Inactivation of the VHL tumor suppressor gene is the signature initiating event in clear cell renal cell carcinoma (ccRCC), which is the most common form of kidney cancer. The VHL tumor suppressor protein marks hypoxia-inducible factor 1 (HIF1) and HIF2 for proteasomal degradation when oxygen is present. The inappropriate accumulation of HIF2 drives tumor formation by VHL tumor suppressor protein (pVHL)–defective ccRCC. Belzutifan, a first-in-class allosteric HIF2 inhibitor, has advanced to phase 3 testing for advanced ccRCC and is approved for ccRCCs arising in patients with VHL disease, which is caused by germline VHL mutations. HIF2 can suppress p53 function in some settings and preliminary data suggested that an intact p53 pathway, as measured by activation in response to DNA damage, was necessary for HIF2 dependence. Here, we correlated HIF2 dependence and p53 status across a broader collection of ccRCC cell lines. We also genetically manipulated p53 function in ccRCC lines that were or were not previously HIF2-dependent and then assessed their subsequent sensitivity to HIF2 ablation using CRISPR-Cas9 or the HIF2 inhibitor PT2399, which is closely related to belzutifan. From these studies, we conclude that p53 status does not dictate HIF2 dependence, at least in preclinical models, and thus is unlikely to be a useful biomarker for predicting which ccRCC patients will respond to HIF2 inhibitors.

ccRCC | HIF | VHL | TP53 | belzutifan

Inactivation of the VHL tumor suppressor protein (pVHL) is the usual initiating or truncal, event in clear cell renal cell carcinoma (ccRCC) (1–7), which is the most common form of kidney cancer. pVHL is the substrate recognition component of an E3 ubiquitin ligase that targets the alpha subunits of the heterodimeric HIF (hypoxia-inducible factor) transcription factor for destruction when oxygen is plentiful. In the absence of pVHL, deregulation of HIF, in particular HIF2, drives the growth of ccRCC (8–13).

Drugs that inhibit the HIF2-responsive growth factor VEGF (vascular endothelial growth factor) are now mainstays of ccRCC treatment (14). However, HIF2 controls many other pathways; including additional genes that are known or suspected of playing pathogenic roles in ccRCC (15, 16). Although HIF2 was classically viewed as undruggable, Richard Bruick and Kevin Gardner identified compounds that can bind to a previously unappreciated pocket in HIF2α and, in so doing, induce an allosteric change in HIF2α that prevents it from binding to its partner protein ARNT and hence to DNA (17–20). These compounds were optimized further by Peloton Therapeutics to produce the HIF2 inhibitors PT2399 (preclinical compound), the first-generation clinical compound PT2385, and the second-generation compound PT2977 (now called MK-6482 or belzutifan) (21–25).

Both PT2385 and PT2977 display significant activity when used to treat ccRCC patients who have failed standard-of-care agents such as VEGF inhibitors and immune checkpoint inhibitors (25–28). However, many heavily pretreated ccRCC patients do not respond to these drugs and most that do respond eventually progress (25–28). Consistent with these observations, some VHL mutant ccRCC cell lines are not dependent on HIF2 (21). There is a pressing need to understand the molecular basis of the HIF2 independence exhibited by some ccRCC cell lines and some ccRCC patients.

We previously identified an acquired p53 mutation in a HIF2-independent subclone of the HIF2-dependent ccRCC cell line 786-O and Brugarolas and coworkers identified a p53 mutation, R273H, in a HIF2-independent ccRCC tumor in a ccRCC patient who had a mixed response to PT2385, suggesting that p53 mutations caused resistance (21, 28). Moreover, in our initial survey of ccRCC cell lines, we noted that an intact p53 pathway, as measured by induction of p53 and p21 after DNA damage, was necessary (although not sufficient) for HIF2 dependence (21). Notably, HIF2 suppression can enhance p53 activation, at least in response to radiation, suggesting that

**Significance**

VHL tumor suppressor gene inactivation is a hallmark of clear cell renal cell carcinoma (ccRCC), the most common form of kidney cancer, and promotes tumor growth by stabilizing the hypoxia-inducible factor 2 (HIF2) transcription factor. HIF2 inhibitors appear to be helpful for some, but not all, ccRCC patients in clinical trials. Previous preclinical and clinical data suggested that only ccRCCs that can activate the p53 tumor suppressor in response to DNA damage would respond to HIF2 inhibitors. Here, we show that an intact p53 pathway is neither necessary nor sufficient for the sensitivity of ccRCCs to HIF2 inhibitors, suggesting that it would be premature to use p53 status to determine which ccRCC patients should be treated with a HIF2 inhibitor.

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Competing interest statement: L.A.S. has financial interests in Blueprint Medicines. S.S. reports receiving commercial research grants from Bristol-Myers Squibb, AstraZeneca, Exelixis, and Novartis; is a consultant/advisory board member for Merck, AstraZeneca, Bristol-Myers Squibb, CRISPR Therapeutics AG, American Association for Cancer Research, and National Cancer Institute; and receives royalties from Biogenex. W.G.K. has financial interests in Lilly Pharmaceuticals, Fibrogen, Cedilla Therapeutics, Nestech Invest, Tango Therapeutics, Circle Pharma, IconOviv Bio, Casdin Capital, and LifeMine Therapeutics. He also has a royalty interest in the HIF2 inhibitor belzutifan, which is currently being commercialized by Merck.

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p53 activation could contribute to the antiproliferative effects of HIF2 inhibitors (29). In this study, we examined an expanded panel of ccRCC cell lines and genetically manipulated TP53 in ccRCC cells to address whether p53 status is, indeed, an important determinant of HIF2 dependence and hence sensitivity to pharmacological HIF2 inhibitors. Our data suggest that p53 activity is neither necessary nor sufficient for dependence on HIF2, at least in the context of ccRCC.

### Results and Discussion

We first examined the Broad Institute DepMap dataset (https://www.depmap.org) with respect to the depletion of EPAS1 single-guide RNAs (sgRNAs) over time in ccRCC cell lines that had been subjected to genome-wide CRISPR-Cas9 screens. EPAS1 sgRNAs were significantly depleted in some ccRCC lines, including TUHR14TKB, OSRC2, 786-O, RCC10RGB, and TUHR4TKB, indicative of HIF2 dependence, but not in many other ccRCC lines, such as CAKI2, 769-P, and SLR23, suggestive of HIF2 independence (Fig. 1). The sensitivity of the 786-O, CAKI2, and 769-P cells to the 769-P, and SLR23, suggestive of HIF2 independence (Fig. 1).

To corroborate this variable HIF2 dependence, we performed low-throughput competition assays with selected ccRCC lines that we infected to produce either a control sgRNA and mCherry or one of two different EPAS1 sgRNAs (or the same control sgRNA) and green fluorescent protein (GFP) (SI Appendix, Fig. S1A), aiming for an ~1:1 ratio of mCherry-positive cells and GFP-positive cells. We then passed the cells for 4 weeks and monitored the GFP:mCherry ratio by fluorescence-activated cell sorting (FACS). The EPAS1 sgRNA cells were dramatically depleted in the HIF2-dependent lines 786-O, OSRC2, and TUHR4TKB, but much less so in the HIF2-independent lines 769-P and CAKI2, and in UMRC2 cells, which we reported before are also largely HIF2-independent (21) (Fig. 2A). Similar results were obtained with both EPAS1 sgRNAs and when the mCherry and GFP fluorescent proteins were swapped in the sgRNA expression vectors and the mCherry-GFP ratio was monitored (SI Appendix, Fig. S2B). Moreover, PT2399 suppressed soft agar growth by the HIF2-dependent lines 786-O and OSRC2 but not by the HIF2-independent lines 769-P, CAKI2, and UMRC2 (Fig. 2 B and C). TUHR4TKB did not form soft agar colonies and therefore could not be studied in this assay. PT2399 reproducibly enhanced the soft agar growth of 769-P cells, although the significance of this finding is not clear. These findings, together with our earlier study, confirm that the HIF2 dependence of ccRCC lines is variable, which provides an opportunity for studying de novo and acquired resistance to HIF2 inhibitors.

We next measured the induction of p53 by the DNA-damaging agent etoposide in a panel of ccRCC cell lines, expanding upon the number of lines we interrogated previously (21) (Fig. 3A). As we reported before, p53 was robustly induced in the HIF2-dependent 786-O cells but not in the HIF2-independent 769-P and UMRC2 cells (21). On the other hand, p53 was not induced in the HIF2-dependent line TUHR4TKB but was induced in the HIF2-dependent line CAKI2. Therefore, an intact p53 pathway appears to be neither necessary nor sufficient for HIF2a dependence.

To investigate this further, we repeated the competition and soft agar assays using 786-O and OSRC2 cells after first inactivating p53 using CRISPR-Cas9. We confirmed that Nutlin treatment induced p53 in 786-O cells expressing a control sgRNA but not a TP53 sgRNA (Fig. 3B). Loss of p53 did not prevent the pharmacodynamic effects of PT2399, as measured by down-regulation of Cyclin D1 (SI Appendix, Fig. S2A). Moreover, eliminating p53 did not significantly mitigate the competitive disadvantage of cells expressing EPAS1 sgRNAs nor did it rescue soft agar growth in the presence of PT2399, despite increasing soft agar growth in the absence of PT2399 (Fig. 3 C–E and SI Appendix, Fig. S2B). Finally, 786-O cells lacking TP53 remained sensitive to PT2399 in orthotopic tumor assays in vivo, as determined by reduced tumor size and Cyclin D1 and Ki67 expression, in mice treated with PT2399 compared with vehicle (Fig. 3 F and G and SI Appendix, Fig. S2 C–E).

We previously identified a 786-O subclone that spontaneously acquired partial HIF2 independence (“786-O HI”) relative to parental 786-O cells (21), which we reconfirmed in competition and soft agar assays (Fig. 4 A–C and SI Appendix, Fig. S3 D and E). These cells, in contrast to parental 786-O, have high basal p53 levels and do not further induce p53 in response to DNA damage (Fig. 3A). Hence, they are phenotypically p53 mutant. Based on sequencing a limited number of complementary DNA (cDNA) clones from these cells, we previously genotyped these cells as p53 R248W (21). To more rigorously genotype these cells, we performed a battery of assays. First, we confirmed that the 786-O HI cells were indeed derived from 786-O cells, as determined by short tandem repeat (STR) analysis (SI Appendix; Table S1). Next, we karyotyped 25 parental 786-O cells and 25 786-O HI cells. This
analysis showed that 786-O are highly heterogeneous and, as reported before, nearly triploid, although some chromosomes are present in two, four, or five copies (SI Appendix, Table S3). Notably, these cells have four copies of chromosome 17, which harbors TP53. The 786-O HI cells appeared to be highly genomically unstable based on the presence of new isochromosomes (SI Appendix, Table S3).

We next determined TP53 mutant allele frequencies by next-generation sequencing of genomic DNA. In the parental 786-O cells, we detected both wild-type (WT) and R248W TP53 alleles at an ∼60:40 ratio. In the 786-O HI cells, we detected 49% P278A, 3% R248W, and 48% WT TP53 (SI Appendix, Fig. S3). In 70 of these 71 cDNAs the sequence corresponding to codon 278 was also evaluable; 8% of these cDNA were WT at codon 278 and 92% were P278A at codon 278. The R248W and P278A mutations were never found in *cis* and no other mutations were detected. We then detected WT TP53 sequences in 25 cDNAs from OSRC2 cells, of which 22 were evaluable for codon 278 in addition to codon 248 (SI Appendix, Fig. S3A). These results indicate that our initial hypothesis that the resistance of 786-O HI to PT2399 was caused by the R248W mutation was incorrect and instead might be caused by the P278A mutation.

TP53 mutations can be loss-of-function, dominant-negative, or neomorphic. The continued sensitivity of the 786-O and OSRC2 cells to PT2399 after CRISPR-Cas9–mediated elimination of TP53 strongly suggested that pure loss-of-function or dominant-negative TP53 mutations would not cause resistance to PT2399. To address the possibility that the mutation induced in the 786-O HI cells through a neomorphic activity, we inactivated TP53, including the P278A allele, in the 786-O HI cells. These cells remained insensitive to PT2399 in competition and soft agar assays, suggesting that expression of the P278A variant is not necessary for the PT2399 insensitivity of the 786-O HI cells (Fig. 4, D–F and SI Appendix, Fig. S3).

To address the issue of sufficient numbers of cells to probe for a possible neomorphic activity, we introduced exogenous WT p53, p53 R248W, p53 R273H, p53 R278A, GFP, or the empty backbone vector into 786-O and OSRC2 cells in which endogenous p53 was or was not first eliminated using CRISPR
R248W was included because it is a well-characterized neomorphic p53 mutant that was mistakenly suspected of causing the resistance of the 786-O HI cells (see above), while the R273H mutant was detected in a kidney cancer patient who developed resistance to PT2399 (28). None of the exogenous p53 mutants caused pharmacodynamic resistance to PT2399, regardless of endogenous TP53 status, as reflected by decreased accumulation of the HIF2-responsive gene products Cyclin D1 and NDRG1 (SI Appendix, Fig. S5) or resistance to PT2399 in soft agar assays.

(Fig. 5A and SI Appendix, Fig. S4A). R248W was included because it is a well-characterized neomorphic p53 mutant that was mistakenly suspected of causing the resistance of the 786-O HI cells (see above), while the R273H mutant was detected in a kidney cancer patient who developed resistance to PT2399 (28). None of the exogenous p53 mutants caused pharmacodynamic resistance to PT2399, regardless of endogenous TP53 status, as reflected by decreased accumulation of the HIF2-responsive gene products Cyclin D1 and NDRG1 (SI Appendix, Fig. S5) or resistance to PT2399 in soft agar assays.
**Fig. 4.** TP53 mutation is not required for the HIF2α independence of 786-O HI cells. (A) Relative ratios of mixtures of 786-O HI cells expressing 1) GFP and either a nontargeting control sgRNA (sgNT) or the indicated sgRNA targeting EPAS1, or 2) mCherry and sgNT, over time, as determined by flow cytometry. Ratios were normalized such that the ratio at $T = 1$ wk after lentiviral introduction of the reporters and sgRNAs was 1. Data represent the mean ± SEM of at least two independent replicates. (B) Representative photomicrographs of soft agar colonies formed by 786-O and 786-O HI cells that were grown in the presence of 2 μM PT2399 or DMSO vehicle control. (C) Quantification of soft agar colonies formed by the cell lines in B. Shown are mean colony numbers in the presence of 2 μM PT2399 relative to DMSO. Data are mean ± SEM of at least two independent replicates. (D) Relative ratio of mixtures of 786-O HI cells (filled circles, cells expressing nontargeting sgRNA; open circles, cells expressing sgTP53 that were then engineered to express 1) GFP and either a nontargeting control sgRNA (sgNT) or the indicated sgRNA targeting EPAS1, or 2) mCherry and sgNT, over time, as determined by flow cytometry. Ratios were normalized such that the ratio at $T = 1$ wk after lentiviral introduction of the reporters and sgRNAs was 1. Data represent the mean ± SEM of at least two independent replicates. (E) Relative ratio of mixtures of 786-O HI cells (filled circles, cells expressing nontargeting sgRNA; open circles, cells expressing sgTP53 that were then engineered to express 1) GFP and either a nontargeting control sgRNA (sgNT) or the indicated sgRNA targeting EPAS1, or 2) mCherry and sgNT, over time, as determined by flow cytometry. Ratios were normalized such that the ratio at $T = 1$ wk after lentiviral introduction of the reporters and sgRNAs was 1. Data represent the mean ± SEM of at least two independent replicates. (F) Quantification of soft agar colonies formed by the cell lines as in D. Shown are mean colony numbers in the presence of 2 μM PT2399 relative to DMSO. Data are mean ± SEM of at least two independent replicates.

(Fig. 5 B and C and SI Appendix; Fig. S4 B and C). Moreover, expression of R273H did not mitigate the survival benefit conferred by PT2399 in ORSC2 orthotopic tumor assays or prevent the suppression of the HIF2 target gene CCND1 by the drug (Fig. 6 and SI Appendix, Fig. S6).

These data, collectively, strongly suggest that an intact p53 pathway is not required for the HIF2 dependence of VHL mutant ccRCC cells, despite early conjecture to the contrary, and also suggest that p53 mutants such as R273H that have been associated with PT2399 resistance do not necessarily cause that resistance. Although we interrogated promoters of various strengths to drive the expression of exogenous mutant p53, it remains formally possible that higher levels of expression would have caused resistance. Moreover, it remains possible that TP53 status could alter the sensitivity of VHL mutant kidney cancers under conditions that were not captured by our preclinical models, such as in conditions created in an immunocompetent host. Nonetheless, our findings argue that it would be premature to use p53 status to exclude kidney cancer patients from trials of HIF2 inhibitors.

The prevalence of TP53 mutations in ccRCC could be as low as 2 to 6% based on analyses primarily of primary nephrectomy specimens, although the true prevalence might be higher in advanced disease or after multiple rounds of therapy (1, 4, 32–37). In one large study that included over 400 samples, ~15% of ccRCC metastases harbored TP53 mutations (37). Another study found that 12% of ccRCC patient-derived xenografts (PDXs) bore TP53 mutations, suggesting that TP53 mutations increase ccRCC fitness and successful PDX propagation, both of which might correlate with more aggressive clinical disease (38). Moreover, other genes in the p53 pathway, such as ATM and MDM2, are also mutated, albeit infrequently, in ccRCC (1, 4, 32–35), and TP53 loss cooperates with VHL loss to promote murine ccRCC (39, 40). Additionally, single specimens might be insufficient to capture the full complement of mutations present in a ccRCC tumor. For example,
Fig. 5. Expression of TP53 mutations previously associated with HIF2α independence is not sufficient to confer HIF2α independence. (A) Immunoblots of 786-O cells expressing control sgRNA (sgAAVS1) or sgTP53 that were then engineered to express GFP, the indicated p53 variants, or an empty vector (EV). The cells were (+) or were not (-) treated with 10 μM Nutlin for 24 h prior to harvest. (B) Representative photomicrographs of soft agar colonies formed by 786-O cells as in A that were grown in the presence of 2 μM PT2399 or DMSO vehicle control. (C) Quantification of soft agar colonies formed by 786-O cells as in B. Shown are mean colony numbers in the presence of 2 μM PT2399 relative to DMSO. Data are mean ± SEM of at least two independent replicates.
multiregion whole-exome sequencing showed that TP53 mutation was subclonal in tumors from 4 out of 10 ccRCC patients examined (35). Notably, increased p53 protein levels, which are often indicative of a compromised p53 pathway, have been observed in up to 50% of ccRCCs and are associated with poor outcomes (41, 42). Therefore it is probable that our findings are relevant for a significant fraction of ccRCC patients who would be eligible for treatment with a HIF2 inhibitor.

Materials and Methods

Cell Lines and Cell Culture. 786-O, 769-P, Caki2, and HEK293FT cells were obtained from the American Type Culture Collection. 786-O HI cells arose from a subcultured of 786-O cells infected with empty plenti-HA backbone (21). OSRC2, TUHR4TKB, TUHR10TKB, and RCC10RG cell lines were obtained from the RIKEN BioResource Research Center Cell Bank. UMRC2 cells were a gift of Bert Zbar and Marston Linehan, National Cancer Institute, Bethesda, MD. 786-O, 769-P, Caki2, TUHR4TKB, RCC10RG, and UMRC2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) ( Gibco; 11995073). Caki2 cells were cultured in McCoy’s medium 5A (Corning; 10050CV), OSRC2, TUHR10TKB, and TUHR4TKB were cultured in RPMI-1640 (Gibco; 11875119). Basal media were supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts; 100-106), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco; 25200056). Cells were maintained in a humidified incubator at 37 °C and 5% CO2.

Cas9-expressing cells were generated by infection with plenti-EF1a-Cas9-Flag-IRES-Neo, a gift of Samuel McBrayer, W.G. K. laboratory, described in ref. 43.

Chemicals. PT2399 was provided by Peloton Therapeutics. Etoposide was purchased from APP Pharmaceuticals. Nutlin-3a was purchased from Selleck Chemicals.

Plasmids. CRISPR-Cas9 plasmids. sgRNAs were cloned into plentiCRISPRv2 (Addgene; 52961), plentiCRISPR_zeo, a gift of Samuel McBrayer, described in ref. 43, or plentiGuide (Addgene; 117986) modified to also express GFP or mCherry, gifts of Vidhyaagar Koduri and Benjamin Lampson, W.G. K. laboratory. Vector backbones were linearized by BsmBI digestion (New England Biolabs; R0580) in NEB buffer 3.1 for 2 h at 55 °C, followed by incubation at 80 °C for 20 min to inactivate the enzyme. The linearized vector backbone was purified by gel electrophoresis using the QIAquick Gel Extraction Kit per the manufacturer’s instructions to introduce attB sites into the fragment of the expected size was confirmed by agarose gel electrophoresis. Full-length attB1 and attB2 sites were added by a second PCR with the attB extension primers listed below, and amplification of the fragment of the expected size was again confirmed by agarose gel electrophoresis. After each PCR, unincorporated primers and dNTPs were removed using the Monarch PCR and DNA Cleanup Kit (New England Biolabs; T1030).

To clone Flag-tagged inserts into pDONR223, 150 ng pDONR223 (Invitrogen) was mixed with 150 ng of the purified, full-length PCR insert and 1 μL BP clonase II (Invitrogen; 11798902). The total reaction volume was brought to 5 μL with TE buffer (10 mM Tris-Cl, 1 mM ethylenediaminetetraacetaate [EDTA]). The BP reaction was incubated overnight at room temperature. Two microliters of the recombination reaction was transformed into 25 μL HB101 chemically competent E. coli (Promega; L2011), and spectinomycin-resistant transformants were isolated by growth on LB-agar plates containing 50 μg/mL spectinomycin overnight at 30 °C. Individual colonies were expanded by growth in LB broth containing 50 μg/mL spectinomycin overnight at 30 °C with shaking. Plasmids were isolated using the QIAprep Spin Plasmid Miniprep Kit (Qiagen; 27106) and incorporation of the desired ORF was validated by Sanger sequencing.

Once validated, the ORFs were shuttled into the plenti-EF1a-Gate-PGK-hypoxymycin destination vector, a gift of Gang Lu, W.G. K. laboratory, by LR recombination reactions. One hundred and fifty nanograms of each entry and destination vector was mixed with 1 μL LR clonase II (Invitrogen; 11791100) and the reaction volume was brought to 5 μL with TE buffer. Recombination reactions were allowed to proceed overnight at room temperature. Two microliters of the reactions was transformed into 25 μL HB101 chemically competent E. coli (Promega; L2011). Kanamycin-resistant transformants were isolated by growth on LB-agar plates containing 50 μg/mL kanamycin overnight at 30 °C. Individual colonies were expanded overnight at 30 °C in a shaking incubator in LB broth containing 50 μg/mL kanamycin. Plasmids were isolated using the
QiAprep Spin Plasmid Miniprep Kit (Qiagen; 27106) and validated by Sanger sequencing. A PDONR223 containing only a short multiple cloning site within the attB-flanked region was also a gift of Gang Lu.

Targeted mutagenesis primers.

TPS2 R273H forward 5’-GCTTGGAGGTGAATTTTGCCGG-3’
TPS2 R273H reverse 5’-CAGCCAAACATGCTCATCAACG-3’
TPS2 P278A forward 5’-GTCGCAGAGCTGTCGGTGGAGACCAGCGCCGC-3’
TPS2 P278A reverse 5’-GGCCGCGCTTCTTCTGAGCAAGGACACGACCACCGC-3’

Primers to amplify Flag-tagged TP53 and GFP for Gateway cloning.

attB1 Flag-GGGGS-TPS2 forward 5’-AAAAACCGCACTTTGAGTATCAGTAAAGGACAAGCA CGTAAACAGG-3’
attB12 Flag-GGGGS-TPS2 GFP forward 5’-AAAAACCGCACTTTGAGTATCAGTAAAGGACAAGCA CGTAAACAGG-3’
attB2 Flag-GGGGS-TPS2 GFP reverse 5’-AAAAACCGCACTTTGAGTATCAGTAAAGGACAAGCA CGTAAACAGG-3’

Generation of Lentivirus. HEK293FT cells were seeded at a density of 45,000 cells per square centimeter in DMEM supplemented with 10% FBS, with the plate size determined by the amount of virus desired. The following day, the cells were cotransfected with the lentiviral plasmid of interest, psPAX2 (Addgene; 13922), along with pMD2.G (Addgene; 12259), at a ratio of 2.1:1 using Lipofectamine 2000 transfection reagent at a ratio of 1 μg DNA:3 μg Lipofectamine (Life Technologies; 11668019). Plasmid DNA and Lipofectamine were diluted separately in Opti-MEM medium and incubated for 5 min at room temperature prior to transfection. Plasmid- and Lipofectamine-containing solutions were then mixed together vigorously and incubated at room temperature for 20 min before drop-wise addition to the plates containing HEK293FT cells. The following day, media were replaced with DMEM supplemented with 30% FBS. Viral supernatants were collected 24 and 48 h later and pooled, spun at 1,200 × g, and filtered with a 0.45-μm SCFA (surfactant-free cellulose acetate) filters (Corning; 431220) before aliquoting and storage at −80 °C.

Generation of Stable Cell Lines by Lentiviral Infection. Three hundred thousand cells per well were seeded into 6-well plates in 2.75 mL of the culture medium normally used for the recipient cell line. Two hundred and fifty microliters of viral supernatant was added per well such that each well received a different virus. One additional well received no virus to serve as an uninfected control. Plates were centrifuged at 4,000 rpm in an A-4-81 rotor for the 5810 R centrifuge (Eppendorf) for 30 min at 37 °C. Rotor buckets were rotated horizontally 180° after which selection antibiotic was removed and cultures were maintained in nonmedium normally used for the recipient cell line. Two hundred and fifty microliters of lentivirus and an mCherry-expressing lentivirus in amounts designed to infect 10% of cells as determined by antibiotic resistance. For competition assays, Cas9-positive cell lines were infected with a mixture of a GFP-expressing lentivirus and an mCherry-expressing lentivirus in amounts designed to infect 10% of the cells with each virus based on the titration data above. Successfully infected cells were selected with 1 μg/mL puromycin until no cells remained in an uninfected control treated in parallel (typically 2 to 3 d). Initial population abundance, measured as the percentage of live cells showing GFP or mCherry positivity, was assessed 1 wk after infection by flow cytometry using an LSRFortessa cell analyzer (BD Biosciences; 649225) with BD FACS Diva software. Population abundance over time was assessed by weekly flow cytometry on the same instrument. Relative abundance was calculated as the ratio of GFP:mCherry or mCherry:GFP as indicated in the figure legends and normalized to the same ratio at the initial time point.

Cellular FACS-Based Competition Assays. Viruses delivering nontargeting sgRNAs or sgRNAs targeting EPAS1 in the p lentGuide_GFP or mCherry backbone were titrated in Cas9-expressing 876-0 cells to determine the volume of virus per well to infect ∼10% of cells as determined by antibiotic resistance. For competition assays, Cas9-positive cell lines were infected with a mixture of a GFP-expressing lentivirus and an mCherry-expressing lentivirus in amounts designed to infect 10% of the cells with each virus based on the titration data above. Successfully infected cells were selected with 1 μg/mL puromycin until no cells remained in an uninfected control treated in parallel (typically 2 to 3 d). Initial population abundance, measured as the percentage of live cells showing GFP or mCherry positivity, was assessed 1 wk after infection by flow cytometry using an LSRFortessa cell analyzer (BD Biosciences; 649225) with BD FACS Diva software. Population abundance over time was assessed by weekly flow cytometry on the same instrument. Relative abundance was calculated as the ratio of GFP:mCherry or mCherry:GFP as indicated in the figure legends and normalized to the same ratio at the initial time point.

Coloncy Formation in Soft Agar. Soft agar assays for all cell lines were carried out in non-tissue culture-treated 6-well plates (Corning; 351146), with the exception of 786-0, which was seeded in ultra-low-adhesion 6-well plates (Corning; 3471). A 3% (weight/volume) SeaPlaque agarose (Lonza; 50100) solution was prepared in PBS (pH 7.4) and sterilized by autoclaving. This stock solution was stored at room temperature, melted before use by gentle microwaving, and maintained in liquid form in a 50 °C water bath during assay setup. Warmed 3% agarose stock solution was diluted 1:2 with growth media to obtain a 1% agarose solution. Two milliliters of 1% agarose was added to each well of the 6-well plate and allowed to solidify at room temperature for at least 20 min. After solidifying, 1 mL of a 0.4% agarose solution containing 50,000 cells and 2 μM PT2399 or dimethyl sulfoxide (DMSO) vehicle control was gently added to each well. The cell-containing layer was allowed to solidify at room temperature for 1 h, after which 1.5 mL of growth media containing 2 μM PT2399 or DMSO vehicle control was added to each well. Plates were incubated in a humidified incubator maintained at 37 °C and 5% CO2. Overall media were changed twice per week until macroscopic colonies formed (2 to 5 wk depending on the cell line). At the termination of the assay, media were aspirated and replaced with...
by oral gavage. Two mice per arm were killed by CO2 asphyxiation after 5 d of dosing. Tumors were fixed in 10% paraformaldehyde for 24 h and then switched to 70% ethanol before embedding in paraffin blocks. Suppression of tumor growth and HIF2α activity was assessed by immunohistochemical staining for K67 and Cyclin D1, respectively, as described in ref. 43. For end-point studies, 28 d after dosing, animals were killed by CO2 asphyxiation and tumors were harvested, weighed, and fixed in 10% paraformaldehyde for histological analysis.

Statistical Methods. Statistical tests were applied as indicated in the figure legends and text and were run using GraphPad Prism software. P values < 0.05 were considered significant.

Data Availability. The plasmids reported in this article have been deposited in Addgene. All study data are included in the article and/or SI Appendix.

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