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Glucose Deprivation Contributes to the Development of *KRAS* Pathway Mutations in Tumor Cells

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Abstract

Tumor progression is driven by genetic mutations, but little is known about the environmental conditions that select for these mutations. Studying the transcriptomes of paired colorectal cancer cell lines that differed only in the mutational status of their *KRAS* or *BRAF* genes, we found that *GLUT1*, encoding glucose transporter-1, was one of three genes consistently upregulated in cells with *KRAS* or *BRAF* mutations. The mutant cells exhibited enhanced glucose uptake and glycolysis and survived in low glucose conditions, phenotypes that all required *GLUT1* expression. In contrast, when cells with wild-type *KRAS* alleles were subjected to a low glucose environment, very few cells survived. Most surviving cells expressed high levels of GLUT1 and 4% of these survivors had acquired new *KRAS* mutations. The glycolysis inhibitor, 3-bromopyruvate preferentially suppressed the growth of cells with *KRAS* or *BRAF* mutations. Together, these data suggest that glucose deprivation can drive the acquisition of *KRAS* pathway mutations in human tumors.

Mutations in oncogenes and tumor suppressor genes endow cancer cells with the ability to outgrow their neighboring cells in situ (1). Though numerous studies have identified the downstream effects of such mutations and their biochemical mediators, there is relatively little known about the microenvironmental conditions that provide the selective advantage that allows cells with such mutations to clonally expand. Mutations in *KRAS* commonly occur in colorectal, pancreatic, and some forms of lung cancer, while *BRAF* mutations occur commonly in melanomas as well as in colorectal tumors without *KRAS* mutations (2–4). *BRAF* and *KRAS* mutations are mutually exclusive, that is, do not occur in the same tumor, suggesting a common origin and effect. Indeed, KRAS binds to and activates BRAF, thereby activating MAPK signaling pathways (5,6). Despite advances in the molecular delineation of the RAS/RAF pathway, the specific environmental pressures that drive *KRAS* and *BRAF* mutations and how *KRAS* and *BRAF* mutations alleviate these pressures are unknown.

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To explore this issue, we developed isogenic colorectal cancer (CRC) cell lines in which the endogenous wild-type (wt) or mutant alleles had been inactivated through targeted homologous recombination (table S1, fig. S1 and fig. S2) (7). We chose to use targeted homologous recombination instead of the more commonly used overexpression or siRNA-dependent systems because only the former permits examination of cells expressing normal or mutant proteins at physiological, normally regulated levels (8). For the investigation of *BRAF* mutations, we used RKO and VACO432, CRC lines with valine to glutamate mutations at codon 600 (V600E) of *BRAF*. This is the most common *BRAF* mutation in human tumors, accounting for over 90% of *BRAF* mutations (3). To analogously investigate *KRAS*, we used HCT116 and DLD1, CRC lines with glycine to aspartate mutations at codon 13 (G13D). This mutation is one of the most common in CRC, accounting for ~20% of *KRAS* mutations (2). These paired lines essentially differ in only one base pair - the base that is mutated or wild-type in *KRAS* or *BRAF*. At least two independent clones of each of the derivatives of each of the four parental cell lines were developed (table S1). In all cases, independent clones with the same genotype behaved similarly in the assays described below.

Based on the mutual exclusivity of *KRAS* and *BRAF* mutations and knowledge of the *KRAS* pathway described above, we reasoned that mutations of both genes would result in common deregulation of a discrete set of transcripts. We performed expression analysis on clones of various genotypes with microarrays as well as with massively parallel sequencing of serial analysis of gene expression (SAGE) tags. Only three genes were found to be more than two-fold upregulated in all four lines containing mutant *KRAS* or *BRAF* alleles compared to their isogenic counterparts containing wt alleles: *GLUT1* (also known as *SLC2A1*), *DUSP5* and *DUSP6* (fig. S3). *DUSP5* and *DUSP6* are known feedback regulators of MAPK signaling pathway, up-regulated when the pathway is active (9) and were thus unlikely to be positive effectors of *KRAS* and *BRAF* tumorigenesis. On the other hand, *GLUT1* was intriguing as it encodes a glucose transporter known to be over-expressed in many types of cancer and its high expression in tumors has been associated with poor prognosis (10,11). We confirmed the results of the microarray and SAGE expression analyses through quantitative-PCR. *GLUT1* transcript expression was always higher, ranging from 3- to 22- fold, in the clones with mutant *KRAS* or *BRAF* alleles compared to the isogenic clones with wt alleles (Fig. 1A and fig. S3). Accordingly, we found that the expression of the GLUT1 protein was markedly higher in cells with mutant *KRAS* or *BRAF* alleles (Fig. 1B). Targeted disruptions of both alleles of *GLUT1* in RKO and DLD1 cells (table S1 and fig. S4) were used as negative controls to ensure the specificity of the antibodies to GLUT1 (Fig. 1B). As expected, the GLUT1 protein was found in the membrane fraction of cells, regardless of *BRAF* or *KRAS* mutational status. Of the 12 human glucose transporter homologs present in the human genome (10), only *GLUT1* was upregulated in the mutant *BRAF* or *KRAS*-containing lines compared to those with wt alleles.

To further test the specificity of the upregulation of *GLUT1*, we evaluated its expression in cell lines in which the mutant or wt alleles of *PIK3CA* had been disrupted by targeted homologous recombination (12). *PIK3CA* has been implicated in the RAS/RAF pathways as well as in metabolic regulation and is commonly mutated in cancers (12,13). Unlike *KRAS* and *BRAF*, the *PIK3CA* genotype did not have a clear effect on GLUT1 protein expression (Fig. 1B). We also tested lines with targeted disruptions of both alleles of *HIF1A* (14) (table S1). Though *HIF1A* has been shown to regulate transcription of *GLUT1* in hypoxic conditions (15–17), GLUT1 expression was found to be largely independent of *HIF1A* status when cells were grown in normal oxygen concentrations (Fig. 1B).

We suspected that the upregulation of *GLUT1* would result in increased glucose uptake in the clones with mutant *KRAS* or *BRAF* alleles. To test this hypothesis, we incubated cells with 2-deoxy-D-[^{3}H] glucose (2-DG), a non-hydrolyzable glucose analog, and measured its uptake. We found that the upregulation of *GLUT1* was accompanied by significant increase in glucose

uptake in all cells with mutant *KRAS* or *BRAF* alleles compared to the isogenic cells with wt alleles (Fig. 2A). Disruption of *GLUT1* substantially inhibited glucose uptake, demonstrating that GLUT1 was the major glucose transporter in these cancer cells (Fig. 2A).

We next determined whether the increased glucose transport was associated with increased lactate production. Lactate production was indeed significantly increased in cells with mutant *KRAS* or *BRAF* alleles, indicating an increased rate of glycolysis and consistent with higher glucose uptake (Fig. 2B). Lactate production was very low in cells without *GLUT1* genes, as would be expected if *GLUT1* was the major glucose transporter in these cells (Fig. 2B). On the other hand, oxygen consumption was not different in cells with mutant *KRAS* or *BRAF* alleles than in cells with wt alleles of these genes, suggesting that mitochondrial function and oxidative respiration were not affected by *KRAS* or *BRAF* mutation (fig. S5). Accordingly, there were no consistent differences in cellular ATP concentrations or ATP/ADP ratios in cells with mutant *KRAS* or *BRAF* alleles compared to their wt counterparts (figs. S6 and S7).

These results suggested that the increase in glucose uptake and glycolysis might provide a growth advantage to cells with *KRAS* or *BRAF* mutations in low glucose environments. When grown in standard, commercially available media (25 mM glucose), all cell lines, including those without *GLUT1* gene, grew reasonably well (fig. S8) and formed colonies when plated at low density. However, when placed in media containing low glucose concentrations (0.5 mM), only cell lines with *KRAS* or *BRAF* mutant alleles were able to survive (Fig. 3A). This growth was dependent on *GLUT1*, as cells in which the *GLUT1* gene was inactivated by targeted homologous recombination lost their ability to form colonies in low glucose, even though they contained mutant *KRAS* or *BRAF* genes (Fig. 3A). In contrast, such growth was independent of *HIF1A*, as cells with mutant *KRAS* or *BRAF* alleles survived in low glucose when the *HIF1A* gene was inactivated by targeted homologous recombination (Fig. 3A).

We then determined whether clones with mutant *KRAS* or *BRAF* genes could selectively outgrow cells without these mutations. For this purpose, cells with mutant *KRAS* or *BRAF* alleles were mixed with an excess of cells containing wt *KRAS* or *BRAF* alleles, respectively, and were incubated in either low glucose (0.5 mM) or standard concentrations of glucose (25 mM). Cells with mutant *KRAS* or *BRAF* alleles preferentially survived in low glucose and overtook the population within two weeks after changing the medium to one containing 25 mM glucose. In contrast, the cells with wt alleles remained predominant when they were not exposed to low glucose conditions (Fig. 3B).

To further mimic situations that might occur in vivo, we subjected cells with only wt alleles (obtained by disrupting the mutant *KRAS* or *BRAF* alleles; figs. S1 and S2) to a low glucose environment in vitro and isolated the few colonies that survived (fig. S10). We reasoned that in the ~35 generations that had elapsed between targeted disruption and this experiment, a small fraction of cells would have spontaneously acquired mutations in genes that could potentially permit them to survive in medium containing low glucose concentrations. The fact that the two cell lines used for this experiment were both mismatch repair deficient should have facilitated the development of such de novo mutations (18). We found that the fraction of DLD1 *KRAS* (−/+) or RKO *BRAF* (−/−/+) cells that could form colonies in low glucose conditions was ~0.05%. Once formed, the colonies were grown in medium containing standard concentrations of glucose (25 mM). We found that more than 75% of the clones derived from either cell line after selection in low glucose stably expressed high levels of GLUT1 protein, even when subsequently grown in standard medium (25 mM glucose; Fig. 3C and fig. S11). Thus, the selection for growth in low glucose resulted in a permanent upregulation of GLUT1 expression in the majority of clones that survived, and this upregulation persisted after normoglycemia was reinstituted, indicating a heritable change. Control clones derived analogously, but with 25 mM glucose substituted for 0.5 mM glucose during the selection

period, did not show elevated GLUT1 expression (Fig. 3C and fig. S11). When the clones derived from DLD1 *KRAS* (−/+) cells were assessed for mutations, 4.4% of the clones arising under hypoglycemic conditions had mutations in *KRAS* (73.5% of these had G12D, 25.2% had G13D, 1.3% had G13C; and 0% had *BRAF* V600E or other mutations in *KRAS* at codon 12 or 13). No *KRAS* or *BRAF* mutations were identified in 2,000 DLD1 *KRAS* (−/+) clones generated in the presence of standard concentrations of glucose ($p<0.000001$, χ^2). In the clones derived from RKO *BRAF*(−/−/+) cells, 0.8% of the clones surviving low glucose exposure had a G12D *KRAS* mutation, while none of 2,000 clones grown in the presence of standard glucose concentrations had such mutations ($p<0.01$, χ^2).

We next attempted to exploit this phenotype to specifically target cancer cells with *KRAS* or *BRAF* mutations. We reasoned that cells with *KRAS* or *BRAF* mutations had stably reprogrammed their metabolic pathways and might be dependent on glycolysis for growth. Accordingly, an agent such as 3-bromopyruvate (3-BrPA), that inhibits glucose metabolism through inhibition of hexokinase (19), might be selectively toxic to cells with *KRAS* or *BRAF* mutations. When this hypothesis was tested experimentally in the paired isogenic cell lines, it was found that 3-BrPA was highly toxic to HCT116, DLD1, VACO432 and RKO cells with *KRAS* or *BRAF* mutations but was much less toxic to the matched cell lines lacking *KRAS* or *BRAF* mutant alleles (Fig. 4A).

We next wished to determine whether this approach might be applicable in experimental tumors in animals. As a prelude, we found that cells with disrupted mutant *KRAS* or *BRAF* alleles grew poorly as xenografts in nude mice compared to their isogenic counterparts with mutant alleles (fig. S12). DLD1 and RKO cells in which the *GLUT1* gene was disrupted also grew poorly in nude mice, even though these cells contained mutant *KRAS* and *BRAF* alleles, respectively (fig. S12). These results indicated that the microenvironment in xenografts in some ways mimicked the low glucose environment in vitro and provided a reasonable system to test the effects of glycolytic inhibitors. Indeed, 3-BrPA significantly inhibited the growth of established xenografts derived from HCT116 and VACO432 cells (Fig. 4B). Though this result was not sufficiently robust to warrant implementation in a clinical setting, it provided proof-of-principle that glycolytic inhibitors can retard tumor growth at doses that are non-toxic to normal tissues in vivo.

Our results led us to investigate glucose metabolism in a completely unbiased way, thereby considerably complementing previous work by other investigators. For example, a role for metabolic abnormalities in cancer has become increasingly recognized (20,21). These metabolic abnormalities often appear to involve abnormal glycolysis, as first demonstrated decades ago by Otto Warburg (22). Insightful hypotheses about the manifold ways in which such metabolic abnormalities can promote tumor progression have been described (23–25). It has also been demonstrated that transformation of rodent fibroblasts by several oncogenes, including *HRAS*, can upregulate glucose transporter expression (16,26–28). However, because transformation by overexpressed oncogenes affects the expression of hundreds of genes and dramatically alters the phenotype of rodent fibroblasts, the relationship between increased glucose transporter expression and tumorigenesis was not clear. In human tumor cells, no obvious relationship between *GLUT1* and *RAS* mutations has been identified (29,30). Moreover, in many previous experimental studies in rodent cells, the increased *GLUT1* expression was ascribed to induction of HIF1A and linked to hypoxia (15,16,31,32). Our results show that the increased *GLUT1* transcription was unrelated to HIF1A because genetic disruption of the *HIF1A* gene did not affect the expression of GLUT1, nor did it affect survival under hypoglycemic conditions. Notably, cells without mutant *KRAS* or *BRAF* alleles were remarkably sensitive to hypoglycemia, but not to hypoxia (Fig. 3 and fig. S9). Furthermore, the changes in *GLUT1* expression and resultant metabolic changes in human colorectal cancer cells were stable phenotypes rather than transient responses to low glucose, as they persisted

under normoglycemic conditions. This stability is consistent with them being the consequence of specific genetic mutations, such as those in *KRAS* or *BRAF*. In aggregate, our results suggest that low glucose environments are a driving force underlying the development of *KRAS* and *BRAF* mutations during tumorigenesis.

F-18-Fluoro-deoxyglucose (FDG)-Positron Emission Tomography (PET) scans are routinely used to image cancers in the clinic. Positive signals in cancers are the result of increased glucose transporter expression or glucose uptake (33). Our data showed that in four different human cancer cell lines, an increase in *GLUT1* expression and glucose uptake was critically dependent on *KRAS* or *BRAF* mutations. It is interesting that abnormal FDG-PET signals can be observed in progressing pre-malignant colorectal neoplasms (adenomas) congruent with the time during tumorigenesis in which *KRAS* or *BRAF* mutations appear (34,35).

The results also raise a variety of as yet unanswered questions. One concerns the relationship between hypoxia and hypoglycemia. Though both these deficiencies are likely to be encountered in tumor microenvironments, it is possible that each condition sets the stage for selection of particular genetic abnormalities (23). For example, hypoglycemic conditions favor the selection of cells with *KRAS* or *BRAF* mutations, while hypoxic conditions may favor the selection of cells with *PIK3CA*, *CMYC* or *TP53* mutations (20,36). Another issue for consideration is that 90% of colorectal cancers exhibit high FDG-PET signals and GLUT1 expression (34,37,38), whereas *KRAS* or *BRAF* mutations are only observed in ~50% of such cancers (4). One possibility to explain this discrepancy is that other genetic alterations that impact the same pathway can substitute for *KRAS* and *BRAF* mutations in upregulating *GLUT1*. This idea is consistent with recent data indicating that the same pathway can be mutationally activated through disparate mutations in numerous genes (1,39,40). It is also consistent with our in vitro selection experiments. Though the majority of clones that survived hypoglycemia upregulated GLUT1, only a minority of these clones had acquired *KRAS* or *BRAF* mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

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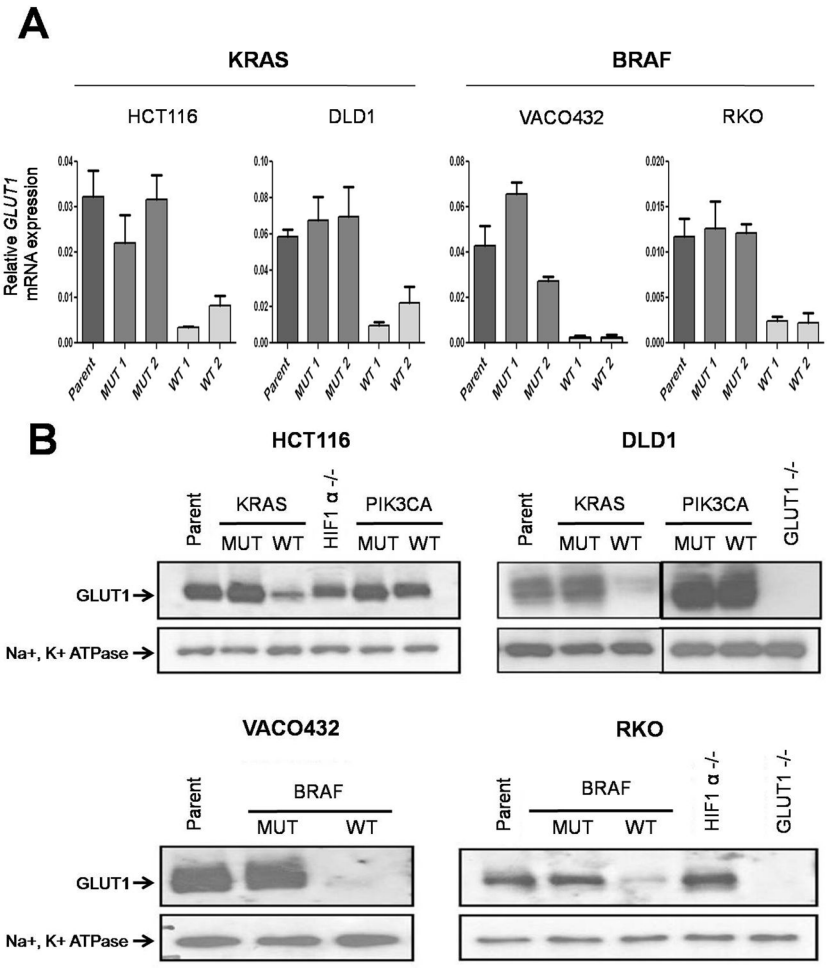

Fig. 1.

Expression of *GLUT1* in matched pairs of isogenic clones. (**A**) Expression levels of *GLUT1* transcripts were determined by real-time PCR and normalized to those of β-actin. Each panel includes the parental line (Parent), which harbors both mutant and wild-type alleles of *KRAS* or *BRAF*, two independent clones with only mutant alleles (MUT1 and MUT2) and two independent clones with only wild-type alleles (WT1 and WT2). The data represent the mean and SD of triplicate experiments. The differences between MUT and WT clones were statistically significant in all cases ($P < 0.05$, Student's t-test). (**B**) Expression of GLUT1 membrane-associated protein levels as determined by immunoblotting. Na^+,K^+-ATPase, a membrane associated protein, was used as a loading control.

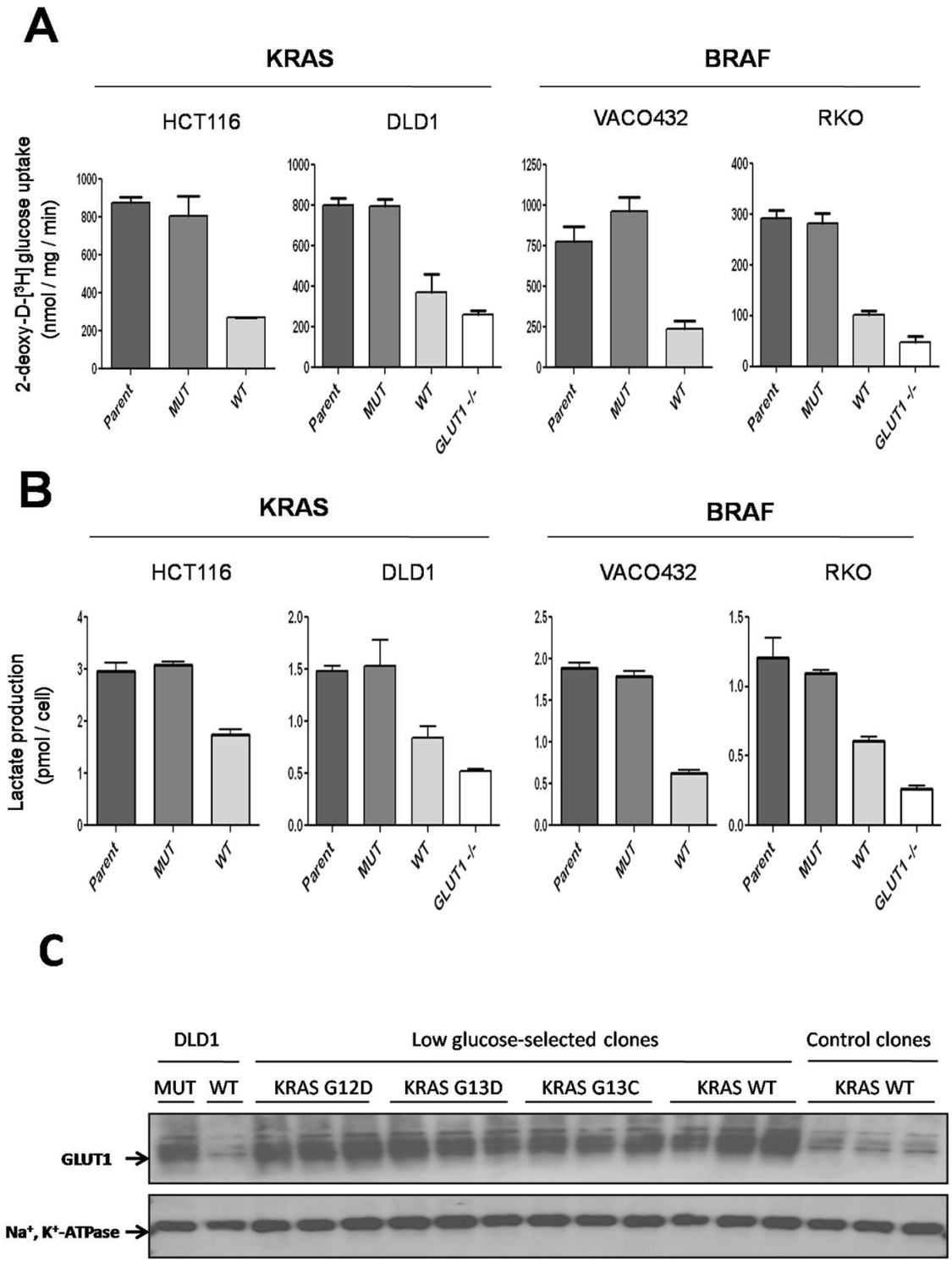

Fig. 2.

Glucose uptake and lactate production in cells with *KRAS* or *BRAF* mutations. (**A**) Glucose uptake, as determined using [^{3}H] 2-deoxyglucose, was normalized to total protein. Differences between MUT and WT clones were statistically significant ($P < 0.01$, Student's t-test). (**B**) Lactate production was normalized to cell number. The differences between MUT and WT

clones were statistically significant in all cases ($P < 0.03$, Student's t-test). The data represent the mean and SD of triplicate experiments.

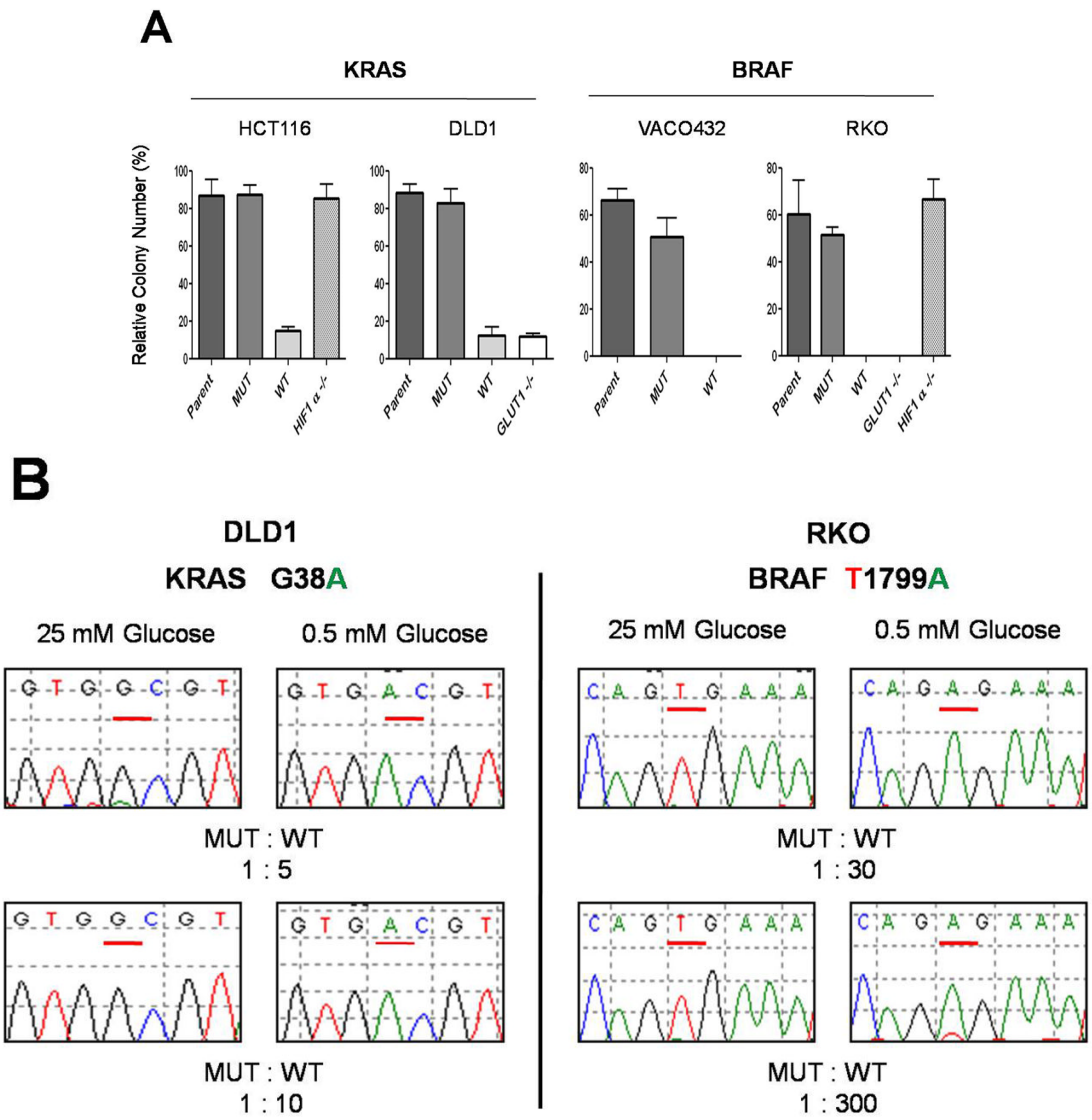

Fig. 3.

KRAS and *BRAF* mutations confer a selective growth advantage in hypoglycemic conditions. (**A**) Cells were subjected to a low glucose environment (0.5 mM) for two (RKO and VACO432) or four (HCT116 and DLD1) days, then dissociated and plated in media containing standard concentrations of glucose (25 mM). Colony counts were normalized to those obtained in cells subjected to the same experimental procedure with the exception that standard glucose levels were substituted for low glucose. See (7) for details. The differences between MUT and WT clones were statistically significant in all cases ($P < 0.004$, Student's t-test). (**B**) MUT and WT clones were mixed at the indicated ratios and grown in media with 0.5 mM glucose for two days (RKO) or five days (DLD1). The media was replaced with one containing 25 mM glucose and the cells incubated for another 10–16 days. RNA was purified from the cells that survived and the *KRAS* or *BRAF* genes were PCR-amplified and sequenced. G and A nucleotides at the underlined positions in the sequencing chromatograms represent wt and mutant alleles of *KRAS*, respectively, in DLD1 cells. T and A nucleotides represent wt and mutant alleles of *BRAF*, respectively, in RKO cells. (**C**) DLD1 cells in which the mutant *KRAS* allele had been deleted by targeted recombination (*KRAS* (−/+)), were plated in low glucose (0.5 mM). After

25–30 days, the few clones that survived were grown in standard glucose (25 mM) and assessed for GLUT1 expression and the sequence of the *KRAS* gene. Clones which harbored mutant alleles of *KRAS* (G12D, G13D, or G13C) are indicated, as are clones in which *KRAS* remained WT. As controls, the same cells (*KRAS* (−/+)) were plated at limiting dilution in media containing 25 mM glucose and individual clones assessed for GLUT1 expression ("Control Clones"). The parental cells used for these experiments (DLD1, WT) are also included, as were their isogenic counterparts in which the wt rather than the mutant allele was disrupted by homologous recombination (DLD1, MUT). All clones had been growing in media containing 25 mM glucose for at least 20 days when harvested for the assessment of GLUT1 expression by immunoblotting. Na^+,K^+-ATPase was used as a loading control. A diagram of the selection scheme is provided in fig. S10 and detailed methods are provided in (7).

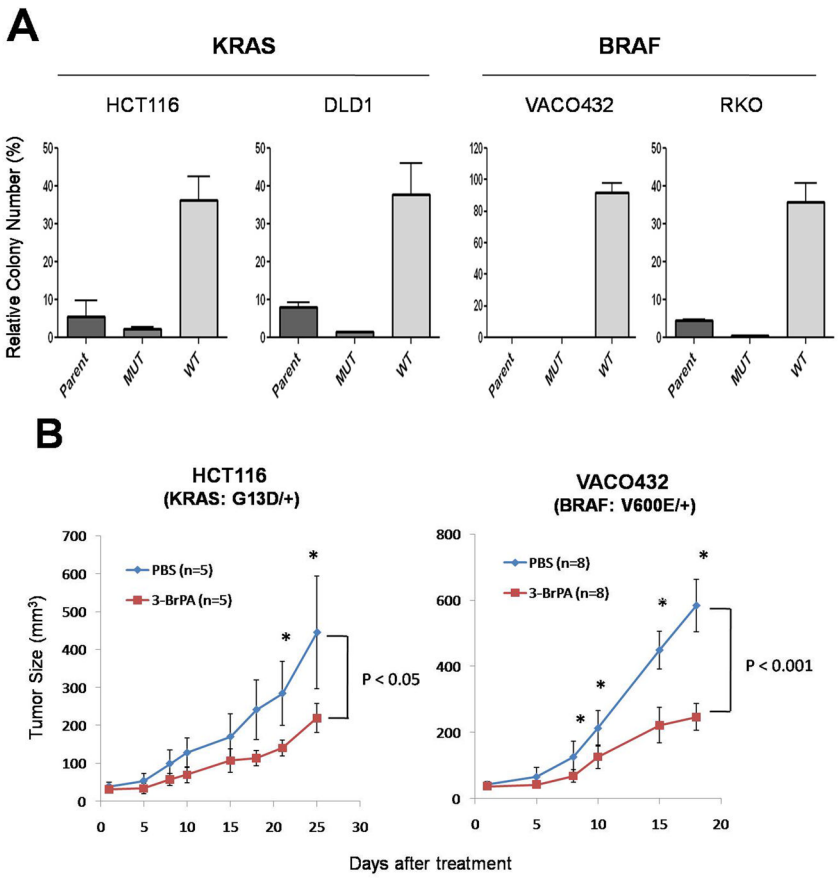

Fig. 4.

The glycolysis inhibitor 3-BrPA is selectively toxic to cells with mutant *KRAS* or *BRAF* alleles. (**A**) Colony formation was assessed after 3-BrPA treatment (110 μM) for three days. Colony counts were normalized to those obtained from cells subjected to the same procedure without exposure to 3-BrPA. The differences between MUT and WT clones were statistically significant in all cases ($P < 0.008$, Student's t-test). (**B**) Mice with subcutaneous tumors established from HCT116 (*KRAS*: G13D/+) or VACO432 (*BRAF*: V600E/+) cells were injected intraperitoneally with 3-BrPA or phosphate buffered saline (PBS) daily for two weeks. "n" represents the number of mice used in each group. Points and error bars represent the means and SD for each group of mice. Asterisks denote times when there were significant differences between the tumor sizes in the PBS vs. 3-BrPA groups ($P < 0.05$, Student's t-test).