Mutant PIK3CA promotes cell growth and invasion of human cancer cells

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Summary

PIK3CA is mutated in diverse human cancers, but the functional effects of these mutations have not been defined. To evaluate the consequences of PIK3CA alterations, the two most common mutations were inactivated by gene targeting in colorectal cancer (CRC) cells. Biochemical analyses of these cells showed that mutant PIK3CA selectively regulated the phosphorylation of AKT and the forkhead transcription factors FKHR and FKHRL1. PIK3CA mutations had little effect on growth under standard conditions, but reduced cellular dependence on growth factors. PIK3CA mutations resulted in attenuation of apoptosis and facilitated tumor invasion. Treatment with the PI3K inhibitor LY294002 abrogated PIK3CA signaling and preferentially inhibited growth of PIK3CA mutant cells. These data have important implications for therapy of cancers harboring PIK3CA alterations.

Introduction

PIK3CA encodes the p110α catalytic subunit of the class IA phosphatidylinositol 3-kinases (PI3Ks). PI3Ks are heterodimers composed of p110 catalytic and p85 regulatory subunits and can be activated by recruitment to the cell surface by growth factor receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and the insulin receptor (Cantley, 2002; Vanhaesebroeck and Waterfield, 1999). Active PI3Ks phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP_2) at the 3-position of the inositol ring, converting it to phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 acts as a docking site for pleckstrin homology (PH)-containing proteins, such as the AKT serine/threonine kinase, and for the 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Vanhaesebroeck and Alessi, 2000). Once at the membrane, AKT is activated by PDK1 and phosphorylates numerous protein targets, including Tuberin (Manning et al., 2002), GSK3β (Cross et al., 1995), BAD (Datta et al., 1997; del Peso et al., 1997), MDM2 (Mayo and Donner, 2001; Zhou et al., 2001), p21 (WAF1/CIP1) (Lawlor and Rotwein, 2000; Rossig et al., 2001), caspase 9 (Cardone et al., 1998), IKK (Romashkova and Makarov, 1999), a subset of forkhead transcription factors (Brunet et al., 1999; Guo et al., 1999; Rena et al., 1999), and mTOR, which in turn regulates phosphorylation of p70-S6K and 4EBP1 (Harris and Lawrence, 2003; Nave et al., 1999). The biological consequences of AKT activation are broad, and can be subdivided into regulation of cell proliferation, survival, and motility (Katso et al., 2001; Vivanco and Sawyers, 2002).

The PI3K pathway is genetically deregulated in human cancer at several levels. The tumor suppressor PTEN, which dephosphorylates PIP_3 to PIP_2, thus antagonizing PI3K activity, is deleted or mutated in several different tumor types (Li et al., 1997; Sansal and Sellers, 2004; Steck et al., 1997), and amplification of genomic regions containing *AKT* or *PIK3CA* genes has also been reported (Bellacosa et al., 1995; Cheng et al., 1992; Cheng et al., 1996; Shayesteh et al., 1999). PIK3CA is somatically mutated in over 25% of colorectal, gastric, breast, and certain brain tumors (Samuels et al., 2004), and is mutated at significant frequencies in other tumor types (Bachman et al., 2004; Broderick et al., 2004; Campbell et al., 2004; Samuels et al., 2004). From these mutation frequencies, *PIK3CA* appears to be one of the most highly mutated oncogenes yet identified in human cancers.

Interestingly, more than 80% of the mutations in *PIK3CA* cluster in two small conserved regions within the helical and

SIGNIFICANCE

PI3K signaling pathways can be deregulated by a variety of mechanisms in human tumors. We have recently discovered that mutations in PIK3CA, a key member of this pathway, occur in a significant fraction of colorectal, breast, brain, and other tumor types. To evaluate the effect of PIK3CA mutation in human cancer cells, we selectively inactivated wild-type and mutant versions of this gene in colorectal cancer cell lines and examined their phenotypes. Our analyses showed that PIK3CA plays an essential role in tumor cell proliferation in adverse conditions as well as in invasion and metastasis. These results suggest that patients with tumors containing PIK3CA mutations may ultimately benefit from therapy directed at mutant PIK3CA or its downstream targets.

kinase domains (Samuels et al., 2004). The high frequency of mutations in these hotspots is reminiscent of mutations found in oncogenes such as *KRAS* and *BRAF* (Bos et al., 1987; Davies et al., 2002; Rajagopalan et al., 2002), and is consistent with the premise that alterations in PIK3CA are activating. Indeed, hotspot mutations have previously been shown to enhance lipid kinase activity in vitro (Samuels et al., 2004; Kang et al., 2005). Additionally, functional analyses of other members of this pathway are consistent with an oncogenic role for PIK3CA: heterozygous PTEN knockout mice show increased tumor development (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998), and overexpression of PIK3CA as well as AKT genes has been shown to be transforming (Aoki et al., 2000; Chang et al., 1997). However, the biochemical and biologic effects of PIK3CA mutations have not been evaluated in human cancer cells.

In this work, we have investigated the role of *PIK3CA* mutation in human colorectal cancer (CRC) cells using a gene targeting approach. Targeted homologous integration was used to specifically disrupt either the mutant or wild-type allele of *PIK3CA* in two colorectal cell lines, each harboring one of the two major hotspot mutations. Because the parental and targeted cells are identical except for the alteration at the *PIK3CA* locus, they serve as a rigorously controlled system with which to examine the effects of mutant *PIK3CA* alleles. Our results indicate that both of the evaluated hotspot mutations constitutively activate the AKT pathway, and that this activation is essential for cellular growth under adverse conditions in vitro, as well as for invasion.

Results

Gene targeting of *PIK3CA* in human cancer cells

To analyze the biologic and tumorigenic effects of PIK3CA, we established isogenic cell lines in which either the wild-type or mutant alleles of this gene were disrupted. The colorectal cancer cell lines HCT116 and DLD1 were selected for gene targeting because each contains a different hotspot mutation of PIK3CA: HCT116 has an H1047R alteration in exon 20 (kinase domain), while DLD1 contains an E545K alteration in exon 9 (helical domain). In addition, both cell lines are diploid at the *PIK3CA* locus and are susceptible to gene targeting through homologous recombination (Shirasawa et al., 1993; Waldman et al., 1995). The adeno-associated virus (AAV) targeting system (Hirata et al., 2002; Kohli et al., 2004) was used to disrupt exon 1 as depicted in Figure 1A, resulting in cells with an endogenous functional allele and a nonfunctional targeted allele. Clones in which the *PIK3CA* locus was appropriately targeted were identified by polymerase chain reaction (PCR) using primers specific to the targeting vector and to adjacent genomic sequences. The *PIK3CA* genotype of the clones was confirmed by reverse transcription (RT)-PCR of the PIK3CA transcript coupled with direct sequencing (Figure 1B). In all cases, clones in which the mutant allele had been targeted expressed only the wild-type allele, and vice versa (Figure 1B). Two clones of each genotype were chosen from each cell line for in-depth analysis, and both clones behaved similarly in all the studies described below. Clones in which the mutant allele had been disrupted and the wild-type allele was intact are referred to as "wild-type" (WT) clones, while clones in which the wild-type allele had been disrupted and the mutant allele was intact are referred to as "mutant" clones.

As previous reports of *PIK3CA* targeting in the mouse resulted in alterations of protein levels of the PI3K regulatory subunit p85α (Bi et al., 1999), we examined whether PIK3CA targeting in our clones might affect levels of other PI3K regulatory or catalytic subunits. Examination of protein levels of p85α, p110β, p110γ, and p110δ showed similar protein levels for all parental, mutant, and WT clones (Supplemental Figure S1).

The AKT pathway is activated in mutant PIK3CA-containing clones

To examine the catalytic activity of mutant PIK3CA, we generated recombinant E545K and H1047R hotspot mutants and examined their lipid kinase activity in vitro. Both mutant proteins showed increased PI3-kinase activity compared to wild-type protein (Supplemental Figure S2), consistent with previous studies showing that mutations in PIK3CA result in increased lipid kinase activity (Samuels et al., 2004; Kang et al., 2005). To determine the effects of mutant PIK3CA on signaling pathways, we first examined AKT phosphorylation, as such phosphorylation appears to be essential for PIK3CA signaling. Levels of phosphorylated AKT in cells growing in normal growth medium were determined using immunoblotting. We found that mutant clones exhibited increased phosphorylation of AKT in comparison to WT clones at residue Thr-308 as well as Ser-473 (Figure 2A). To determine which of the three AKT proteins, AKT1, AKT2, or AKT3, was affected by PIK3CA mutation, each AKT protein was individually immunoprecipitated and subjected to immunoblotting with the same phosphospecific antibodies. These analyses showed that AKT1 is the predominant AKT isoform expressed in CRC and is activated in cells with mutant PIK3CA (Figure 2B). To examine if the increased activation of AKT was due to increased PIK3CA kinase activity or increased stability of mutant protein, we evaluated p110α protein levels and found that these were similar in all WT and mutant clones (Supplemental Figure S1). These results suggest that increased AKT phosphorylation is due to constitutive activation of PIK3CA rather than alteration in its protein levels.

AKT is known to directly phosphorylate a number of proteins (Vivanco and Sawyers, 2002). To determine which AKT targets are mediators of PI3K signaling in CRC cells, the phosphorylation of candidate targets was assessed using specific antibodies. Increased phosphorylation of two forkhead transcription factors of the FOXO family, FKHRL1 and FKHR, was detected in the mutant clones (Figure 2C). Reduced levels of FKHR protein were observed in both mutant clones (Figure 2C), consistent with a recent study suggesting that FKHR is regulated by phosphorylation-dependent proteosome degradation (Aoki et al., 2004). Surprisingly, analysis of other AKT downstream targets, such as mTOR, 4E-BP1, p70-S6K, Tuberin, GSK3β, and AFX showed no consistent differences in phosphorylation among the WT and mutant clones (Supplemental Figure S3 and data not shown). Thus, in CRCs, mutant PIK3CA appears to constitutively activate signaling through AKT1, and this signal appears to be propagated, at least in part, by its FKHR and FKHRL1 substrates.

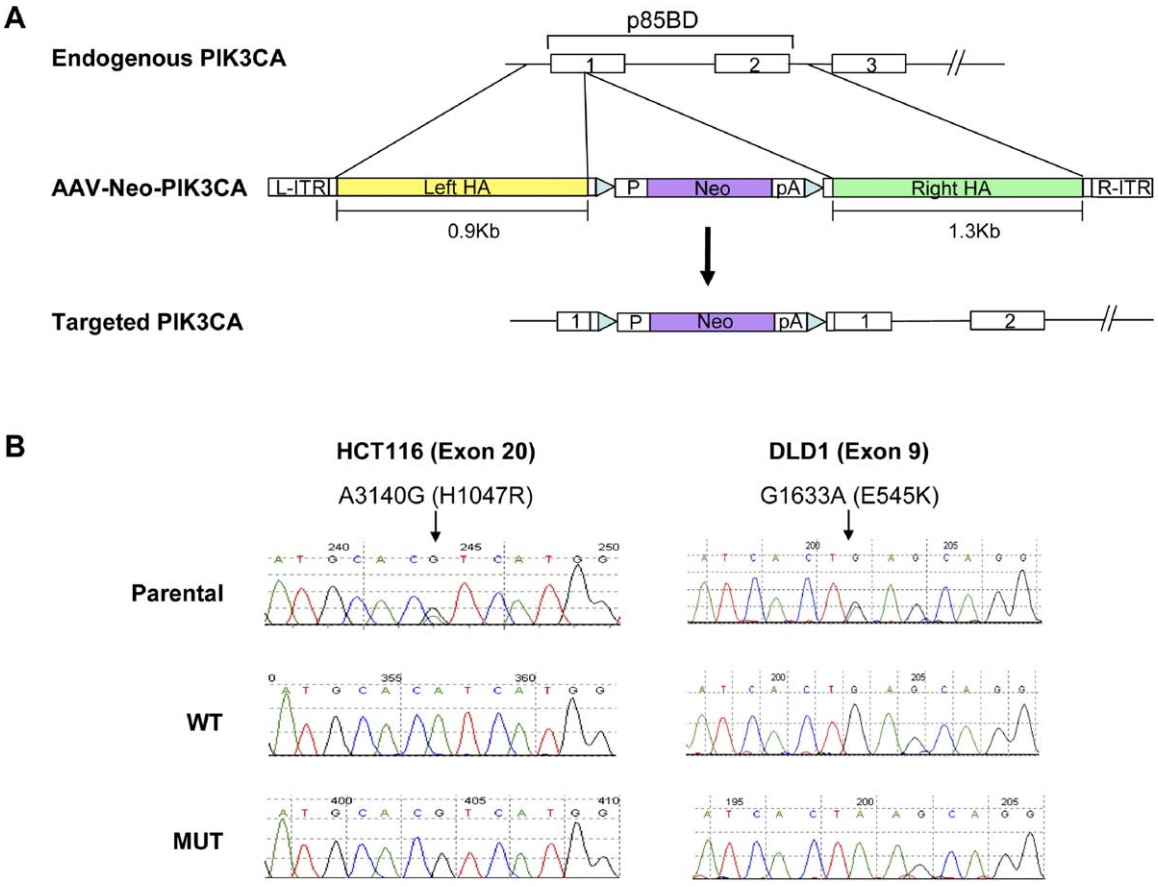


Figure 1. Disruption of the *PIK3CA* gene in human colorectal cancer cells

A: A portion of the *PIK3CA* locus is shown before and after targeting with the AAV targeting construct. A targeted insertion was made in exon 1 by homologous recombination. p85BD, p85 binding domain; AAV-Neo-PIK3CA, the targeting construct; HA, homology arm; P, SV40 promoter; Neo, geneticin-resistance gene; R-ITR, right inverted terminal repeat; L-ITR, left inverted terminal repeat; triangles, loxP sites; pA, polyadenylation signal. Three STOP codons were added at the end of the *Neo* gene to ensure premature termination of the transcript.

B: The *PIK3CA* genotype of targeted DLD1 and HCT116 clones was determined by RT-PCR and sequencing of the PIK3CA transcript. The nucleotide and amino acid alterations are indicated above the arrow. HCT116 cells contain a PIK3CA kinase domain mutation, while DLD1 cells contain a helical domain mutation. Clones in which the mutant allele has been disrupted and the wild-type allele is intact are referred to as wild-type (WT) clones, while clones in which the wild-type allele has been disrupted and mutant allele is intact are referred to as mutant (MUT) clones.

PIK3CA mutation promotes growth factor-independent cell proliferation

Mutations in oncogenes like PIK3CA presumably confer some net growth advantage on the cancer cells in which they occur, thereby facilitating clonal expansion. To assess the effects of PIK3CA mutations on growth, we first investigated growth rate on plastic tissue culture plates and in soft agar (Figures 3A–3C). In the presence of media with 10% serum, all clones grew similarly both on plastic and in soft agar. Consistent with this result, no differences in growth of cells injected subcutaneously in athymic nude mice were observed among various clones (Figure 3D). However, if the serum concentration was reduced to 0.5%, wild-type clones grew at a lower rate than mutant clones on plastic (Figure 3B). This difference was also observed when the cells were assessed for anchorage independence in 0.5% serum. Although all clones formed a similar number of colonies in soft agar under reduced serum concentrations, WT clones formed smaller colonies than mutant clones (Figure 3C).

To determine the biochemical basis for these growth disparities, we examined phosphorylation of the downstream mediators activated by PIK3CA. The most dramatic differences were observed in AKT and its targets, FKHRL1 and FKHR. In 0.5% serum, the level of phosphorylation of AKT and the two forkhead proteins was much higher in the mutant clones than in the WT clones, and was in fact as high as in the WT clones grown in normal serum conditions (Figure 4A). In contrast, other AKT targets such as GSK3β were not affected in this manner in low serum concentrations (Supplemental Figure S4). These results suggested that high serum concentration activates the pathway in the WT clones, and that mutant clones are less sensitive to these factors because the pathway is already constitutively activated. To determine what serum factors might be responsible for the activation observed in WT cells, we tested the effects of various cellular growth factors. When the cells were grown in 0.5% serum supplemented with either EGF or insulin, the phosphorylation of FKHRL1 and FKHR in WT clones was increased to levels similar to those observed in

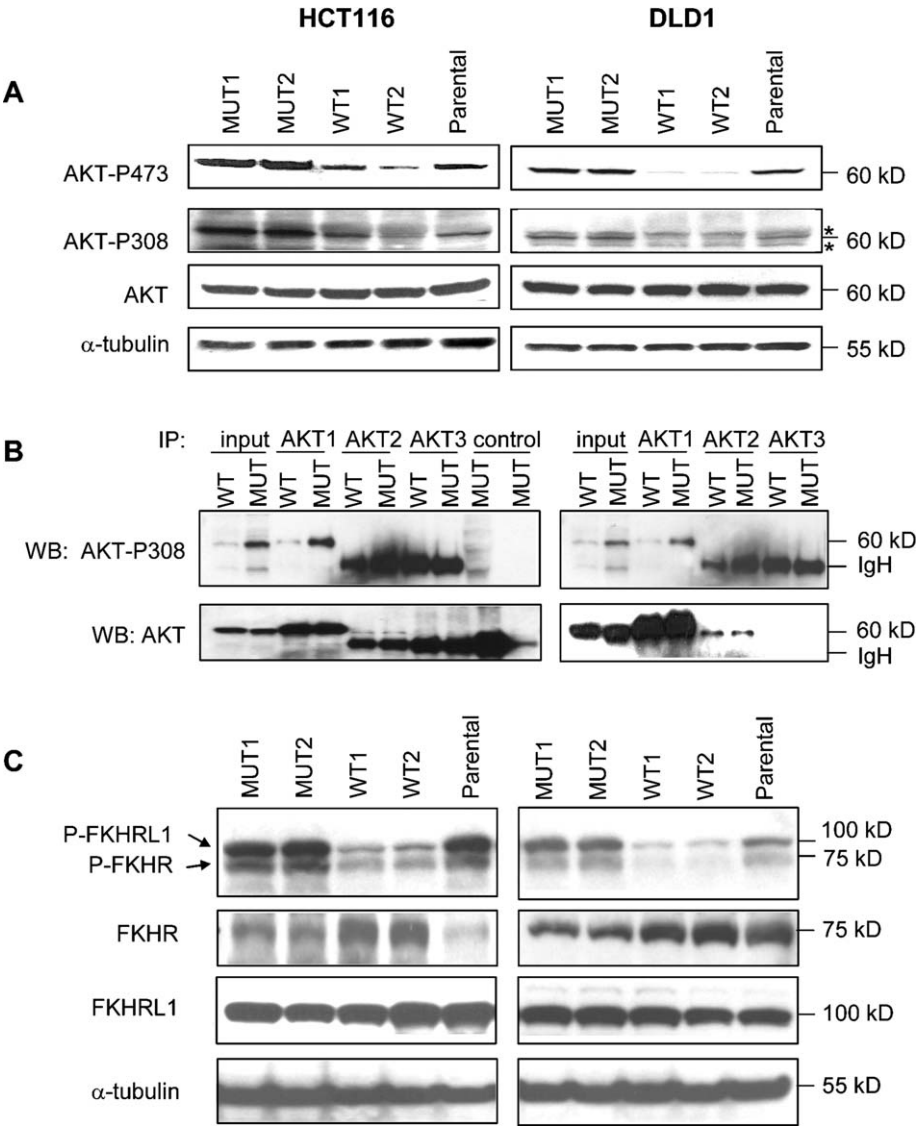


Figure 2. Effects of PIK3CA mutation on AKT, FKHRL1, and FKHR phosphorylation

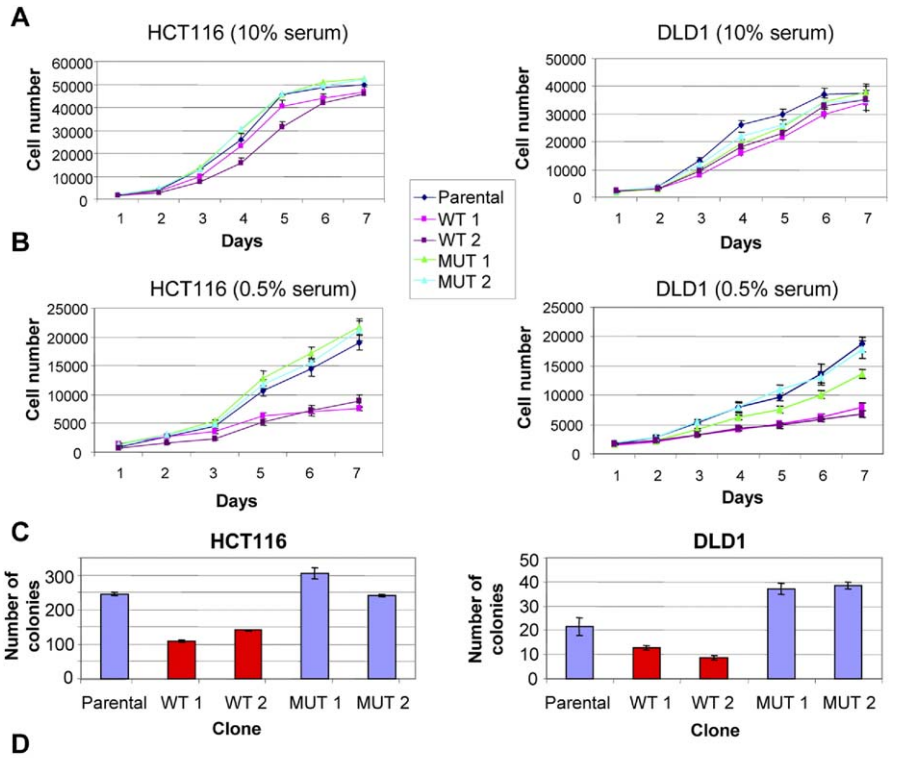
A: Lysates from the indicated cells were immunoblotted with anti-phospho-AKT (Ser473), anti-phospho-AKT (Thr308), and phosphorylation-independent anti-AKT (AKT). Cell lysates contained similar amounts of total protein as determined by immunoblotting with the anti-α-tubulin antibody.

B: Lysates from mutant clone 1 (MUT) and WT clone 1 (WT) were used for immunoprecipitation with the indicated antibodies. Immunoprecipitates were analyzed by Western blotting with an anti-phospho-AKT antibody. The same blot was stripped and reprobed with a pan-AKT antibody (bottom).

C: Lysates from the indicated clones were immunoblotted with anti-phospho-FKHRL1/phospho-FKHR (Thr24/Thr32), anti-FKHR, anti-FKHRL1, and anti-α-tubulin antibodies.

mutant clones in the absence of EGF or insulin (Figure 4B). Identical results were obtained when the cells were activated with growth factors in the complete absence of serum (Supplemental Figure S5). In contrast, neither of these growth factors had much effect on the constitutively activated proteins in the mutant clones.

The decreased growth rate of the WT clones in reduced serum could have arisen either through perturbations in cell cycle progression (e.g., longer G1 phase) or through increased cell death. To determine which mechanism was responsible, we assessed cell cycle parameters via flow cytometry and apoptosis via morphology. No differences in cell cycle parameters were observed, regardless of the serum concentration in the growth medium (data not shown). Similarly, when grown in 10% serum, WT and mutant clones were indistinguishable with respect to the level of apoptosis (<4% in all cases). However, in reduced serum, there was a dramatic difference in apoptosis between WT and mutant clones in both the HCT116 and DLD1 cell lines (Figure 5). These results are consistent with previous results showing that AKT can function in an antiapoptotic manner and that phosphorylation of FKHR and FKHRL1 by AKT prevents their transcriptional activation of various proapoptotic genes (Brunet et al., 1999; Modur et al., 2002; Nakamura et al., 2000; Rokudai et al., 2002). To directly test whether inhibition of FKHR would have the same effect on cellular survival as PIK3CA mutation, we used siRNA to knock down FKHR protein levels in HCT116 WT and mutant clones. We examined the effects of five different siRNA duplexes against FKHR: all showed significant reduction of apoptosis of WT cells grown in 0.5% serum, resulting in apoptosis levels similar to those observed in mutant cells (Supplemental Figure S6). As expected, FKHR knockdown in mutant cells had no effect on apoptosis levels (Supplemental Figure S6). Taken together, these results suggest that mutant PIK3CA is essential for growth factor-independent cellular proliferation, and that this is achieved by AKT-mediated resistance to apoptosis through inhibition of forkhead transcription factors.



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HCT116	Number of mice with tumors	DLD1	Number of mice with tumors
Parental	6/6	Parental	5/5
WT1	5/5	WT1	5/5
WT2	5/5	WT2	5/5
MUT1	5/5	MUT1	5/5
MUT2	7/7	MUT2	5/5

Figure 3. Effect of PIK3CA mutations on cell growth

A and B: Cellular proliferation was assessed in plastic culture plates using media containing either 10% (**A**) or 0.5% (**B**) serum. Average cell number at each time point was measured by determining DNA content in ten replicate wells using SYBR Green I.

C: Anchorage-independent proliferation of cell clones was assessed by measuring colony growth in soft agar in the presence of 0.5% serum. Graphs indicate number of colonies greater than 2 mm in diameter observed after two weeks of growth.

D: Athymic nude mice were injected subcutaneously with the indicated clones and were examined for subcutaneous tumor growth two weeks later.

Mutant PIK3CA confers resistance to TRAIL-induced apoptosis

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands that can initiate apoptosis by a pathway triggered by its interaction with death receptors (Wang and El-Deiry, 2003). It has been shown that T cells expressing TRAIL play a role in killing tumors (Smyth et al., 2003), and previous data has indicated that activation of the AKT pathway can modulate this process in tumor cells (Modur et al., 2002). To test the sensitivity of PIK3CA mutant clones to TRAIL-induced apoptosis, time course studies were performed in the presence of media with 10% serum. Untreated cultures of WT and mutant PIK3CA clones contained very few (0%–2%) apoptotic cells. Within 18 hr of exposure to TRAIL, massive apoptosis occurred in the WT cells, while the mutant cells were spared (Figure 6). In contrast to TRAIL, exposure to the proapoptotic FAS ligand did not induce significant apoptosis in either the WT or mutant clones (data not shown).

Mutant PIK3CA promotes cell migration and invasion

Previous experiments have shown that disruption of the PTEN tumor suppressor gene increases the ability of the cells to migrate (Tamura et al., 1998). Because PIK3CA activation and PTEN disruption are predicted to have similar effects on accumulation of PIP_3, we evaluated migration and related phenotypes of the PIK3CA clones. Boyden chamber assays showed that HCT116 or DLD1 mutant clones had a 6- to 8-fold increased ability to migrate through a porous membrane or to invade through Matrigel (Figures 7A and 7B). To determine whether these differences resulted in altered capacities for invasive phenotypes in vivo, parental, WT, and mutant HCT116 clones were administered to athymic nude mice by tail vein injection. Eight weeks after injection, mice with cells from parental or mutant clones were obviously cachectic, while mice receiving WT clones appeared healthy (Figure 7C). Accordingly, the former mice had lost an average of ~15% of their body weight compared to the latter (Figure 7D) ($p < 0.01$, t test). At necropsy, none of the mice injected with the WT clones had tumors, while all but one of the mice that received parental or mutant clones had tumors in various locations ranging in size from 2 mm to 12 mm (Figure 7D) (difference between WT and mutant clones was statistically significant, $p < 0.001$ by chi-square test). Microscopic examination was performed on all mice, and no additional tumors were found in the mice injected with WT clones. However, numerous tumor micrometastases and evidence of tumor invasion were observed in mice receiving mutant clones (Figure 7E).

Effects of LY294002 on wild-type and mutant PIK3CA signaling and cell growth

LY294002 is a small molecule that competitively and reversibly inhibits the ATP binding site of several different PI3Ks (Vlahos

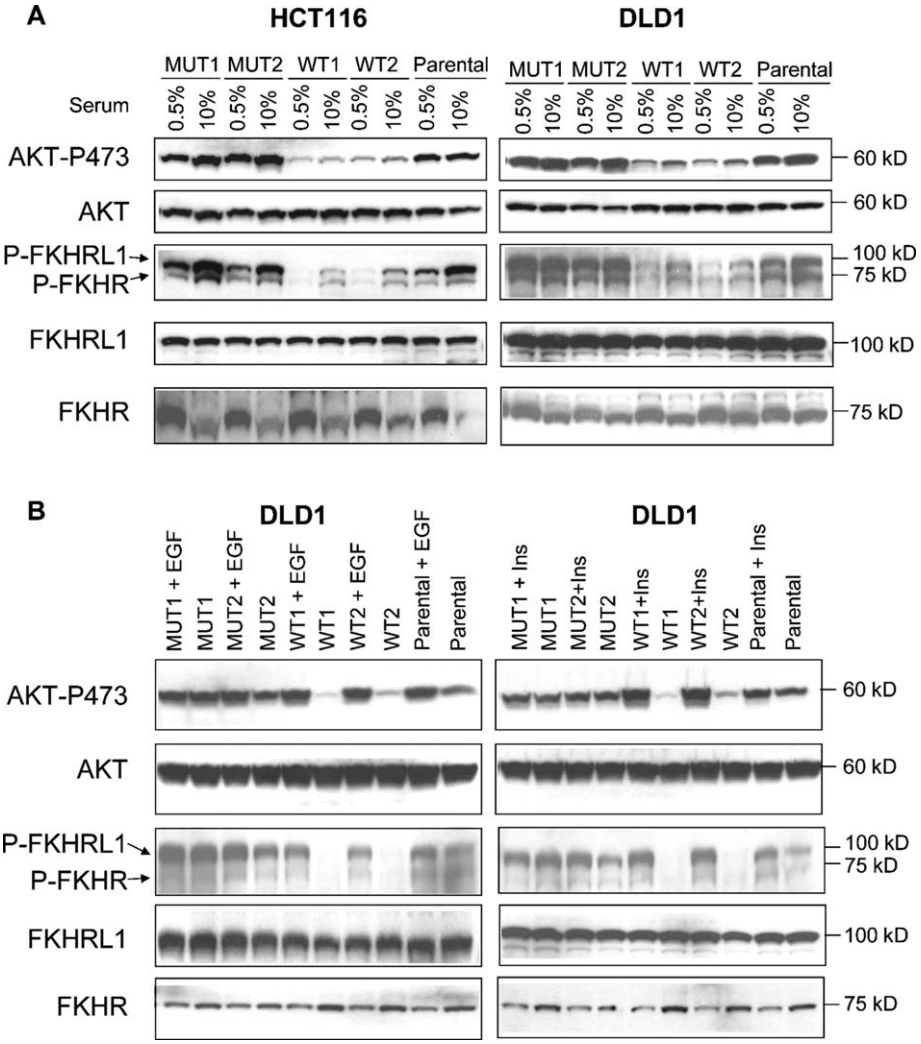


Figure 4. PIK3CA mutation abrogates serum and specific growth factor dependence of AKT, FKHR, and FKHRL1 phosphorylation

A: Cells were grown in 10% or 0.5% serum, and lysates prepared from them were immunoblotted with the indicated antibodies.

B: Cells were grown in 0.5% serum for 19 hr and then stimulated with epidermal growth factor (EGF) or insulin (Ins). Similar results were obtained for HCT116 cells (data not shown).

et al., 1994). To test whether LY294002 would inhibit mutant forms of PIK3CA, cells were grown in 0.5% serum and treated with 10 μM LY294002 for two hours. In all cases, LY294002 inhibited AKT, FKHRL1, and FKHR phosphorylation in mutant as well as WT clones (Figure 8A). The relative degree of inhibition was greater for the mutant clones than it was for the WT clones. In fact, the level of phosphorylation of AKT, FKHR, and FKHRL1 in the mutant clones following treatment with LY294002 was reduced to that occurring in WT clones in the absence of LY294002 treatment (Figure 8A). This result suggested that WT clones would grow better than mutant cells in the presence of LY294002. These predictions were verified by

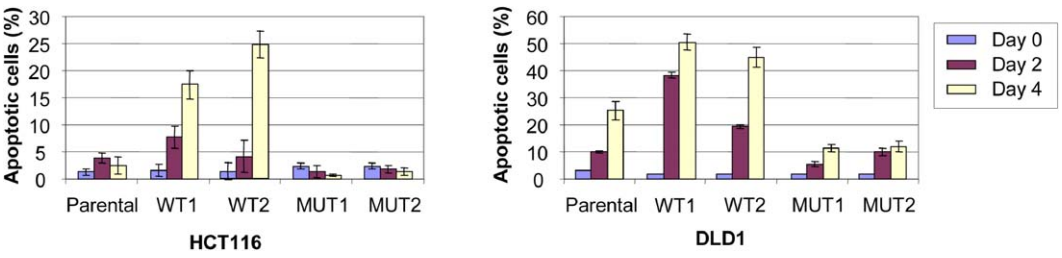


Figure 5. PIK3CA mutation confers resistance to apoptosis induced by growth factor depletion

Cells were grown in growth medium containing 0.5% serum for the indicated times. Apoptosis was assessed by fluorescence microscopy of Hoechst 33258-stained cells.

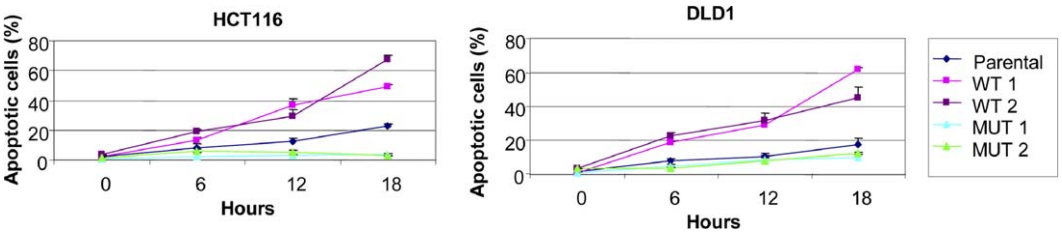


Figure 6. Effect of PIK3CA mutation on TRAIL-induced apoptosis

Cells were grown on plastic in the presence of TRAIL, and apoptosis was assessed by fluorescence microscopy of Hoechst 33258-stained cells at the indicated times.

exposure of cells to LY294002, which resulted in reduced cell proliferation to a greater extent in mutant cells than in WT cells (Figures 8B and 8C). These results suggest that LY294002, despite its limited specificity for PI3KCA (Davies et al., 2000; Fruman et al., 1998), preferentially inhibits mutant PIK3CA signaling.

Discussion

The results reported above lead to several conclusions relevant to the role of PIK3CA in colorectal neoplasia. First, the results provide strong evidence that endogenous PIK3CA mutations promote cellular phenotypes typical of neoplastic cells. Second, the biochemical and biological effects of a mutation in the helical domain, a region of unknown function, were identical to those resulting from mutation in the catalytic kinase domain. Third, the effects of PIK3CA mutations were generally more discernable under unfavorable conditions than under conventional in vitro conditions of growth. Fourth, PIK3CA mutations conferred resistance to apoptosis, as evident upon growth in reduced serum concentrations or after exposure to TRAIL. Fifth, PIK3CA mutations conferred a dramatic ability to migrate and invade in vitro as well as in vivo. And sixth, PIK3CA mutations clearly activated one particular downstream pathway involving AKT1, FKHR, and FKHRL1. These conclusions are discussed in more detail below.

Helical versus kinase domain mutations

One of the most remarkable observations made in this study is that the two different mutations in PIK3CA conferred virtually identical phenotypes, despite the fact that these phenotypes were assessed in two unrelated CRC cell lines. Though the mechanism through which kinase domain mutations exert their effects are in principle clear (increased specific activity of the enzyme), the mechanism(s) underlying the increased kinase activity and enhanced pathway activation resulting from helical domain mutations are not. It will be of interest to investigate this issue further through structural studies of PIK3CA proteins containing engineered mutations.

Selective growth conditions in vivo

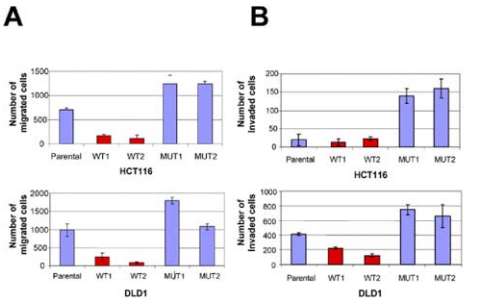
Mutations in cancer genes must provide a growth advantage in order for cells containing those mutations to become the predominant clone within the population. In general, it is difficult to determine the environmental conditions that are responsible for such growth selection in vivo. Our results provide some clues as to what might have occurred during the development of tumors with PIK3CA mutations in the human host. Although there were relatively minor differences in growth between mutant and WT cells when cultured in conventional conditions (10% serum), there were striking differences in growth when cells were cultured in suboptimal conditions (0.5% serum) (Figures 3–5). These differences in net growth rate were accompanied by involvement of specific downstream mediators (e.g., AKT1 and forkhead proteins). The effects of serum were mimicked by EGF or insulin, so that WT, but not mutant, cells were dependent on these growth factors for pathway activation (Figure 4B and Supplemental Figure S5). These experiments suggest a model where mutant PIK3CA tumor cells in an environment with suboptimal growth factors, perhaps due to inadequate vascularization, would be able to survive, and perhaps even proliferate, while cells with WT PIK3CA would die through apoptosis (Figure 5).

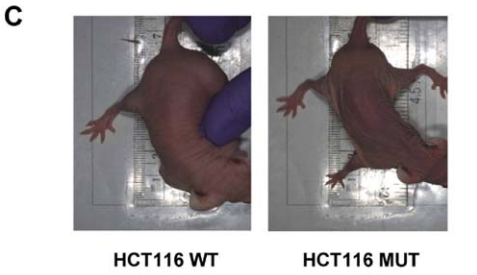
Migration, invasion, and metastasis

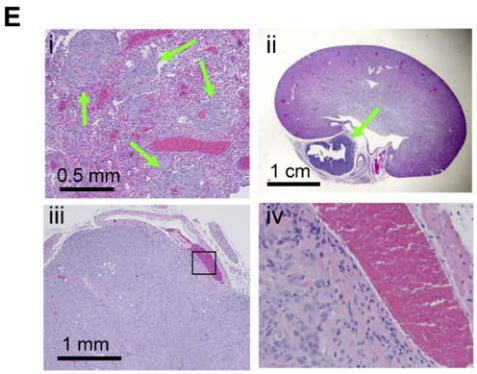
Though metastases are responsible for most cancer mortality, the biochemical and genetic basis of this process is still being investigated. Our results suggest that PIK3CA mutations not only result in the capacity to migrate and invade in vitro, but are also essential for forming metastases in a mouse model when cells are intravenously injected (Figure 7). As invasion in patients is initiated during the stage of tumorigenesis when PIK3CA mutations generally occur (adenoma to carcinoma transition [Samuels et al., 2004]), it is possible that PIK3CA is one of the genes responsible for the invasive and metastatic phenotypes in naturally occurring tumors. The hypothesis that PIK3CA mutations contribute to invasion is consistent with previous studies indicating that PIK3CA and PTEN mutations occur late in glioma progression (Broderick et al., 2004; Wang et al., 1997). Additionally, functional analyses have shown that AKTs can regulate migration and invasive processes (Kim et al., 2001; Park et al., 2001; Suzuki et al., 2004; Tamura et al., 1998; Vasko et al., 2004), and that PTEN ablation enhances migration and integrin-mediated cell spreading associated with formation of focal adhesions (Tamura et al., 1998; Tamura et al., 1999). Although the underlying mechanism for the invasive phenotype is unclear, the striking differences between the WT and mutant clones reported here should prove useful for evaluating this issue in the future.

Downstream pathways

AKTs are hubs at the center of several major cell signaling pathways, and AKT activation has been associated with a myr-







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Mouse	Clone	Initial weight (g)	final Weight (g)	Tumor Location
1	WT1	ND	18.1	None
2	WT1	ND	22.6	None
3	WT1	ND	25.8	None
4	WT1	ND	26.6	None
5	WT1	20.4	24.4	None
6	WT1	18.9	22.0	None
7	WT1	21.9	22.8	None
8	WT1	21.0	21.2	None
9	WT1	18.4	21.7	None
10	WT2	19.2	20.8	None
11	WT2	19.5	20.9	None
12	WT2	20.8	22.3	None
13	WT2	21.3	22.3	None
14	WT2	22.9	24.4	None
15	MUT1	ND	21.1	Abdomen, pelvis, chest wall, lung, anterior dorsum
16	MUT1	ND	20.8	Anterior dorsum
17	MUT1	ND	15.8	Anterior mediastinum
18	MUT1	ND	18.6	Anterior mediastinum
19	MUT1	20.4	16.6	Abdomen, chest wall, lung
20	MUT1	18.9	15.3	Pelvis, chest wall, anterior dorsum, anterior mediastinum, lung
21	MUT1	21.9	21.7	Kidney, lung
22	MUT1	21.0	15.9	Pelvis, thoracic vertebral column, anterior mediastinum, lung
23	MUT2	22.5	17.7	Kidney, lung
24	MUT2	19.9	24.0	Cranium, chest wall, lung
25	MUT2	20.5	19.1	Lung
26	MUT2	19.5	19.2	Kidney, chest wall, lung
27	MUT2	21.5	21.9	Anterior dorsum, lung
28	Parental	22.8	25.8	None
29	Parental	22.4	14.7	Anterior dorsum, thoracic vertebral column, lung
30	Parental	19.6	18.7	Anterior dorsum, thoracic vertebral column, lung
31	Parental	22.0	19.9	Kidney

Figure 7. Effect of PIK3CA mutation on tumor cell invasion in vitro and in vivo

A and B: Cells of the indicated genotypes were grown in Boyden chambers and assessed for their ability to migrate through porous 8 μm membranes (**A**) or invade through Matrigel (**B**). Each graph indicates the number of cells that migrated or invaded 24 hr after seeding.

C–E: Athymic nude mice were intravenously injected with cells from HCT116 parental, WT clones, or mutant clones and examined after eight weeks. **C:** Severe cachexia was observed in mice injected with mutant or parental cells, whereas all mice containing WT cells appeared healthy. **D:** Mice injected with mutant or parental cells developed tumors and lost weight, whereas none of the mice injected with WT cells had any pathologic abnormalities. **E:** Representative hematoxylin and eosin-stained images of histopathological sections from mice injected with MUT1 clones: (i) numerous parenchymal micrometases were present in the lung (arrows); (ii) a large tumor adjacent to the kidney (arrow); (iii) a tumor of the anterior mediastinum, which at a higher magnification (iv) showed apparent distortion and invasion of the muscular layer surrounding a large pulmonary vessel.

iad of downstream mediators in different cell types and organisms. Which of these downstream signaling molecules are actually responsible for transducing the mutant PIK3CA signal can only be determined by examining pathway activation in cells with those mutations. In the CRCs analyzed, AKT1 was the predominant AKT isoform observed and found to be activated downstream of mutant PIK3CA (Figure 2B). This is consistent with the fact that expression of AKT isoforms varies among different tissues and cancer types (Altomare et al., 1995; Nakatani et al., 1999) and that AKT1 has been reported to be highly expressed in CRCs (Dufour et al., 2004). Our finding that activation of AKT1 is involved in resistance to apoptosis in CRCs is consistent with data demonstrating AKT1 is essential for cell survival (Dufour et al., 2004), that cells from AKT1 knockout mice are susceptible to serum withdrawal induced apoptosis (Chen et al., 2001), and that AKT1 is essential for PTEN-controlled tumorigenesis (Stiles et al., 2002). Further work will be required to evaluate AKT signaling mediated by mutant PIK3CA in additional tumor types where other AKT isoforms may be highly expressed.

A surprising result was that only a selected subset of AKT substrates was altered by PIK3CA mutation. Phosphorylation of FKHRL1 and FKHR was dramatically affected, while the phosphorylation of other substrates or mediators of AKT function was not consistently altered in mutant clones (i.e., observed in all clones of both cell lines tested). Increased phosphorylation of FKHR has been shown to result in its proteosomal degradation (Aoki et al., 2004), and phosphorylation of FKHR and FKHRL1 induces their exclusion from the nucleus and their binding to 14-3-3 proteins in the cytoplasm (Biggs et al., 1999; Brunet et al., 1999; Cahill et al., 2001; Kops et al., 1999). Consistent with these reports, total FKHR protein

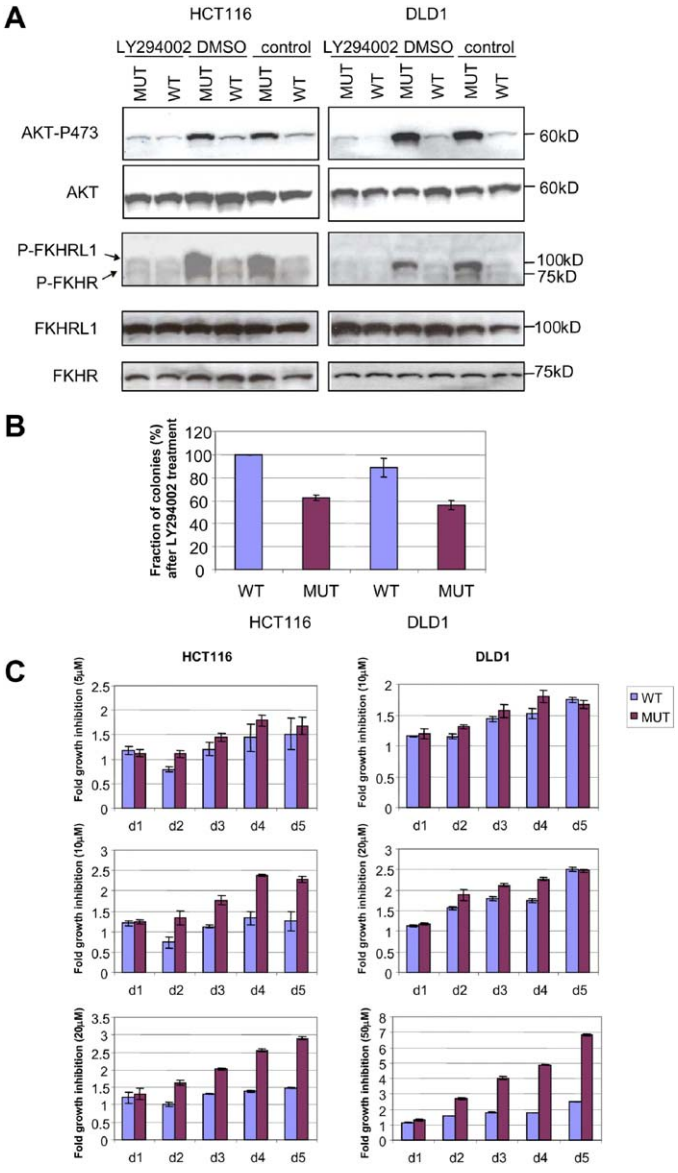

Figure 8. Effect of the PI3K inhibitor LY294002 on PIK3CA signaling and cell growth

For these analyses HCT116 and DLD1 WT clone 1 (WT) and mutant clone 1 (MUT) were examined.

A: Cells were grown in 0.5% serum and were treated with 10 μM LY294002 diluted in DMSO or in DMSO alone for two hours or were left untreated. Cell lysates were analyzed by immunoblotting using the indicated antibodies.

B: The effect of LY294002 on clonogenic growth was evaluated by treatment of cells with 10 μM LY294002 in 1% serum for two weeks. Results are normalized to the number of colonies formed in control cultures treated with DMSO alone.

C: The effect of LY294002 on cellular proliferation was assessed in plastic culture plates. The average cell number at each time point was measured by determining DNA content in four replicate wells using SYBR Green I after treatment with either 5 μM, 10 μM, 20 μM, or 50 μM LY294002. Results are normalized to cell growth treated with corresponding amounts of the DMSO.

levels were reduced in mutant clones compared to WT clones in the presence of serum, insulin, or EGF. These growth factors increased the phosphorylation of FKHR and FKHRL1 in WT clones, as expected, but their phosphorylation was constitutively high in mutant clones, thereby allowing such cells to proliferate under adverse conditions. The importance of this pathway is bolstered by its evolutionary conservation. For example, the *C. elegans* genome encodes a forkhead transcription factor, DAF-16, which is a downstream target of insulin/AKT whose inactivation results in an increased life span (Lin et al., 1997; Ogg et al., 1997). Our data also strengthen the evidence that this pathway plays a critical role in neoplasia, first suggested by the discovery of chromosomal translocations involving FKHR (Barr et al., 1993; Galili et al., 1993).

In addition to the forkhead transcription factors, other targets of AKT have also been shown to be important for cell growth and proliferation. For example, AKT-mediated inactivation of GSK3β promotes cyclin D1 accumulation and cell cycle progression (Cross et al., 1995; Diehl et al., 1998). Additionally, AKT phosphorylation of the protein kinase mTOR leads to phosphorylation of p70-S6K, which enhances the translation of specific mRNAs and inhibition of 4EBP1, a negative regulator of translation (Harris and Lawrence, 2003; Nave et al., 1999). Interestingly, the selective involvement of FKHR and FKHRL1 observed in CRCs is different from the effects of overexpression of mutant PIK3CA in chicken embryonic fibroblasts (Kang et al., 2005) and the deletion of PTEN in mouse embryonic fibroblasts (Neshat et al., 2001), where other members of the AKT pathway (including mTOR targets) appear to be affected. Future work will be needed to determine if the targets of AKT activation depend on the cell type, species, or experimental system analyzed.

What happens downstream of FKHR and FKHRL1 phosphorylation in CRC cells with PIK3CA mutations is still unknown. Our data suggest that PIK3CA mutations inhibit CRC apoptosis under specific circumstances and promote tumor invasion. Numerous mechanisms by which AKTs suppress apoptosis or facilitate invasion have been suggested (Luo et al., 2003; Majewski et al., 2004; Vivanco and Sawyers, 2002). The observation that inhibition of FKHR resulted in apoptosis levels similar to those of PIK3CA mutant cells (Supplemental Figure S6) suggests that forkhead transcription factors play a major role in bringing about these effects. Evaluation of transcripts induced by FKHR and FKHRL1 in these cell lines through microarray (Chee et al., 1996; Schena et al., 1995) or SAGE technologies (Saha et al., 2002; Velculescu et al., 1995) should therefore be particularly informative.

Finally, our results have potential therapeutic implications. For one, they suggest that TRAIL, a TNF family member that has been considered for clinical trials, is not likely to be very useful in patients with PIK3CA mutations, and by extension, in patients with AKT pathway activation caused by other mutations. Second, the data in Figure 8 demonstrate that mutant forms of PIK3CA can still be inhibited by broadly acting PI3K inhibitors such as LY294002. This suggests that cells with PIK3CA mutations are subject to "oncogenic addiction" and that PI3K signaling is required for continued cell proliferation in cancer cells (Kaelin, 1999; Lu et al., 2003; Neshat et al., 2001; Sansal and Sellers, 2004; Weinstein, 2002). Interestingly, cells deficient for PTEN have been shown to be more sensitive to inhibitors of the PI3K pathway, and LY294002 has been shown

to inhibit ovarian tumors with an activated PI3K pathway in vivo (Hu et al., 2000; Neshat et al., 2001). One can imagine the development of inhibitors that would specifically target one or both of the two major mutant forms of PIK3CA (i.e., those in the kinase and helical domains). As there are hundreds of thousands of individuals each year who present with tumors containing these specific PIK3CA mutations, this treatment approach could in theory be applicable to a large number of patients.

Experimental procedures

Targeted deletion of the human *PIK3CA* locus

The approach for generating knockouts with AAV vectors was performed as described (Hirata et al., 2002; Kohli et al., 2004). The targeting construct pAAV-Neo-PIK3CA was constructed by PCR, using bacterial artificial chromosome clone RPCI11.C (Invitrogen, Carlsbad, CA) as the template for the homology arms. Constructs and primer sequences are available upon request. Stable G418-resistant clones were selected in the presence of either 0.4 mg/ml or 1 mg/ml for HCT116 and DLD1 cells, respectively (Invitrogen, Carlsbad, CA), and were propagated in the absence of selective agents. Genotypes of each clone were determined by RT-PCR and sequencing of the coding region of *PIK3CA*.

Cell culture and reagents

The colon cancer cell lines HCT116 and DLD1 (ATCC, Manassas, Virginia) were grown as described (Cummins et al., 2004a). Cell proliferation on plastic was measured as described (Cummins et al., 2004b). For serum starvation and growth factor stimulation, cells were grown to 70%–80% confluency, washed once with PBS, and incubated for 19 hr in McCoy's 5A modified medium containing 0.5% FCS. The cells were stimulated with 100 ng/ml insulin (Santa Cruz Biotechnology, Santa Cruz, CA) for 10 min or with 60 ng/ml EGF for 2.5 min (Cell Signaling, Beverly, MA). TRAIL treatment was performed as described (Cummins et al., 2004a). LY294002 (Cell Signaling, Beverly, MA) was applied at concentrations of 10 μM.

Expression of p85α and p110α in Sf9 cells

Human recombinant *PIK3R1*, *PIK3CA* wild-type, and *PIK3CA* E545K and H1047R mutants were expressed using the Invitrogen Bac-to-Bac Baculovirus Expression System (Invitrogen, Carisbad, CA) (for detailed procedures, see Supplemental Data).

Immunoblotting

Protein extracts from HCT116 and DLD1 clones were prepared by washing the cells once in ice-cold phosphate-buffered saline and then lysing them on ice in Laemelli sample buffer. The extracts were then briefly sonicated and boiled, and debris was removed by centrifugation at 10,000 × g for 15 min at 4°C. Immunoblotting was performed with Immobilon P membranes (MilliPore, Bedford, MA) (for details on antibodies used, see Supplemental Data).

Immunoprecipitation

Immunoprecipitation was performed as described (Samuels et al., 2004) (for detailed procedures and antibodies used, see Supplemental Data).

Focus formation assay in soft agar

HCT116 and DLD1 clones were plated in duplicate at 5000 cells/ml in top plugs consisting of McCoy's 5A modified medium containing various FCS concentrations and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine). After two weeks, the colonies were photographed, and the colonies were digitally counted using Metamorph software. When all colony sizes were considered, the total number of colonies formed by WT and mutant clones was essentially the same. However, when colonies above the threshold of 2 mm were counted, differences between the number of WT and mutant clones were observed.

Cell cycle and apoptosis assays

Apoptosis assays were performed as described (Cummins et al., 2004a). For analyses of cell cycle parameters, harvested cells were stained as for apoptosis assays and evaluated by flow cytometry on a Beckman Coulter EPICS XL according to the manufacturer's instructions.

Knockdown of FKHR protein using siRNAs

siRNA analyses were performed using 27 bp RNA duplexes (Kim et al., 2005). Five siRNA duplexes were used against FKHR: (1) 5′-CCAGGC ATCTCATAACAAAATGATGAA-3′, (2) 5′-CCAGATGCCTATACAAACACTTC AGGA-3′, (3) 5′-GGAGGTATGAGTCAGTATAACTGTGCG-3′, (4) GAGGTAT GAGTCAGTATAACTGTGCGC, and (5) 5′-GGTCTGTCGCCCTTATCCTTCA GCTCG-3′ (IDT, Coralville, IA). A mixture of 4 siRNA duplexes (siControl) each designed to have ≥ 4 mismatches to all human genes was used as a control (Dharmacon Research Inc., Lafayette, CO). HCT116 WT and mutant clones were cultured in 96-well plates until 60% confluent and were transiently transfected with 1 nM siRNA duplexes using Lipofectamine 2000 (Invitrogen, Carisbad, CA). Both adherent cells and those in suspension were collected and analyzed by immunoblotting and apoptosis assays.

Colony formation assay

Cell lines were plated at 1000 cells per T-25 flask in 1% serum concentration, and 48 hr later, medium containing 10 μM LY294002 was added to each flask. Plates were incubated for 14 days and stained with Hema3 (Sigma, St. Louis, MO).

In vitro migration and invasion assays and tumor growth and invasion in mice

In vitro migration and invasion assays were performed using either 8 μm pore size transwell migration plates or Matrigel matrix-coated polycarbonate filters, respectively (Biocoat, Beckton Dickinson, Bedford, MA). For tumor growth assays, female athymic nude mice, obtained from Harlan (Indianapolis, IN) at 4 to 6 weeks of age, were injected subcutaneously in the right foreleg with 5×10^6 HCT116 or DLD1 clones resuspended in 100 μl of PBS. Two weeks after injection, tumor size was measured. In vivo invasion experiments were performed using 6- to 8-week-old female athymic nude mice (Harlan, Indianapolis, IN), using tail vein injection of HCT116 clones. For detailed procedures, see Supplemental Data. Animal protocols were designed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/6/561/DC1/.

Acknowledgments

The authors thank Zhenghe Wang, Marcelo Reis, and Mark Behlke for advice, and Natalie Silliman, Janine Ptak, Steve Szabo, and Latice Watson for technical assistance. This work was supported by the Ludwig Trust, The Pew Charitable Trusts, and NIH grants CA43460, CA62924, and CA057345. Y.S. is an EMBO fellow.

Received: December 17, 2004
Revised: April 6, 2005
Accepted: May 20, 2005
Published: June 13, 2005

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