

Knockin of mutant PIK3CA activates multiple oncogenic pathways

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The phosphatidylinositol 3-kinase subunit PIK3CA is frequently mutated in human cancers. Here we used gene targeting to “knock in” PIK3CA mutations into human breast epithelial cells to identify new therapeutic targets associated with oncogenic PIK3CA. Mutant PIK3CA knockin cells were capable of epidermal growth factor and mTOR-independent cell proliferation that was associated with AKT, ERK, and GSK3 β phosphorylation. Paradoxically, the GSK3 β inhibitors lithium chloride and SB216763 selectively decreased the proliferation of human breast and colorectal cancer cell lines with oncogenic PIK3CA mutations and led to a decrease in the GSK3 β target gene *CYCLIN D1*. Oral treatment with lithium preferentially inhibited the growth of nude mouse xenografts of HCT-116 colon cancer cells with mutant PIK3CA compared with isogenic HCT-116 knockout cells containing only wild-type PIK3CA. Our findings suggest GSK3 β is an important effector of mutant PIK3CA, and that lithium, an FDA-approved therapy for bipolar disorders, has selective antineoplastic properties against cancers that harbor these mutations.

GSK3 β | lithium | mTOR | phosphatidylinositol 3-kinase | cancer

An attractive target for cancer therapy is the phosphatidylinositol 3-kinase (PI3K) enzyme, as its activity is often dysregulated in many human malignancies. PI3K, along with other key downstream effectors, such as AKT1 (AKT) and mammalian target of rapamycin (mTOR), have been the subject of therapeutic exploitation in many studies. However, cell signaling through the PI3K-AKT-mTOR pathway occurs in many normal tissues as well as other disease states. Thus, the development of inhibitors that are selective for cancer cells with a tolerable side effect profile has remained difficult.

Recently the p110 α catalytic subunit of PI3K, PIK3CA, has been shown to be frequently mutated in a number of different human cancers (1, 2). Notably, 3 recurrent oncogenic “hotspot” mutations comprise the majority of somatic PIK3CA mutations. Two of these mutations, E542K and E545K, occur in the helical domain found in exon 9, and the third mutation, H1047R, affects the kinase domain located within exon 20 (3). Although many studies have implicated PIK3CA mutations with features of transformation (4–6), definitive mechanisms describing how these mutations lead to increased cell growth and proliferation have not been fully elucidated. Knowledge of these molecular mediators would greatly assist in the development of inhibitors capable of specifically targeting cancer cells that carry oncogenic PIK3CA mutations.

Although the biological phenotypes of mutant PIK3CA in various cellular systems have been demonstrated, we have recently shown that overexpression of mutant oncogenes via transgene methods may not accurately represent the biologic changes seen when mutant alleles are expressed under the control of a gene’s native promoter (7). Thus, similar to knockin mice, we have used gene targeting to knock in pathogenic

mutations into human cell lines to further elucidate their physiologic consequences and to validate these mutated genes as bona fide targets for cancer therapy. In the present study, knockin of oncogenic PIK3CA mutations was performed using the nontumorigenic human breast epithelial cell line MCF-10A. Our findings reveal that mutant PIK3CA alters several distinct downstream effectors, and that in addition to AKT/mTOR, other signaling pathways contribute to mutant PIK3CA-mediated cellular proliferation, including increased phosphorylation of GSK3 β . Surprisingly, pharmacologic modulation of GSK3 β with lithium, an FDA-approved drug for the treatment of bipolar disorder, led to selective growth inhibition of cells harboring mutant PIK3CA. Thus, our study validates the use of human isogenic cell lines for target identification and drug discovery, and suggests the need for targeting several key growth-promoting pathways for optimal antineoplastic therapy against human cancers containing oncogenic PIK3CA.

Results

Gene Targeting of Oncogenic PIK3CA Leads to Growth Factor Independence. To create models of human epithelial cells harboring PIK3CA mutations expressed in a physiologic manner, we used gene targeting in the nontumorigenic human breast epithelial cell line MCF-10A as previously described (7, 8). Two targeting vectors were created: one designed to introduce the E545K mutation, and the other for knocking in the H1047R mutation (supporting information (SI) Fig. S1). Three E545K and 2 H1047R clones were independently isolated. Further characterization of these clones is described in SI Methods.

Removal of epidermal growth factor (EGF) from the culture

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Conflict of interest statement: Under an agreement between The Johns Hopkins University and Genzyme Corporation, Dr. Vogelstein is entitled to a share of royalty received by the University on sales of products related to PIK3CA mutations. Johns Hopkins University has also licensed HCT-116 cell lines with particular mutations of PIK3CA to Horizon Discovery Corporation and other pharmaceutical companies. The terms of these arrangements are being managed by The Johns Hopkins University in accordance with its conflict of interest policies. B.H.P. receives research support from GlaxoSmithKline, though the studies reported here were not supported by this contract. K.E.B. is currently an employee of GlaxoSmithKline, though all of the work reported here was during his faculty appointment at the University of Maryland.

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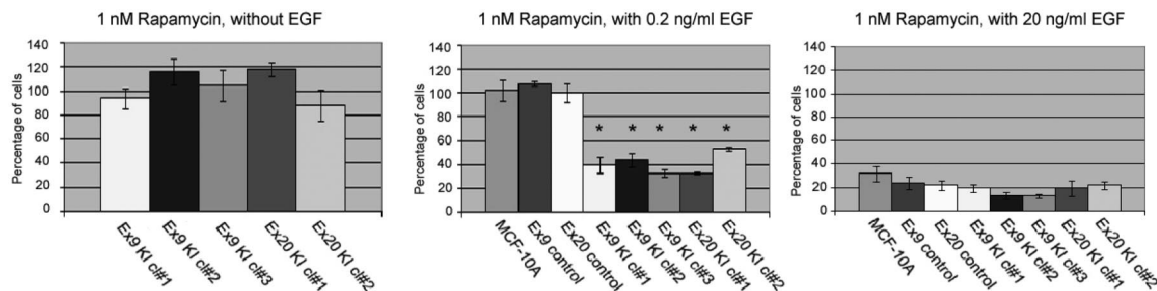


Fig. 1. Sensitivity of MCF-10A mutant *PIK3CA* knockin cell lines to rapamycin. Cell proliferation assays were performed with MCF-10A cells and/or their knockin derivatives. EGF and rapamycin concentrations are shown above each graph. Bars represent the percentage of cell proliferation relative to vehicle only (DMSO) controls. Error bars represent SEM from triplicate samples. * $P < 0.0011$ for all clones vs. control cell lines.

medium results in G1 arrest of MCF-10A cells, and the ability of these cells to proliferate in the absence of this growth factor is often associated with an increased malignant potential (9). Cell proliferation assays showed that mutant *PIK3CA* knockin cells, but not their control counterparts or parental cells, were capable of growth in media without EGF similar to previous studies with forced mutant *PIK3CA* overexpression (Fig. S2) (5). However, other features of transformation seen with overexpression of mutant *PIK3CA*, such as abnormal acini formation in Matrigel (5), were not present (Fig. S3). In addition, mutant *PIK3CA* knockin cells did not form colonies in soft agar or tumors in nude mouse xenograft assays (data not shown). Collectively these studies suggest that mutant *PIK3CA* imparts some features of transformation but is not sufficient for tumorigenicity.

Oncogenic *PIK3CA* and mTOR Activation. To identify and validate molecular targets of therapy, we used our knockin cell lines to analyze pathways previously implicated in PI3K-AKT-mTOR signal transduction. The dependence of MCF-10A on EGF for cell proliferation allows for a clean analysis of the molecular pathways involved with oncogene-induced cell proliferation, because removal of EGF from the culture medium effectively ablates signaling through the epidermal growth factor receptor (EGFR). We therefore initially evaluated mutant *PIK3CA* knockin cells for their response to the mTOR pathway inhibitor rapamycin as cell growth mediated by mutant *PIK3CA* is thought to be regulated partially through activation of the AKT-mTOR pathway (4, 10, 11). Unexpectedly, rapamycin at pharmacological doses of 1 nM had no effect on the proliferation of knockin cells in the absence of EGF, as depicted in Fig. 1. In contrast, the weakly selective PI3K inhibitors LY294002 and wortmannin were capable of inhibiting proliferation of mutant *PIK3CA* knockin cells in the absence of EGF similar to other studies (Fig. S4) (5, 12). The effect of rapamycin on the proliferation of control cell lines could not be determined because they do not propagate in the absence of EGF, as previously noted. For this reason, we evaluated the effects of rapamycin using EGF at a standard concentration of 20 ng/ml (13). As illustrated in Fig. 1, *PIK3CA* knockin clones, parental MCF-10A, and control cell lines showed equivalent sensitivity to rapamycin when grown in the presence of 20 ng/ml EGF. We then repeated these assays at 0.2 ng/ml, which represents a more physiologic concentration of EGF (14). These experiments showed a differential sensitivity toward rapamycin between *PIK3CA* knockin cell lines vs. control and parental cells (Fig. 1). However, the weakly selective PI3K inhibitors showed equal toxicity for both parental MCF-10A and mutant *PIK3CA* knockin cell lines at this concentration of EGF (Fig. S4).

To uncover the mechanism of this effect, Western blotting was performed on *PIK3CA* mutant knockin cell lines and control cells. As shown in Fig. 2A, mutant *PIK3CA* knockin cells did not

exhibit any appreciable mTOR activation in the absence of EGF as depicted by the lack of p70S6Kinase (S6K) phosphorylation (Thr-389), despite the cells clearly having the capacity to proliferate under these culture conditions. However, in the presence of 20 ng/ml EGF, MCF-10A cells, controls, and *PIK3CA* mutant knockin cells all exhibited mTOR activation. Western blotting confirmed that mutant *PIK3CA* knockin cells exhibited a reduced threshold for mTOR activation by EGF compared with parental and control clones, as seen by the increased S6K phosphorylation (Thr-389) only in the knockin cell lines when cultured in 0.2 ng/ml EGF (Fig. 2B and Fig. S5). Importantly, in all of these assays, sensitivity to rapamycin correlated with S6K phosphorylation (Thr-389), which was reversible upon addition of this mTOR inhibitor. Collectively, our findings suggest that oncogenic *PIK3CA* is neither necessary nor sufficient for mTOR activation. However, cells containing mutant *PIK3CA* do have a reduced threshold for mTOR activation by EGF as measured by S6K phosphorylation (Thr-389) and sensitivity to rapamycin. Moreover, these cells are capable of proliferation that is independent of EGF and mTOR activation, suggesting that cell growth and proliferation mediated by mutant *PIK3CA* involves one or more additional pathways that may need to be targeted for effective antineoplastic therapy.

Oncogenic *PIK3CA* Leads to Phosphorylated ERK. Although it has been established that Ras signaling via *PIK3CA* is necessary for Ras' full oncogenic function (15), mutant *PIK3CA* leading to MEK/ERK signaling has only recently been reported (5, 16), and the mechanism of this crosstalk has not been fully elucidated. To determine if knockin of oncogenic *PIK3CA* also exhibited increased ERK signaling, mutant *PIK3CA* knockin cell lines and control cells were subjected to Western blotting using total and phospho-specific anti-ERK antibodies. As shown in Fig. 2A (Left), in the absence of EGF, ERK was also phosphorylated (Thr-202/Tyr-204) in mutant *PIK3CA* knockin cell lines relative to control cells. However, upon exposure to 20 ng/ml EGF, control and parental cell lines showed approximately equal amounts of ERK phosphorylation (Thr-202/Tyr-204) relative to total ERK (Fig. 2A Right) when compared with mutant *PIK3CA* knockin cell lines. In addition, similar to S6K phosphorylation, mutant *PIK3CA* knockin clones have a relatively lower threshold for MAP kinase pathway activation by EGF, as seen by the increased ERK phosphorylation (Thr-202/Tyr-204) in the presence of 0.2 ng/ml EGF relative to control cell lines (Fig. 2B and Fig. S5). Accordingly, in the absence of EGF, mutant *PIK3CA* knockin cell lines were sensitive to the MEK inhibitor U0126, but in the presence of 0.2 ng/ml EGF, both parental MCF-10A cells and mutant *PIK3CA* knockin cells displayed sensitivity to this compound, similar to findings with PI3K inhibitors, although there was slight preferential toxicity for mutant *PIK3CA* knockin cell lines (Fig. S4).

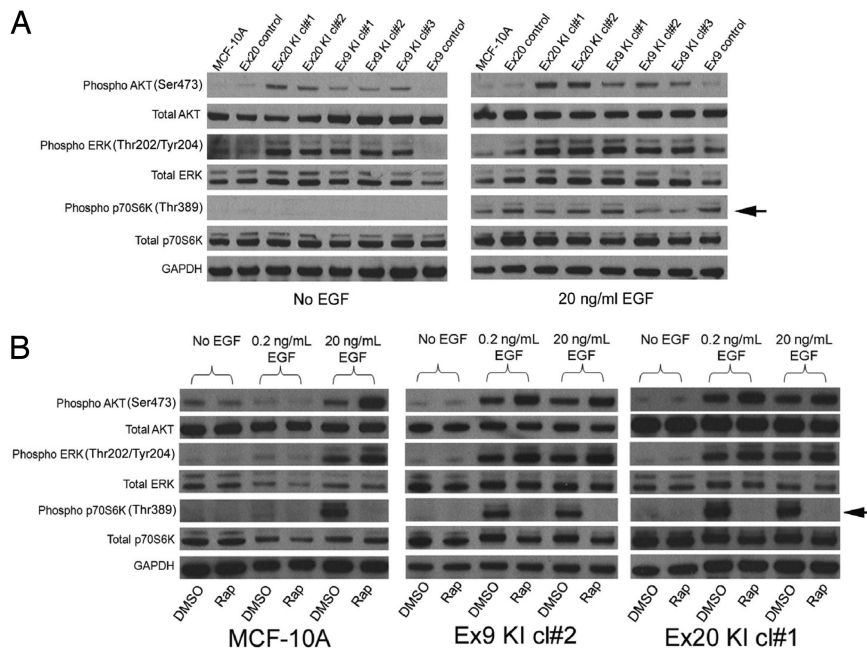


Fig. 2. Mutant *PIK3CA* is not sufficient for mTOR activation in MCF-10A cells. (A) Western blot illustrating levels of phosphorylated AKT (Ser-473), total AKT, phosphorylated ERK (Thr-202/Tyr-204), total ERK, phosphorylated p70S6K (Thr-389), and total p70S6K in mutant *PIK3CA* knockin cell lines and controls in the absence of EGF (Left) or presence of 20 ng/ml EGF (Right). Arrow denotes differences in p70S6K (Thr-389) phosphorylation. GAPDH is shown as a loading control. Note that shorter exposure times were used for AKT phosphorylation (Ser-473) with 20 ng/ml EGF as described in the text. (B) Western blot illustrating levels of phosphorylated AKT (Ser-473), total AKT, phosphorylated ERK (Thr-202/Tyr-204), total ERK, phosphorylated p70S6K (Thr-389), and total p70S6K in MCF-10A parental cells and 2 mutant *PIK3CA* knockin cell lines in the presence of no EGF, 0.2 ng/ml EGF, and 20 ng/ml EGF in the presence (Rap) and absence (DMSO) of 1 nM rapamycin. Arrow denotes differences in p70S6K (Thr-389) phosphorylation. GAPDH is shown as a loading control. Identical results were obtained for all *PIK3CA* knockin clones and controls. Note that shorter exposure times were used for AKT phosphorylation (Ser-473) for mutant *PIK3CA* knockin clones compared with parental and control cells as described in the text.

Oncogenic *PIK3CA* Leads to Phosphorylated AKT and GSK3 β . To further characterize mutant *PIK3CA*-mediated growth, we next analyzed levels of phosphorylated AKT. AKT is a substrate of PI3K, and mediates several distinct signaling pathways involved with cell proliferation and apoptosis (17). Phosphorylated AKT (Ser-473) was consistently elevated in *PIK3CA* mutant knockin clones relative to parental and control cells regardless of EGF concentration (Fig. 2A, Left vs. Right). Indeed, shorter exposure times were necessary when cells were treated with 20 ng/ml EGF compared with no EGF, to appreciate differences in AKT phosphorylation (Ser-473). In addition, shorter exposure times with mutant *PIK3CA* knockin clones were also necessary compared with parental and control clones, to fully appreciate the differences in increased AKT phosphorylation (Ser-473) using 0, 0.2, and 20 ng/ml EGF (Fig. 2B and Fig. S5). Thus, although baseline levels of phosphorylated AKT (Ser-473) were elevated in mutant *PIK3CA* knockin clones relative to parental and control cell lines, further AKT phosphorylation (Ser-473) was seen at relatively lower levels of EGF stimulation compared with parental and control cell lines, akin to the situation with ERK and S6K. Because AKT is itself a protein kinase, we next analyzed downstream targets of AKT to determine if differences in protein phosphorylation of these effectors could account for the increased cellular proliferation observed in mutant *PIK3CA* cells. For example, phosphorylation of the cyclin-dependent kinase inhibitors (CDKIs) p21 and p27 via AKT results in nuclear exclusion of these CDKIs, rendering them nonfunctional in mediating G1 arrest (18–21). However, Western blotting for phosphorylated p21 and p27 did not reveal any differences between mutant and wild-type *PIK3CA*-containing cell lines (data not shown). We next examined phosphorylation of GSK3 β , a tumor suppressor in the WNT-APC signaling pathway, which has also been shown to be phosphorylated at serine

9 and functionally inhibited by AKT (22). As seen in Fig. 3A and Fig. S6, all mutant *PIK3CA* knockin clones exhibited increased GSK3 β phosphorylation (Ser-9) relative to parental and control cells. This was accompanied by an increase in cyclin D1 levels, a gene known to be regulated by GSK3 β (23).

Mutant *PIK3CA* Sensitizes Cells to Lithium in Vitro and in Vivo. Our findings presented the intriguing possibility that mutant *PIK3CA* cells may be more sensitive to the effects of GSK3 β inhibitors such as lithium chloride (24) and SB216763 (25). Although inhibition of GSK3 β would theoretically lead to an increase in mitotic activity, previous studies have shown that lithium and other GSK3 β inhibitors can in fact inhibit cell proliferation (26–28). Though this effect is paradoxical, it has been postulated that inhibition of GSK3 β initially leads to cellular proliferation, but further GSK3 β inhibition by lithium and SB216763 beyond a critical threshold results in an antiproliferative effect, as it has been demonstrated that complete inhibition of GSK3 β is deleterious for cells because GSK3 β knockout mice are embryonic lethal (29). Moreover, depending on the genetic context of the cell, recent reports suggest that GSK3 β may have a pro-oncogenic effect in mixed lineage leukemias (MLL) leukemias (28). Thus, our rationale for testing lithium and SB216763 on our cell lines was based on these prior studies. Treatment with lithium chloride led to a decrease in cell proliferation in both EGF-free and EGF-containing medium (0.2 ng/ml) with high selectivity for mutant *PIK3CA* knockin cells (Fig. 3B). Control and parental cell lines grown in 0.2 ng/ml EGF proliferate at approximately the same rate as mutant *PIK3CA* knockin cells in the absence of EGF (Figs. S2 and S7). Thus, the antiproliferative effects of lithium are not simply the result of increased cell proliferation seen in mutant *PIK3CA* knockin clones. Although lithium is known to have other cellular effects, the small-

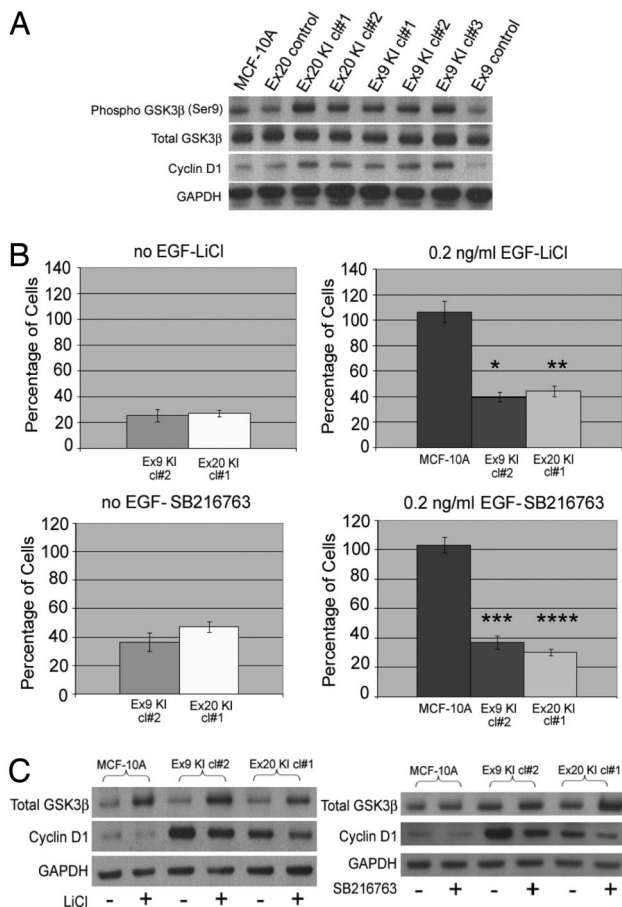


Fig. 3. Mutant *PIK3CA* leads to increased GSK3 β phosphorylation and sensitizes MCF-10A human breast epithelial cells to lithium and SB216763. (A) Western blot illustrating levels of phosphorylated GSK3 β (Ser-9), total GSK3 β , and cyclin D1 in mutant *PIK3CA* knockin cell lines and controls cultured for 24 h in the absence of EGF. GAPDH is shown as a loading control. Results are representative of 3 independent experiments. (B) Cell proliferation assays were performed as described in *Methods*, with MCF-10A cells and their knockin derivatives grown in the absence of EGF (*Left*) or presence of 0.2 ng/ml EGF (*Right*). Bars represent the percentage of cell proliferation in 10 mM lithium chloride (LiCl) (*Upper*) or 10 μ M SB216763 (*Lower*) relative to cells grown in control medium (water for LiCl, DMSO for SB216763) after 6 days in culture. Error bars represent SEM from triplicate samples. * $P < 0.0001$, ** $P < 0.0001$, *** $P < 0.0002$, **** $P < 0.0001$ compared with parental MCF-10A cells. (C) Western blot illustrating levels of total GSK3 β , cyclin D1, and GAPDH in MCF-10A parental cells and 2 mutant *PIK3CA* knockin cell lines in the presence (+) and absence (-) of 10 mM lithium chloride (LiCl) (*Left*), and in the presence (+) and absence (-) of 10 μ M SB216763 (*Right*). Cells were cultured in 0.2 ng/ml EGF and harvested for cell lysates after 24 h.

molecule competitive ATP inhibitor SB216763 has been shown to be a highly specific GSK3 kinase inhibitor (25). Using a relatively lower concentration of SB216763 than previously reported in the literature, identical effects of decreased cell proliferation were found in mutant *PIK3CA* knockin cell lines but not control cell lines (Fig. 3*B*). Western blotting revealed that lithium and SB216763 led to an increase in total GSK3 β , with a concomitant decrease in cyclin D1 (Fig. 3*C*), suggesting that in epithelial cells these compounds decrease cell proliferation via upregulating GSK3 β rather than inhibiting its activity, an effect more consistent with the known role of GSK3 β as a tumor suppressor. In accord with these findings, GSK3 β phosphorylation (Ser-9) was decreased in mutant *PIK3CA* knockin clones treated with lithium and SB216763, although the kinetics of this decrease varied between both of these agents (data not shown).

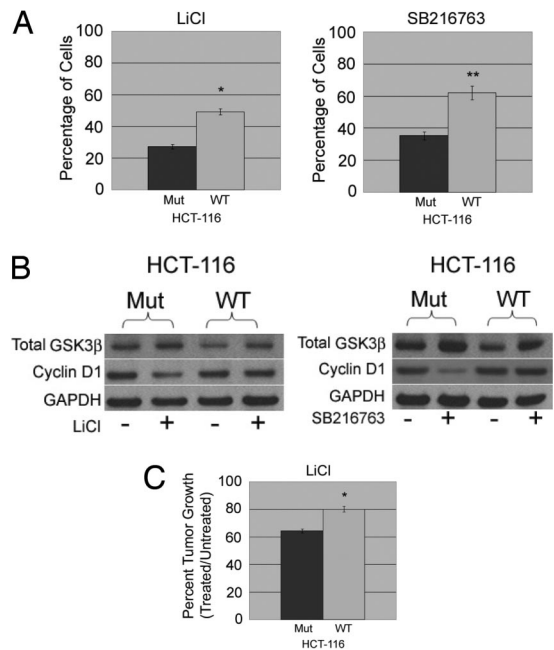


Fig. 4. Mutant *PIK3CA* sensitizes HCT-116 human colon cancer cells to lithium and GSK3 β inhibitors in vitro and in vivo. (A) Cell proliferation assays were performed as described in *Methods* using the HCT-116 somatic cell knockout derivatives containing either a single mutant (Mut) or wild-type (WT) *PIK3CA* allele in the presence of either 10 mM lithium chloride (LiCl) (*Left*) or 1 μ M SB216763 (*Right*). Bars represent the percentage of cell proliferation in LiCl or SB216763 relative to cells grown without these inhibitors. Error bars represent SEM from triplicate samples. * $P < 0.0003$, ** $P < 0.0005$. (B) Western blot illustrating levels of total GSK3 β and cyclin D1 in the HCT-116 somatic cell knockout derivatives containing either a single mutant (Mut) or wild-type (WT) *PIK3CA* allele in the presence of either 10 mM lithium chloride (LiCl) (*Left*) or 1 μ M SB216763 (*Right*) cultured for 24 h. GAPDH is shown as a loading control. (C) LiCl treatment and xenograft assays were conducted, with percentage of average tumor volumes ($n = 10$ for each group) shown for lithium- and sham-treated mice at day 18 postinoculation. * $P < 0.04$.

These findings suggest that lithium and SB216763 can selectively modulate GSK3 β levels, resulting in decreased cell proliferation preferentially in cells with mutant *PIK3CA*.

The generality of our findings was then rigorously tested. We first analyzed the effects of lithium and SB216763 using the breast cancer cell lines MCF-7 and ZR-75-1, which are mutant (E545K) and wild type for *PIK3CA*, respectively. As illustrated in Fig. S8*A*, MCF-7 cells also exhibited sensitivity toward the toxic effects of lithium and SB216763, whereas ZR-75-1 cells were relatively resistant against both reagents. Western blotting confirmed that lithium and SB216763 led to an increase in total GSK3 β and a decrease in cyclin D1 in MCF-7 cells, similar to mutant *PIK3CA* knockin clones (Fig. S8*B*). We then verified our findings using another paired isogenic system: HCT-116 human colorectal cancer somatic cell knockouts, containing either a single wild-type or mutant *PIK3CA* allele (12). Similar to our breast epithelial knockin clones, cell proliferation assays showed that lithium and SB216763 had selective toxicity for HCT-116 knockout cells harboring a mutant *PIK3CA* gene, compared with knockout derivatives carrying a single *PIK3CA* wild-type allele (Fig. 4*A*). Western blotting of treated vs. untreated cells showed identical results seen with MCF-10A mutant *PIK3CA* knockin clones (Fig. 4*B*). However, the magnitude of the difference in cell proliferation was not as pronounced as in our breast epithelial knockin cell lines, nor the breast cancer cell lines, because wild-type *PIK3CA* HCT-116 cells also displayed partial sensitivity toward GSK3 β inhibitors. This may reflect the fact

independent cellular proliferation induced by mutant PIK3CA, as evidenced by our findings with lithium chloride and SB216763. Our study has important consequences for the design of clinical trials, as combinations of drugs targeting multiple pathways may be necessary to achieve optimal effective antineoplastic therapy against cancers that contain *PIK3CA* mutations. The identification of GSK3 β as a potential therapeutic target may allow for the emergence of more efficacious drugs to treat the significant number of human malignancies that harbor mutant PIK3CA.

Methods

Targeted Knockin of the PIK3CA Oncogene. Targeting vectors were designed to introduce a single oncogenic mutation within PIK3CA to separately create E545K and H1047R knockin clones. Vector transduction, colony selection, clone screening, and Cre recombinase removal of the neomycin resistance gene were done as previously described (7, 35).

Cell Proliferation Assays. Cell proliferation assays were performed as previously described (7, 13) with and without pathway inhibitors.

Xenograft Assays. Lithium chloride (1,200 mg/l) in 5% sucrose (wt/vol) and water was administered orally 10 days before xenograft inoculation as pre-

viously described (30). Sham-treated mice received 5% sucrose in their drinking water. Drinking water was monitored to ensure no differences in consumption between treatment and control groups. Xenograft assays were performed as previously described (7).

Additional details and methods are described in *SI Methods*.

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