.** For stable transfection, KAP shRNA and control shRNA plasmids were first transiently transfected into cells, and then mixed populations were selected in puromycin for use in further experiments.

Lentiviruses were generated according to the manufacturer's protocol using the ViralPower Lentivirus Expression System (Invitrogen) and the IRES-EGFP lentiviral vectors described above. Transduction efficiency was monitored with *EGFP* as a reporter gene.

**Western blots and immunoprecipitation.** The anti-myc antibody (clone 9E10) was purchased from Upstate Biotechnology (Charlottesville, VA). Antibodies against KAP and Cdk2 were purchased from BD Transduction Laboratories (San Jose, CA). Antibodies directed against the COOH-terminal 18 amino acids of KAP and against cdc2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A phosphospecific antibody directed against Cdk2-T160 was purchased from Cell Signaling Technology (Beverly, MA). An anti-β-actin antibody was purchased from Sigma (St. Louis, MO).

For Western blots, cells were lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate with 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich), and boiled briefly in SDS sample buffer. Protein isolates were then subjected to SDS gel electrophoresis and transferred to nitrocellulose membranes. Immunoreactive bands were detected by chemiluminescence.

For immunoprecipitation, whole-cell lysates prepared from cultured cells or from frozen human brain tissues (glioblastoma or nontumor brain) were immunoprecipitated with 1 μg of specific antibody that had been prebound to 30 μL of Protein A-Sepharose 4B beads (Amersham Biosciences Corp., Piscataway, NJ). The beads were washed twice and the cold immunoprecipitates were boiled in SDS and processed for Western blot using the appropriate antibodies as described above.

**Bromodeoxyuridine cell proliferation assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** U87 glioblastoma cells were maintained in DMEM supplemented with 10% FCS. For bromodeoxyuridine (BrdUrd) ELISA, $5 \times 10^3$ cells were plated in 96-well plates and maintained in serum-supplemented medium. BrdUrd was added to the medium at a final concentration of 10 μmol/L, and the cells were incubated for an additional 6 h. Newly synthesized BrdUrd-DNA was measured using colorimetric detection on an ELISA plate reader at 370 nm according to the manufacturer's protocol. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, $1 \times 10^3$ cells were plated in 96-well plates and cell proliferation was measured after 72 h at 590 nm according to the manufacturer's protocol. Five wells were assayed for each condition and each experiment was repeated in triplicate.

**Cell migration assays.** A fluorescent phagokinetic migration assay was done as previously described (12). Briefly, 12-well plates were pretreated with poly-D-ornithine (Sigma) for 1 h at 37° C and then coated with a 0.01% suspension of 1-μm-diameter fluorescent microspheres (Molecular Probes, Carlsbad, CA) for 2 h at room temperature. U87 glioblastoma cells ($1.6 \times 10^3$) were plated in DMEM containing 20% serum, incubated at 37°C for 18 h, and subsequently fixed with 4% paraformaldehyde in PBS. Random fields containing fluorescent cells were collected using a 10× phase objective, and the area of migration for at least 50 individual cells per condition was quantified using the Spot imaging software program. Three independent experiments were done.

The transwell migration assay was done as previously described (13). Briefly, $5 \times 10^4$ cells were plated into a transwell containing an 8-μm-pore polycarbonate membrane (Nalge Nunc International, Rochester, NY) with DMEM containing 10% FBS in the top chamber and 20% FBS in the lower chamber. After 18 h, cells on the top of the membrane were removed. Cells that migrated through to the bottom surface of the membrane were stained using the Quick Cell Staining kit (Richard-Allan Scientific, Kalamazoo, MI). Cell number was counted in 10 random fields for each condition using a 20× phase objective. Two independent experiments were done.

## Results

**KAP mRNA is overexpressed in primary human glioblastomas and correlates with histologic grade and patient survival.** Several studies have reported increased expression of KAP mRNA

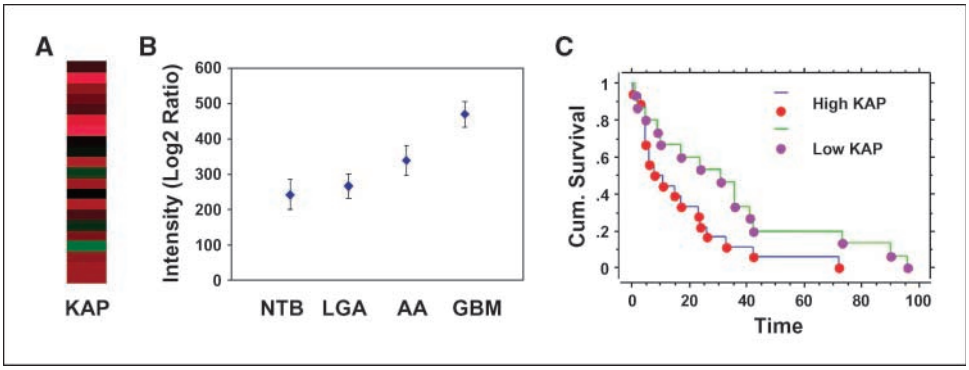


**Figure 1.** KAP mRNA is overexpressed and correlates with survival and malignancy in human astrocytomas. *A,* KAP mRNA expression profile from 21 primary human glioblastoma samples is shown as a heat map. *Red,* increased expression; *green,* decreased expression relative to nontumor brain. The Affymetrix HG-U133a *CDKN3* probe set (209714_s_at) contains 11 probes that cover the extent of the *CDKN3* gene. A subpopulation of glioblastomas showed a significant increase in KAP mRNA expression when compared with nontumor brain ($P < 0.006$, *t* test). *B,* Affymetrix microarray data were obtained for KAP mRNA expression levels using five nontumor brain samples (*NTB*), 15 WHO grade 2/low-grade diffuse astrocytomas (*LGA*), 7 WHO grade 3/anaplastic astrocytomas (*AA*), and 33 WHO grade 4/glioblastomas (*GBM*). Mean intensity values for KAP mRNA expression were calculated and plotted as a function of tumor grade. *Points,* mean; *bars,* SE. The mean intensity value for KAP mRNA expression in glioblastomas differed significantly from that of nontumor brain ($P < 0.02$, unpaired *t* test) and LGA ($P < 0.0011$, unpaired *t* test). *C,* Kaplan-Meier survival analysis using 33 malignant astrocytomas [6 anaplastic astrocytomas (WHO grade 3) and 27 glioblastomas (WHO grade 4 astrocytomas)] for which both expression and survival data were available. The tumors were divided into two groups based on the level of KAP expression: those with high KAP expression ($n$ =18) and those with low KAP expression ($n$ = 15). Increased KAP mRNA expression correlated significantly with decreased survival in patients with malignant astrocytomas ($P < 0.038$, Mantel-Cox log-rank test). Time of survival was measured in months.

in breast, prostate, liver, and brain cancers (9, 11, 14, 15). To confirm these observations, we analyzed microarray expression data from 21 primary malignant astrocytomas (glioblastomas) and 5 nontumor brain samples to determine KAP mRNA expression in glioblastoma. The analysis revealed evidence of KAP mRNA overexpression in a majority of the tumor samples tested when compared with nontumor brain (Fig. 1*A*).

We next examined the relationship between KAP mRNA expression and histologic grade in human astrocytomas. Microarray expression data for 5 nontumor brain samples, 15 grade 2 astrocytomas, 7 grade 3 astrocytomas, and 31 grade 4 astrocytomas were first analyzed for KAP mRNA expression and were then correlated with histologic tumor grade. As shown in Fig. 1*B*, increased KAP mRNA expression levels were associated with increasing histologic tumor grade in astrocytomas.

The correlation between KAP mRNA expression and histologic grade suggested a possible relationship between KAP mRNA expression and patient outcome. We therefore did a Kaplan-Meier survival analysis of 33 malignant gliomas for which survival data were available (27 glioblastomas and 6 anaplastic astrocytomas). Increased KAP mRNA expression was significantly associated with decreased patient survival in malignant astrocytomas (Fig. 1*C*; $P < 0.038$, Mantel-Cox log-rank test). We validated this result using Cox regression analysis of an independent, publicly available mRNA expression data set from 50 glioblastomas,[1] which also indicated a statistically significant relationship between *CDKN3* mRNA expression and survival ($P < 0.012$).

**Aberrant splicing of KAP in glioblastoma.** The finding that KAP mRNA is overexpressed in a subpopulation of glioblastomas and in other cancers (11, 14, 15) seemed paradoxical (given the reported inhibitory effect of KAP on cell cycle progression) and raised the possibility that the expressed KAP mRNA or protein may not be normal in these tumors. We therefore used RT-PCR to examine KAP mRNA transcripts in human glioblastomas in greater detail. As shown in Fig. 2*A*, nontumor brain samples (*lanes 10–13*) contained two KAP transcripts: the full-length KAP transcript (a variant, 755 bp) and a truncated transcript (c variant, 672 bp) lacking exon 2. The absence of exon 2 in variant c generates a frame shift and stop codon (TAA) ~60 bp downstream, thereby creating a 23-amino-acid peptide with minimal amino acid sequence homology to KAP (Fig. 2*C*).

In addition, a subpopulation of primary glioblastoma samples (*lanes 1, 3*, and *9*) contained several additional KAP-related transcripts when compared with nontumor brain samples. Sequencing indicated that these variants involved aberrant splicing of exons 2 and 3 and resulted in the generation of premature stop codons leading to truncated KAP protein or to short mRNA variants with alterations near the $NH_2$-terminal portion of the protein (Fig. 2*B* and *C*). One of these variants (b variant) contained an excision of 5 bp at the beginning of exon 2, leading to the creation of a stop codon 15 bp downstream and the generation of a truncated 8-amino-acid peptide (Fig. 2*B* and *C*). Another variant (d variant) arose via use of an alternative splice site within exon 2, creating a 179-amino-acid protein (~20 kDa) lacking a portion of exon 2 and all of exon 3 (Fig. 2*B* and *C*). We also sequenced KAP transcripts from human U87 and U343 glioblastoma cells and HEK 293T cells. These cells contained only the a and c KAP mRNA variants, a pattern identical to that observed in nontumor brain tissues (data not shown).

Based on the sequencing data described above, we predicted that the aberrant KAP mRNA splice variants would fail to generate full-length KAP protein. To investigate this possibility, we first did real-time PCR to quantitate KAP mRNA expression levels in those glioblastoma samples with aberrant KAP splicing, and compared the results to those obtained from nontumor brain. As shown in Fig. 2*D*, two of three glioblastoma samples containing aberrant KAP transcripts showed a >5-fold increase in KAP mRNA expression when compared with nontumor brain (*top* and *middle*). Importantly, the glioblastoma samples with aberrant splicing displayed reduced KAP protein expression when compared with nontumor brain (Fig. 2*D, bottom*). Thus, the increased mRNA expression observed in a subpopulation of glioblastomas

[1] http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4271.

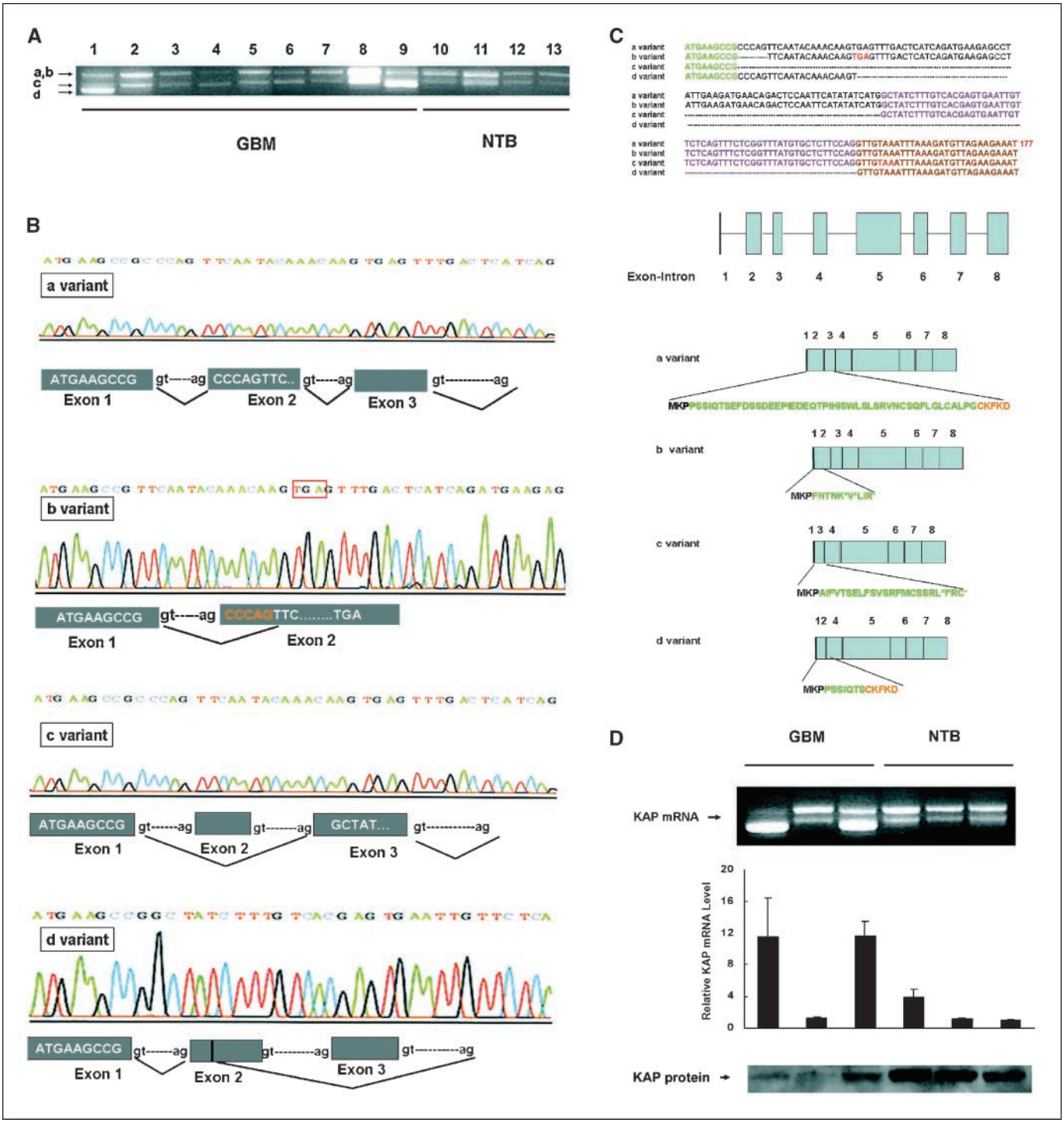


**Figure 2.** Aberrant splicing of KAP mRNA in glioblastoma leads to decreased KAP protein. *A,* RT-PCR for full-length human KAP was done using total RNA isolated from 10 human glioblastoma and 6 nontumor brain samples. Representative data from nine glioblastoma and four nontumor brain samples. All nontumor brain samples and 7 of 10 glioblastomas analyzed contained KAP splice variants a and c only. In three glioblastomas, RT-PCR revealed several additional bands that corresponded to mRNA transcript variants b and d (*lanes 1, 3*, and *9*). *Lanes 1* and *9,* glioblastoma samples contained transcript variants a, b, c, and d; *lane 3,* sample contained variants a, b, and c. Note that KAP splice variants a and b differ by only 5 bp and run at the same place on the gel. *B,* RT-PCR products for KAP transcripts obtained from glioblastomas and nontumor brain samples. DNA sequences near the relevant splice junctions are illustrated. *C,* sequence of KAP splice variants. *Top,* the sequence results of KAP transcript variants are shown from bp 1 to 177. Exons contributing to the sequence of different KAP variants are color coded (*green,* exon 1; *black,* exon 2; *purple,* exon 3; *brown,* exon 4). *Red,* in-frame stop codons within each sequence. *Bottom,* exon-intron structure of KAP mRNA splice variants identified in glioblastoma. *D,* RT-PCR analysis of KAP mRNA obtained from three primary human glioblastomas containing the b or d splice variants and from three nontumor human brain samples (*top*) was compared with TaqMan real-time PCR quantitative measurements of KAP mRNA expression (*middle*), and with endogenous KAP protein expression measured by Western blots of immunoprecipitated protein (*bottom*) obtained from the same tissue samples. Note that although the second glioblastoma sample contains a normal total level of expression of KAP-related mRNA, the frequency of occurrence of sequenced variants suggests that roughly half of the mRNA in the upper band corresponds to the aberrantly spliced b variant, which fails to generate full-length KAP protein.

correlated with the production of aberrant KAP transcripts and a decrease in full-length functional KAP protein.

**KAP inhibits proliferation in glioblastoma.** To determine the role of KAP in glioblastomas, cultured human U87 glioblastoma cells were transduced with a lentivirus containing full-length KAP with a myc tag attached to the $NH_2$ terminus, and stable clones were selected in puromycin. Control cells were transduced with a control lentivirus containing an empty expression vector. A mixed pool of stable clones was then analyzed for proliferation using BrdUrd incorporation to measure DNA synthesis. Overexpression of KAP significantly inhibited BrdUrd incorporation in U87 glioblastoma cells (Fig. 3*A*).

To confirm a role for KAP in regulating the proliferation of glioblastoma cells, we used an RNA interference strategy to decrease KAP expression. U87 glioblastoma cells were transfected with an shRNA expression vector directed against human KAP, and a mixed pool of stable clones was again selected. Knockdown of KAP expression increased BrdUrd incorporation in U87 glioblastoma cells (Fig. 3*A*). Similar inhibitory effects of KAP on proliferation were observed in human U343 and D566 glioblastoma cells (data not shown).

**KAP inhibits migration in glioblastoma.** Although KAP binds to cdc2, Cdk2, and Cdk3, only Cdk2 has been identified as a direct target of KAP phosphatase activity (4, 5). Thus, the only known function of KAP is to inhibit cell cycle progression. To investigate whether KAP may regulate other cellular functions, we examined the role of KAP in cell migration. Human U87 glioblastoma cells were stably transfected with a KAP shRNA vector and cell migration was assayed *in vitro* with a fluorescent phagokinetic motility assay (12). As shown in Fig. 3*B*, knockdown of endogenous KAP expression increased cell migration by almost 2-fold.

We confirmed the inhibitory effect of KAP on cell migration by overexpressing wild-type KAP in human glioblastoma cells using lentiviral-mediated transduction. As predicted from the shRNA knockdown experiments, enforced overexpression of KAP significantly decreased glioblastoma cell migration (Fig. 3*B*). We also used the transwell cell migration assay to examine the effects of KAP on cell migration. shRNA-mediated knockdown of KAP protein expression increased U87 glioblastoma cell migration through the membrane by nearly 3-fold (Fig. 3*C*).

**KAP decreases cdc2 protein and mRNA expression.** As previously mentioned, KAP binds both cdc2 and Cdk2, but it is unclear whether KAP regulates cdc2 in a functional manner. To

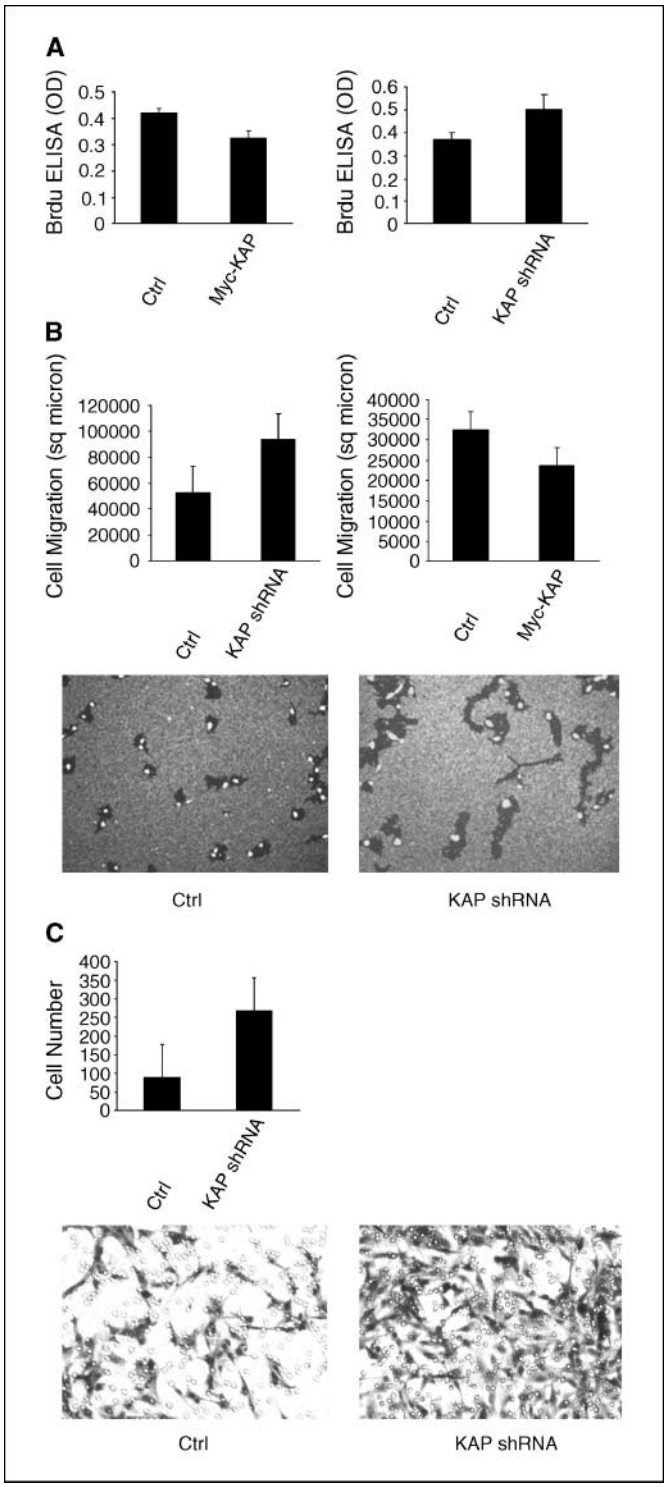


**Figure 3.** KAP inhibits proliferation and migration of glioblastoma cells. *A,* U87 glioblastoma cells were transfected with a KAP shRNA plasmid or an empty shRNA control vector (*Ctrl; right*), or transduced with a lentivirus containing myc-tagged KAP (*Myc-KAP*) or with a control lentivirus containing an empty vector (*Ctrl*; *left*). A mixed pool of stable clones was obtained using antibiotic selection, and a BrdUrd cell proliferation assay was done as described in Materials and Methods. *Columns,* mean; *bars,* SE. Western blots showed increased KAP protein after overexpression and knockdown of KAP protein in the stable cell lines used (see Fig. 4*A*). Knockdown of KAP led to a significant increase in glioblastoma cell proliferation ($P < 0.002$, unpaired *t* test), whereas overexpression of KAP significantly decreased BrdUrd incorporation into DNA ($P < 0.0004$, unpaired *t* test). *B,* U87 glioblastoma cells were stably transfected with a KAP shRNA vector or an empty shRNA control vector (*left*), or transduced with a lentivirus containing myc-tagged KAP or with a control lentivirus containing an empty control vector (*right*). A fluorescent phagokinetic migration assay was then done as described in Materials and Methods. Quantitative analysis revealed an ~1.8-fold increase in the area of migration after knockdown of KAP expression in glioma cells ($P < 1.3 \times 10^{-8}$, unpaired *t* test), whereas overexpression of KAP decreased the area of migration by ~30% ($P < 7.0 \times 10^{-6}$, unpaired *t* test). *Columns,* mean; *bars,* SE. Micrographs illustrate the effect of KAP knockdown on migration using the fluorescent phagokinetic migration assay. *C,* U87 glioblastoma cells stably transfected with either KAP shRNA expression vector or an empty control vector were plated in a transwell chamber and a cell migration assay was done as described in Materials and Methods. A significantly larger number of cells with decreased KAP expression migrated through the membrane when compared with control cells. Quantitation of this result revealed a >3-fold increase in the number of cells that migrated across the membrane ($P < 0.008$, unpaired *t* test). *Columns,* mean; *bars,* SE. Each experiment was repeated in duplicate. Micrographs illustrate the effect of KAP knockdown on migration using the transwell migration assay.

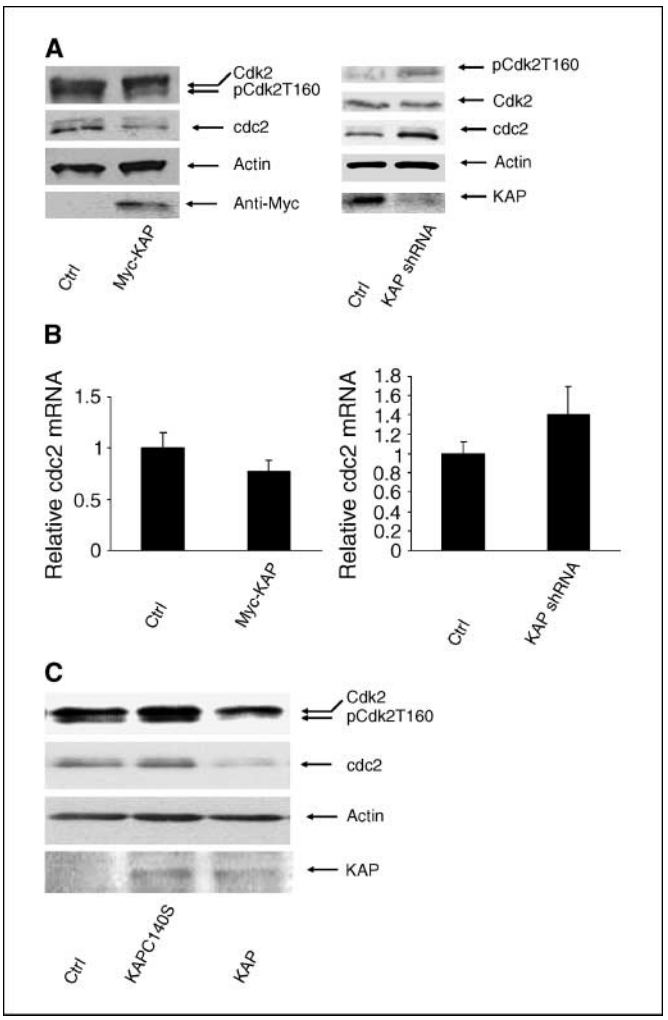


**Figure 4.** KAP decreases Cdk2 phosphorylation and cdc2 expression via its phosphatase activity. *A,* Western blot analysis of Cdk2 and cdc2 expression in U87 glioblastoma cells overexpressing myc-KAP, KAP shRNA, or the appropriate empty control vectors. Myc-KAP overexpression (*left*) decreased Cdk2 phosphorylation (*pCdk2T160*) and cdc2 protein expression, whereas KAP knockdown (*right*) increased Cdk2 phosphorylation (*pCdk2T160*) and cdc2 protein expression. *Left,* Cdk2 protein was immunoprecipitated with an anti-Cdk2 antibody and analyzed for $Thr^{160}$ phosphorylation with a phosphospecific anti-pCdk2T160 antibody. For confirmation of shRNA-mediated KAP knockdown, endogenous KAP protein was first immunoprecipitated with a specific anti-KAP antibody directed against the COOH terminus of the protein, and the blot was then probed using a different anti-KAP antibody. β-Actin was used as an endogenous control. *B,* U87 glioblastoma cells expressing either myc-KAP or an empty control vector were analyzed using real-time PCR to measure *cdc2* mRNA expression levels. *Columns,* mean of four replicates per condition; *bars,* SE. Overexpression of myc-KAP decreased *cdc2* mRNA expression ($P < 0.01$, unpaired *t* test) whereas knockdown of KAP increased *cdc2* mRNA expression ($P < 0.01$, unpaired *t* test). *C,* Western blot analysis of Cdk2 and cdc2 protein expression in U87 glioblastoma cells overexpressing KAP, the phosphatase-dead KAPC140S mutant, or an empty control vector (*Ctrl*). β-Actin was used as an endogenous reference control. In contrast to wild-type KAP, the phosphatase-dead KAPC140S mutant failed to decrease cdc2 protein expression or Cdk2 phosphorylation.

investigate this matter, we did Western blot analyses of Cdk2 and cdc2 proteins isolated from U87 glioblastoma cells transduced with a KAP lentivirus or with a control virus. As shown in Fig. 4*A*, overexpression of KAP reduced phosphorylation of Cdk2 on $Thr^{160}$ without altering the total amount of Cdk2 protein. In contrast, cdc2 protein levels were decreased, although no specific effect on cdc2 phosphorylation was detected. To confirm the effects of KAP on Cdk2 and cdc2, we again used U87 glioblastoma cells that were stably transfected with an shRNA vector directed against KAP or with a control vector. Western blot analyses indicated that knockdown of KAP protein expression led not only to increased phosphorylation of Cdk2 but also to increased expression of cdc2 protein (Fig. 4*A*).

We wondered whether the regulation of cdc2 protein expression by KAP was mediated through direct binding interactions, through KAP phosphatase activity, or through an effect at the mRNA level. Real-time PCR of *cdc2* mRNA indicated that enforced overexpression of KAP in U87 glioblastoma cells decreased *cdc2* mRNA expression (Fig. 4*B*). Conversely, knockdown of KAP using an anti-KAP shRNA increased *cdc2* mRNA expression (Fig. 4*B*). These data indicate that KAP regulates cdc2 protein levels through regulation of mRNA expression.

To determine whether the phosphatase activity of KAP was required for the inhibition of *cdc2* mRNA expression, we used site-specific mutagenesis to generate a phosphatase-dead mutant of KAP (KAPC140S) in which $Cys^{140}$ (located at the catalytic site) was converted to serine. This point mutant has been shown to bind to Cdk2 in crystallographic studies but to lack phosphatase activity (16). Overexpression of phosphatase-dead KAP by lentiviral transduction in U87 glioblastoma cells increased the phosphorylation of Cdk2, indicating that this mutant displayed dominant negative activity (Fig. 3*C*). Importantly, phosphatase-dead KAP failed to decrease cdc2 protein expression. Thus, the phosphatase activity of KAP is required for regulation of cdc2 expression.

**KAP-mediated inhibition of migration is cdc2 dependent.** A recent study indicates that cdc2 plays an important role in cell migration (13). We thus considered the possibility that the effect of KAP on cell migration was mediated through cdc2. To investigate this possibility, we first tested the effect of specific cell-permeable cdc2 inhibitors on migration in nontransfected U87 glioblastoma cells. In cell-free assays, purvalanol A inhibits Cdk1/Cdk2 and DYRK1A at 1 μmol/L concentration, whereas alsterpaullone inhibits Cdk1/Cdk2/Cdk5, GSK3β, and LCK (17, 18). Thus, the use of this combination of inhibitors at low concentrations is useful for identifying Cdk-mediated effects in cell-based assays. Exposure to either purvalanol A (1 μmol/L) or alsterpaullone (1 μmol/L) significantly inhibited glioblastoma cell migration, indicating that this process is Cdk dependent (Fig. 5*A*). Because cdc2 has been implicated in the control of proliferation (19), we examined the effects of cdc2 inhibition on DNA synthesis in glioma cells. Although alsterpaullone increased BrdUrd incorporation by ~18%, purvalanol A had no effect on BrdUrd incorporation in U87 glioblastoma cells at this concentration (Fig. 5*A*). This was in contrast to the potent inhibitory effect of purvalanol A on migration at the same concentration. These data indicated that migration is a cdc2-dependent process in human glioblastoma cells, and that cdc2 activity is not essential for proliferation, as has been clearly shown in other cell types (13, 20).

We next examined the effect of cdc2 inhibitors on KAP-mediated regulation of cell migration. KAP protein expression was first reduced in U87 glioblastoma cells by overexpression of a KAP shRNA expression vector. Control cells were transfected with a control shRNA expression vector. Cell migration was then assayed in the presence or absence of purvalanol A (1 μmol/L) using the phagokinetic migration assay. As shown in Fig. 5*B*, cdc2 inhibition completely abrogated the stimulatory effect of KAP knockdown on glioblastoma cell migration.

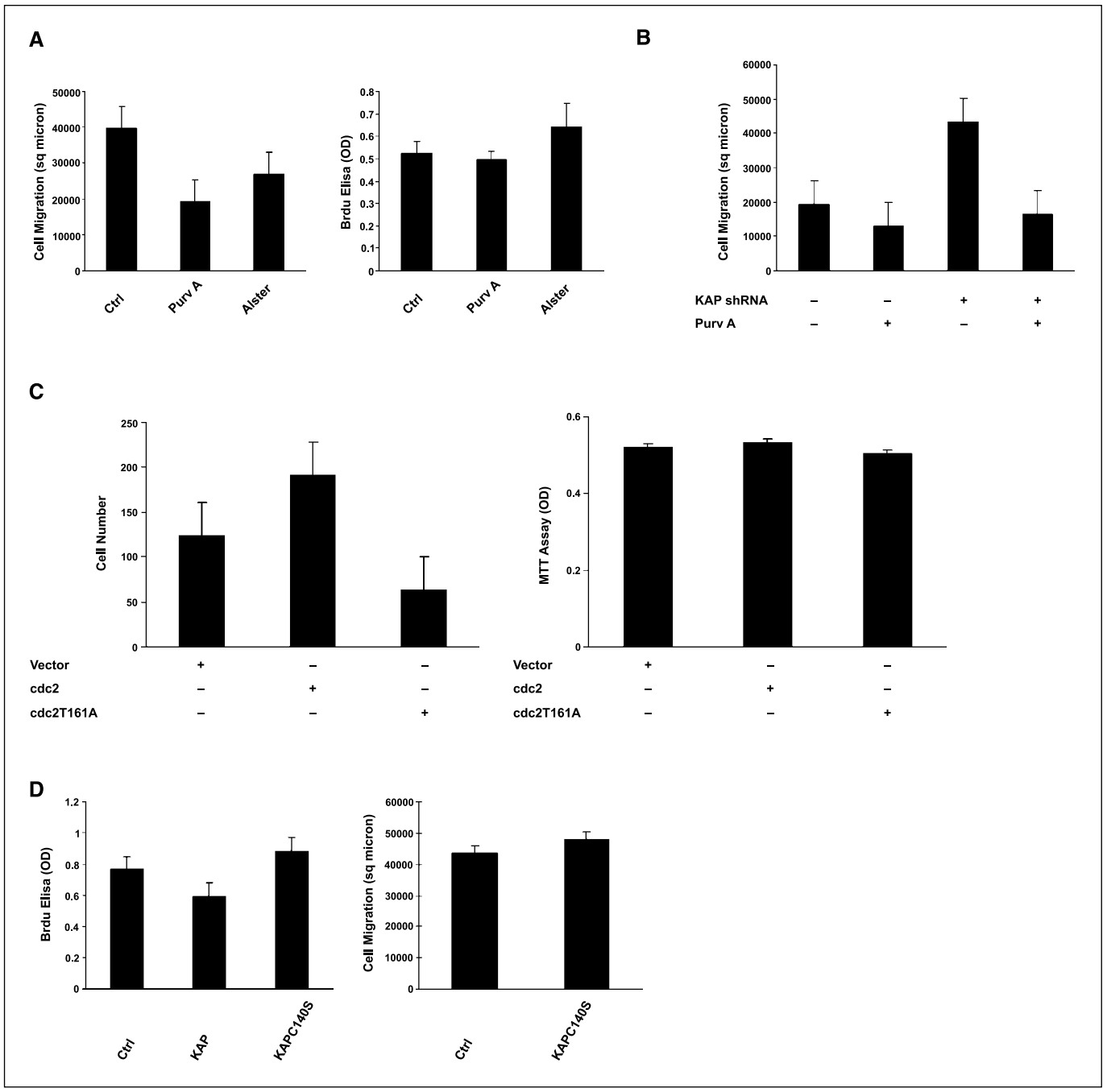


**Figure 5.** KAP inhibits migration in a cdc2-dependent manner. *A, left,* U87 glioblastoma cells were plated in the presence or absence of the specific cell-permeable cdc2 inhibitors purvalanol A (*Purv A*; 1 μmol/L) or alsterpaullone (*Alster*; 1 μmol/L). A fluorescent phagokinetic migration assay was then done as described in Materials and Methods. *Columns,* mean; *bars,* SE. Inhibition of cdc2 by purvalone A ($P < 2.2 \times 10^{-11}$, unpaired *t* test) or by alsterpauline ($P < 1.87 \times 10^{-11}$, unpaired *t* test) significantly decreased glioblastoma cell migration. *Right,* U87 glioblastoma cells were cultured in the presence or absence of purvalanol A (1 μmol/L) or alsterpaullone (1 μmol/L) for 72 h. Proliferation was determined by measuring BrdUrd incorporation into DNA. *Columns,* mean; *bars,* SE. Inhibition of cdc2 either had no effect (purvalanol A; $P < 0.25$, unpaired *t* test) or slightly increased (alsterpaullone; $P < 0.03$, unpaired *t* test) DNA synthesis in U87 glioblastoma cells. *B,* U87 glioblastoma cells transfected with a KAP shRNA vector or an empty control vector were maintained in the presence or absence of purvalanol A (1 μmol/L), and the fluorescent phagokinetic migration assay was done as described. *Columns,* mean; *bars,* SE. Purvalone A alone decreased cell migration by ~35% in control transfected cells ($P < 3.3 \times 10^{-6}$, unpaired *t* test). Knockdown of KAP expression increased cell migration by >2-fold ($P < 6.67 \times 10^{-11}$, unpaired *t* test), and this effect was abrogated in the presence of purvalanol A ($P < 3.19 \times 10^{-12}$, unpaired *t* test). *C, left,* transwell migration assay for U87 glioblastoma cells stably expressing KAP shRNA and transduced with a lentivirus containing wild-type cdc2, a dominant negative cdc2T161A mutant, or an empty control vector. *Columns,* mean; *bars,* SE. Overexpression of cdc2 increased migration when compared with cells overexpressing the control vector ($P < 1.48 \times 10^{-7}$, unpaired *t* test), whereas the dominant negative cdc2T161A mutant reduced migration ($P < 8.43 \times 10^{-9}$, unpaired *t* test). *Right,* MTT growth assay for U87 glioblastoma cells stably expressing KAP shRNA and transduced with a lentivirus containing wild-type cdc2, cdc2T161A, or an empty control vector. *Columns,* mean; *bars,* SE. Neither cdc2 nor cdc2T161A altered cell growth ($P < 0.25$, unpaired *t* test). *D, left,* proliferation assay for U87 cells transduced with a lentivirus containing the phosphatase-dead KAP mutant KAPC140S or an empty control vector. *Columns,* mean; *bars,* SE. Whereas KAP overexpression inhibited glioma cell proliferation ($P < 2.5 \times 10^{-5}$, unpaired *t* test), the phosphatase-dead KAPC140S mutant slightly increased proliferation ($P < 0.001$, unpaired *t* test). *Right,* phagokinetic migration assay U87 glioblastoma cells transduced with a lentivirus containing the KAPC140S phosphatase-dead mutant or an empty control vector. *Columns,* mean; *bars,* SE. Overexpression of the phosphatase-dead KAPC140S mutant failed to inhibit U87 glioblastoma cell migration ($P < 0.248$, unpaired *t* test).

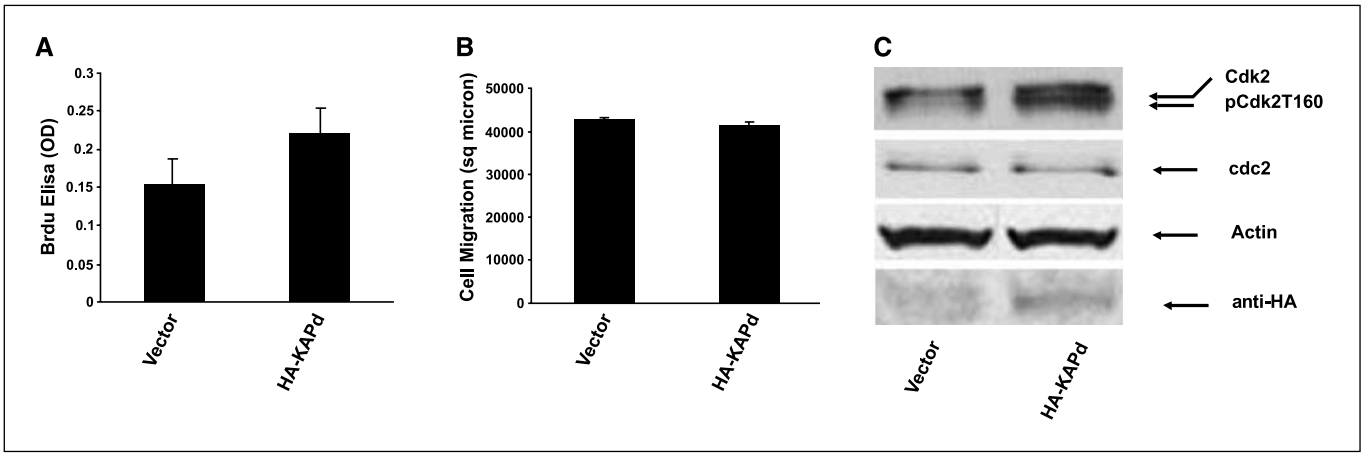


**Figure 6.** Dominant negative activity of aberrant KAP splice variant d. *A,* proliferation assay for U87 glioblastoma cells transduced with a lentivirus containing the d splice variant of KAP with a hemagglutinin tag attached to the $NH_2$ terminus (*HA-KAPd*) or an empty control expression vector. *Columns,* mean; *bars,* SE. Overexpression of KAP mRNA splice variant d significantly increased glioblastoma cell proliferation ($P < 0.0006$, unpaired *t* test). *B,* phagokinetic migration assay for U87 glioblastoma cells transduced with a lentivirus containing the d splice variant of KAP or an empty control expression vector. *Columns,* mean; *bars,* SE. No difference in cell migration was observed after overexpression of HA-KAPd ($P < 0.699$, unpaired *t* test). *C,* Western blot analysis of Cdk2 and cdc2 expression in U87 glioblastoma cells transduced with a lentivirus containing the d splice variant of KAP (*HA-KAPd*). Control cells were transduced with a lentivirus containing an empty control vector. Overexpression of HA-KAPd was confirmed using a specific anti-hemagglutinin antibody. β-Actin was used as an internal reference.

To further show the involvement of cdc2 in KAP-regulated migration, we examined the effect of overexpressing wild-type cdc2 or a dominant negative cdc2 mutant (cdc2T161A) in U87 cells in which migration was stimulated by KAP knockdown. As shown in Fig. 5*C*, wild-type cdc2 increased migration by 1.5-fold, whereas the cdc2 dominant negative mutant cdc2T161A reduced cell migration by ~2 fold. Neither wild-type cdc2 nor cdc2T161A affected proliferation in these cells (Fig. 5*C*). These data indicate that cdc2 mediates cell migration regulated by KAP.

Additional evidence for a role of cdc2 in the KAP-mediated inhibition of cell migration was obtained using the phosphatase-dead KAP mutant KAPC140S. As previously mentioned, overexpression of this mutant failed to alter cdc2 protein levels but nevertheless increased Cdk2 phosphorylation. Based on these findings, one might predict that this phosphatase-dead KAP mutant would increase proliferation but would fail to inhibit migration. As shown in Fig. 5*D*, overexpression of the mutant KAPC140S caused a small but significant increase in BrdUrd incorporation in U87 glioblastoma cells. As predicted, however, this mutant failed to inhibit cell migration. Taken together, these data indicate that the inhibitory effect of KAP on migration is mediated through phosphatase-dependent inhibition of cdc2 expression.

**Aberrant mRNA splicing generates a dominant negative variant.** Aberrant or alternative splicing has been reported to generate dominant negative proteins in other cancers (21–24). KAP splice variant d has previously been identified in hepatocellular carcinoma (9, 10). However, a biological role for this variant in intact cells has not been determined. We therefore examined whether KAP splice variant d affects cell migration or proliferation. As shown in Fig. 6*A*, overexpression of the d variant of KAP significantly increased glioblastoma cell proliferation. Interestingly, no effect of the d variant of KAP on cell migration was observed (Fig. 6*B*). Western blot analysis (Fig. 6*C*) indicated that the d variant of KAP increased Cdk2 phosphorylation but did not alter cdc2 protein expression. The increase in Cdk2 phosphorylation was consistent with the observed dominant negative effect of the d splice variant on proliferation, whereas the absence of a change in cdc2 protein levels was consistent with the observation that the d variant had no effect on cdc2-dependent cell migration. One possible explanation for this result is that the truncated KAP variant d interacts with other KAP-binding proteins (e.g., Htm4) to selectively inhibit KAP-mediated Cdk2-dephosphorylation, thereby promoting proliferation but not migration (8).

## Discussion

We and others have reported that KAP mRNA is overexpressed in malignant astrocytomas, and one of these studies also found a relationship between KAP mRNA expression and patient survival (14, 15). In addition, KAP is overexpressed in breast, prostate, and liver cancers (9–11). The overexpression of KAP in cancer is paradoxical, given the evidence that KAP inhibits proliferation and migration. We have resolved this apparent paradox in glioblastomas by showing that the increased KAP mRNA expression is associated with aberrant splicing and reduced KAP protein expression. This leads to increased cell cycle progression and increased cell migration, which is a prerequisite for tumor invasion. Moreover, we show that a dominant negative form of KAP (d variant) that increases cell proliferation is generated in glioblastomas.

Aberrant splicing may promote tumorigenesis in a variety of cancers (21–27). In gliomas, expression of an invasion inhibitory gene, *IIp45*, was decreased due to alternative splicing, generating a less stable protein and contributing to tumor malignancy (26). Several mechanisms have been identified that could contribute to the development of aberrant splicing in glioblastomas and other cancers. For example, overexpression of the splicing regulator polypyrimidine tract binding protein (*PTB*) in glioblastoma leads to fibroblast growth factor receptor-1 α exon exclusion, thereby contributing to tumor aggressiveness (28). Intronic mutations have also been shown to promote aberrant splicing in cancer (22). Such inappropriately spliced transcripts often create proteins that are rapidly degraded or that act as dominant negative forms (21–27), as shown in this report. Additional studies are needed to determine the mechanisms contributing to the aberrant splicing of KAP in glioblastomas.

Our finding that KAP regulates both cdc2 expression and Cdk2 activity places it squarely at the center of pathways controlling cell cycle progression and migration. Other members of this pathway, including RB1, E2F, p53, p21, and p27/Kip1, have all been implicated in carcinogenesis (3). Similar to these proteins, we find that a loss of KAP expression due to aberrant splicing is associated with increased malignancy in astrocytomas, suggesting that this dual-specificity phosphatase may also play a role in suppressing tumorigenesis. A role for KAP in cancer has been implied in other studies. For example, KAP mRNA is overexpressed in glioblastomas (14, 15) and is aberrantly spliced in hepatocellular carcinoma (9, 10). KAP mRNA is also overexpressed in breast and prostate cancers (11). The authors of this latter study used an antisense approach to conclude that KAP promotes malignant behavior in these tumors. It is difficult to reconcile this conclusion with the well-established inhibitory effect of KAP on cell proliferation and with our current observation that KAP inhibits cell migration. One difficulty with this latter report was that it relied on the visualization of KAP at 34 kDa on Western blot, even though the predicted size of KAP is 23.5 kDa. We have obtained extensive evidence that this 34-kDa band is nonspecific and does not represent KAP (see Supplementary Fig. S1). Another possible explanation for this discrepancy would be the presence of aberrant splicing in breast and prostate cancers (as is observed in brain and liver cancers), leading to the production of a dominant negative form of KAP that promotes malignancy (21–24). In this latter scenario, knockdown of a dominant negative KAP variant could lead to decreased proliferation in the tumor cells.

Recent studies indicate that $\alpha_v\beta_3$ integrin–mediated activation of cdc2 enhances migration in several cell lines through interactions with cyclin B2, then cdc2 substrate caldesmon, and consequent actin rearrangement (13). We report here that KAP inhibits cell migration via a cdc2-dependent mechanism. This is in addition to the previously known inhibitory effect of KAP on Cdk2 activity and cell cycle progression. In this study, KAP decreased *cdc2* mRNA and protein levels via its phosphatase activity. It is possible that the change in cdc2 expression is cell cycle dependent and is not directly related to KAP expression. Arguing against such an effect here, however, is the observation that the d variant of KAP altered proliferation without altering cdc2 expression. Additional studies are under way to determine whether this effect is mediated through the dephosphorylation of Cdk2 or another, as yet unidentified, target of KAP.

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