Genome-Wide Interrogation of Human Cancers Identifies EGLN1 Dependency in Clear Cell Ovarian Cancers

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Abstract

We hypothesized that candidate dependencies for which there are small molecules that are either approved or in advanced development for a nononcology indication may represent potential therapeutic targets. To test this hypothesis, we performed genome-scale loss-of-function screens in hundreds of cancer cell lines. We found that knockout of EGLN1, which encodes prolyl hydroxylase domain-containing protein 2 (PHD2), reduced the proliferation of a subset of clear cell ovarian cancer cell lines in vitro. EGLN1-dependent cells exhibited sensitivity to the pan-EGLN inhibitor FG-4592. The response to FG-4592 was reversed by deletion of HIF1A, demonstrating that EGLN1 dependency was related to negative regulation of HIF1A. We also found that ovarian clear cell tumors susceptible to both genetic and pharmacologic inhibition of EGLN1 required intact HIF1A. Collectively, these observations identify EGLN1 as a cancer target with therapeutic potential.

Significance: These findings reveal a differential dependency of clear cell ovarian cancers on EGLN1, thus identifying EGLN1 as a potential therapeutic target in clear cell ovarian cancer patients.

Introduction

The development of genome-scale methods to interrogate the function of genes now provides a path to systematically identify genes that are essential for cell survival. Several studies using RNAi and more recently CRISPR-Cas9 to suppress or delete genes have identified cell-essential genes in human cancer cell lines (1–8). Compared with similar experiments in yeast (9, 10), these studies have identified a larger number of essential genes in human cells (7, 10–12).

We and others have used similar approaches to identify genes that are essential for subsets of cancer cell lines (7, 10–13). A number of screens involving a modest number of cell lines have identified several cancer dependencies related to oncogenic mutation (12) or genes that become required in the setting of loss of a paralog (11, 13). In recent work, we and others have expanded such efforts to hundreds of cancer cell lines and defined several types of dependencies that occur in subsets of cancers (2, 5, 7, 12, 13). Such efforts will eventually lead to a comprehensive map of cancer dependencies.

A direct downstream sensor of oxygen tension is the family of prolyl hydroxylases called EGLN (Egl9 family hypoxia-inducible factor 1). When oxygen is present, EGLN, also known as hypoxia-inducible factor prolyl hydroxylase (HIF-PH) and prolyl hydroxylase domain-containing protein (PHD), hydroxylates HIF1, which results in a binding site for a ubiquitin ligase complex that includes VHL (von Hippel-Lindau tumor suppressor), leading to HIF1 degradation (14–17). This leads to HIF1 degradation by forming a binding site for a ubiquitin ligase complex that includes VHL (14–17). Among the three EGLN family members, EGLN1 is generally regarded as the primary HIF1 hydroxylase, while EGLN2 and EGLN3 regulate HIF1 under specific conditions (15–17).

With decreased concentrations of oxygen, EGLN-mediated hydroxylation of HIF1 is reduced, which leads to increased levels of HIF1 (17). When HIF1 is increased under low oxygen conditions, the α subunit heterodimerizes with the β subunit and translocates to the nucleus to activate genes that promote glucose uptake, glycolysis, and decreases oxidative phosphorylation (17).

We initiated an effort to identify differential dependencies by interrogating the data derived from screening a large number of cell lines with genome-scale RNAi and CRISPR-Cas9 libraries. From a list of nominated strong preferential dependencies, we identified EGLN1 as a dependency preferentially required for the viability of a subset of cancer cell lines.

Materials and Methods

Analysis of dependency data

We used gene dependency data from Project Achilles including data from the screening of 501 cancer cell lines by RNAi (~49k shRNAs, ~5 shRNAs/gene) and 436 cancer cell lines with CRISPR-Cas9 (~70k sgRNAs, ~4 sgRNAs/gene; refs. 1–5). EGLN1 was originally identified as an interesting target to pursue using the DEMETER six sigma dependencies (2). Further analyses were...
performed using CERES CRISPR/Cas9 dependency data (7, 18–22) and DEMETER2 RNAi (20, 21). All significant findings are summarized in the Supplementary Tables.

**Identifying features associated with EGLN1 dependency**

We used multiple approaches to find relationships between EGLN1 dependency and mRNA expression, copy number, mutation, or dependency data [see Cancer Cell Line Encyclopedia (CCLE) Omics Data section for details]. To identify genes whose mutation was associated with EGLN1 dependency, we used two-sample t tests to measure the mean difference of dependency between mutant and WT cell lines for each gene and type of mutation (damaging or missense). Only mutations present in at least 5 cell lines were included in the analysis. To identify other continuous-valued (expression, copy number, dependency) features associated with EGLN1 dependency, we used the R package Limma. Linear model coefficients were used to assess the strength of association between each feature and EGLN1 dependency. P values were determined using empirical Bayes moderated t-statistics (analyzing each data type separately). Note that we also used this method to identify top dependencies associated with HIF1A expression in Supplementary Fig. S1M.

**CCLE omics data**

We used the following CCLE (22) datasets available for download at https://depmap.org/portal/download/all/:
- mRNA Expression: CCLE_DepMap_18Q2_RNAseq_RPKM_20180502.gct
- Copy number: public_18Q2_gene_cn_v2.csv
- Damaging/nondamaging mutations: CCLE_DepMap_18Q2_maf_20180502.txt (genes are classified as having a "damaging mutation" if isDeleterious is "TRUE" for any of its mutations).

**Identifying gene sets associated with EGLN1 dependency**

Using the GenePattern software platform developed by the Broad Institute, we ran single-sample Gene Set Enrichment Analysis (ssGSEA) on ovarian mRNA expression to calculate the activity level of each HALLMARK gene set in each ovarian cell line (using the Hallmark collection from the Molecular Signatures Database). We then found gene sets whose ssGSEA values were associated with EGLN1 dependency using linear model analysis as mentioned above.

**Lineage enrichment analysis**

Using a one-sided Fisher exact test, we measured the positive enrichment of EGLN1-dependent lines in a given lineage compared with all other lineages. We defined CRISPR EGLN1 dependencies as cell lines with at least a 50% probability of being dependent, leveraging the CERES probabilities of dependency that were published alongside the CERES effect scores (19). Lineages represented by fewer than 5 lines were filtered out and false discovery rates were calculated to correct for multiple hypothesis testing.

**Cell culture conditions**

All cell lines were provided from stocks curated by the Cancer Dependency Map project at the Broad Institute and originally obtained from the CCLE (www.broadinstitute.org/ccle) unless otherwise indicated. All cell lines were fingerprinted using one of two genotyping platforms, Sequenom or Fluidigm. Mycoplasma testing was performed when cell lines were obtained from CCLE. Cell lines expressing pLX_311-cas9 were generated by the Project Achilles for use in the genome-wide pooled CRISPR screening data used to inform this project. ES2, OVTOKO, TOV112D, HEYA8, OVISE were cultured in RPMI1640 + 10% FBS. OVK18 was cultured in MEM + 10% FBS. COV434 was cultured in DMEM + 10% FBS + 2 mmol/L glutamine. TOV21G was cultured in MCDB105:Medium199 (1:1): 15% FBS. JHOC5 was cultured in DMEM:F12 (1:1): 10% FBS + 0.1 mmol/L NEAA. All media were supplemented with penicillin–streptomycin.

**Vectors and constructs**

All vectors and plasmids used for these experiments, where they were obtained and sequences for all single-guide RNAs (sgRNA) designed are listed in Supplementary Table S1.

**Arrayed lentivirus production**

293T cells were seeded in 6-well plates at 1.5 × 10^6 per well (2-ml volume) 24 hours pretransfection. Transfection was performed using TransIT-LTI Transfection Reagent (Mirus). 8.25 µL of LT1 was diluted in 75 µL of Opti-MEM (Corning) for each well and incubated at room temperature for 5 minutes. The diluted LT1 mixture was added to a mixture of 1,250 ng PsPAX2, 250 ng VSV-G, and 1,250 ng lentivector DNA suspended in Opti-MEM and incubated at room temperature for 20–30 minutes. The transfection mixture was then added to the plate of cells, spun at 1,000 × g for 30 minutes, and incubated at standard cell culture conditions (37°C, 5% CO2) for 6–8 hours. Media were then changed to high serum media (DMEM + 30% FBS) and incubated until harvest after 48–72 hours. Viral supernatants were stored at −80°C until use in the experiments.

**Immunoblots**

Cells were infected with lentiviruses expressing sgRNAs targeting EGLN1 and selected with puromycin at a concentration of 4 µg/mL for 48–72 hours or until all uninfected cells were dead. Whole-cell lysates were prepared using RIPA buffer (Sigma-Aldrich) supplemented with EDTA-free Protease Inhibitor Cocktail (Roche). 1 mmol/L sodium orthovanadate (NEB), and 5 mmol/L sodium fluoride (NEB). Protein levels were quantified using the Pierce BCA assay kit (Thermo Fisher Scientific). Protein lysates were run on 4%–12% Bis-Tris Pre-Cast gels (Thermo Fisher Scientific) and proteins transferred to a polyvinylidene difluoride membrane using the iBlot 2 system (Thermo Fisher Scientific). HIF1α levels were detected using a mouse monoclonal anti-HIF1α antibody at 1:1,000 dilution (BD Biosciences, catalog no. #610958) and a LI-COR-compatible anti-mouse IR secondary antibody (LI-COR, catalog no. #926-68020) at 1:5,000 dilution. EGLN1 levels were detected using a rabbit monoclonal EGLN1 antibody (Cell Signaling Technology, catalog no. #3293) at 1:1,000 and a LI-COR-compatible anti-rabbit IR secondary antibody (#926-32211) at 1:5,000 dilution. VHL levels were detected using a rabbit monoclonal VHL antibody (Cell Signaling Technology, catalog no. #685475) and a LI-COR-compatible anti-rabbit IR secondary antibody. GAPDH levels were detected using a rabbit monoclonal GAPDH antibody (Cell Signaling Technology, 14C10 #2118) at 1:1,000 and the same LI-COR-compatible anti-rabbit IR secondary antibody at 1:5,000 dilution.
EGLN1 and VHL inhibitors

EGLN1 inhibitors IOX2 (catalog no. S2919), roxadustat FG-4592 (catalog no. S1007), Molidustat Bay-85-3934 (catalog no. S8138), FG-2216 (catalog no. S7979), and Daprodustat GSK1278863 (catalog no. S8171) were obtained from Selleck Chemicals (www.selleckchem.com). The VHL inhibitor VH298 (Promega) was obtained from Tocris Bioscience (catalog no. 6156). Compounds were dissolved in DMSO per manufacturer’s instructions to an initial stock concentration of 10 mmol/L or formulated in 10% ethanol. Additional EGLN1 inhibitors IOX2 (catalog no. S2919), roxadustat FG-4592, IOX2, BAY85-3934, VH298 (Promega). The other plate continues until the next time point. Freshly seeded plates were spun at 2,000 rpm for 30 minutes and centrifuged. After mixing the cell population, we added sgRNA delivery vector (xPR_003) and averaged across six biological replicates. Results are representative of two independent experiments.

Luciferase competition assay

Infections to make cell lines expressing open reading frames (ORF) for Firefly luciferase 2P (gateway cloned into pLX_313 from Promega pG4.11[FLuc2P]) and Renilla luciferase (pLX_313) were performed by centrifuging freshly seeded plates containing cells with lentiviral particles and 4 μg/mL polybrene for 2 hours at 2,000 rpm. Cell lines expressing pLX_313-cas9 were infected with pLX_313-FireflyLuc2P and parental cell lines were infected with pLX_313-RenillaLuc. Firefly- and Renilla-expressing cells were mixed 1:1 in wells of a 96-well plate containing 4 μg/mL polybrene. After mixing the cell population, we added sgRNA lentivirus. Both firefly- and Renilla-expressing cells were infected with the sgRNA, but only the firefly expressing cells have Cas9. Freshly seeded plates were spun at 2,000 rpm for 30 minutes and incubated overnight at standard cell culture conditions. The following day, infected cells were selected using 4 μg/mL puromycin for 48 hours. Every 3–4 days following the selection period, the plate is split into two new plates. One plate is read 24 hours later using reagents from a Dual-Glo Luciferase Assay Kit (Promega). The other plate continues until the next time point.

Luciferase signal was quantified using an Envision Plate Reader. Data was expressed as the fold change of the ratio of Firefly luciferase signal to Renilla luciferase signal normalized to the signal ratio postselection from an infection with the empty sgRNA delivery vector (xPR_003) and averaged across six biological replicates. Results are representative of two independent experiments.

CellTiter-Glo viability assays

We performed CellTiter-Glo (CTG) viability assays per the manufacturer’s instructions. Briefly, we seeded 5,000–10,000 cells per well in a 96-well plate then treated with either DMSO or EGLN1 inhibitor in doses indicated. After 3–6 days treatment (retreated at day 4), we added CTG compound and measured viability. Data presented are the average of three technical replicates. Data are representative of three independent experiments.

Proliferation assays

Non-Cas9–expressing cells were plated on a 12-well or 6-well plate in triplicate with varying concentrations of FG-4592. Every 3–4 days cells were trypsinized, counted using Nexcelom Cellometer, and reseeded at equal densities with the same concentrations of FG-4592, IOX2, BAY85-3934, or VH298. Data presented are the average of three technical replicate wells when available, excluding wells where there are no live cells to passage. Data are representative of two independent experiments.

Flow cytometry analysis

Flow cytometry was performed at the Broad Institute Flow Cytometry core using CFSE [S(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester] Cell Labeling Kit (Abcam ab113853) to label dividing cells with CFSE and analyzed on FL-1 Excitation(max) 492 nm, Emission(max) 517 nm. Apoptosis was detected using PE Annexin V Apoptosis Detection Kit 1 (BD Biosciences 559763). Dual labeling Annexin V with 7-Amino-Acridine (7-AAD) allowed quantification of apoptosis. Data are presented as the average of two technical replicates and is representative of three independent experiments. Data were analyzed using FlowJo data analysis.

Hypoxia response element

The hypoxia response element (HRE) was designed on the basis of previous publications (18, 19) and was cloned with a luciferase reporter. The construct was then stably transfected to EGLN1-dependent and independent cell lines. After transfection, cells were treated with EGLN1 inhibitor for up to 6 days and luciferase activity was measured as a readout of HIF1A activity.

Colonies formation assay and dose–response curves

Cells were plated at a low density (250–1,000 cells/well) with varying concentrations of FG-4592 (Selleck) on 24-well plates. Media and drug were refreshed every 3–4 days for 10 days. Cells were fixed on the plate with 10% neutral buffered formalin (Thermo Fisher Scientific) and stained with 0.1% crystal violet in 10% ethanol. After plates were washed and dried, dye was extracted using 10% acetic acid. Absorbance of extracted dye samples at 595 nm was quantified using a Molecular Devices SpectraMax M5 Plate Reader. Data presented are normalized to the DMSO-treated sample. Data are representative of three independent experiments.

Animal models

Female AthymicNu/Nu mice (8–10 weeks old) were used and obtained from Jackson Laboratories and had access to food and water in appropriate housing conditions. All animal procedures performed at the Broad Institute were approved by the Institutional Animal Care and Use Committee and according to institutional regulations.

In vivo transplantation

Cells were infected with lentivirus expression control sgChr2-1 or sgEGLN1-9. After 24 hours of selection, cells were subcutaneously implanted into recipient mice (AthymicNu/Nu, Jackson Laboratories) in 50% Matrigel at a concentration of 250,000 cells (ES2) or 400,000 cells (OVISE). Mice were implanted with sgChr2-1 and sgEGLN1-9 at opposite side flanks to control for any variability between mice. Following the detection of tumors, tumor size was quantified at least once per week using a caliper measuring tumor dimensions. Mice were euthanized at the end of the experiment. Tumors were harvested from the euthanized mice and dissociated in cell culture and further saved for RNA, protein, or frozen cell storage.

RT-PCR

ES2-formed tumors harvested from implanted cells were isolated from total tumor. Total RNA was isolated using QIAGEN miRNeasy Mini Kits. Reverse transcription for RNA samples was performed using Thermo Fisher Superscript III First-Strand Synthesis System (Thermo Fisher Scientific: #18080-051). RT-PCR was performed on the QuantStudio 6 Flex (Applied Biosystems) using SYBR Green Master Mix available from Thermo Fisher.
Identification of EGLN1 as a preferential cancer cell dependency. A, Identification of EGLN1 dependency in RNAi data from Project Achilles. From the initial approximately 17k genes tested, we found 762 were strong (Six Sigma) dependencies using DEMETER scores. From these dependencies, we found 153 were currently druggable, while 15 of them had compounds in clinical trials. We identified EGLN1 as one of these 15 clinically druggable dependencies. B, Identification of cancer cells dependent on EGLN1 using CRISPR-Cas9 data from Project Achilles. Histogram shows the distribution of EGLN1 CERES dependencies (x-axis) across 456 cancer cell lines screened with CRISPR. The left tail shows that a subset of lines are preferentially dependent on EGLN1. C, Concordance between RNAi and CRISPR-Cas9 datasets. EGLN1 DEMETER2 scores are graphed against EGLN1 CERES scores (CRISPR, x-axis) for the 243 cell lines screened in both datasets. The correlation between the datasets was strong and highly significant. Pearson $r = 0.512$; $n = 243$; $P < 10^{-15}$. D, Volcano plot showing cancer dependencies associated with EGLN1 dependency graphed as $P$ value ($-\log_{10}$, y-axis) against effect size (x-axis). Red, other members of the EGLN1 pathway. E, EGLN1 and VHL are the strongest correlated dependencies within the EGLN1 pathway while EGLN1 and HIF1AN are the second strongest correlated dependencies. $P$ values were adjusted using the Benjamini and Hochberg FDR method. FDR $< 0.05$ (*), $0.01$ (**), $0.001$ (***). F, Cell lines that express low levels of HIF1A (y-axis) are not dependent on EGLN1 (x-axis).
Our earlier analysis of the data derived from screening 501 human Cas9 technologies in hundreds of human cancer cell lines (2, 3, 5) to identify genes that have similar function (2, 11–13). We found that among the codependencies derived from the Achilles datasets to identify cancer dependencies. To identify such genes, we have performed manner, in contrast to pan-essential genes, represent potential cancer dependencies. To identify such genes, we have performed identification of EGLN1 as a druggable preferential dependency (21, 22). Indeed, the concordance between EGLN1 dependency in cell lines screened by CRISPR and RNAi was highly significant (Fig. 1C; Pearson correlation 0.512; P < 10−17).

Because EGLN1 is one of three family members, we queried whether the other family members, EGLN2 and EGLN3, were also dependencies in any of the cell lines. We found that among the EGLN1 family members, EGLN1 was the strongest preferential dependency in both CRISPR and RNAi datasets (Supplementary Fig. S1A–1C). Furthermore, we found that there were few cell lines dependent on EGLN1 that were also dependent on EGLN2 or EGLN3.

We and others have previously shown that we could use codependencies derived from the Achilles datasets to identify genes that have similar function (2, 11–13, 24, 25). Here, we applied this method to gain insight into the molecular basis of EGLN1 dependency. Specifically, we built linear models to identify codependency relationships between EGLN1 and every other gene. We found that VHL was the strongest and most significantly associated dependency in the CRISPR-Cas9 screens, while HIF1AN were among the top hits in both CRISPR-Cas9 and RNAi and HIF1A was one of the strongest negatively associated hits (Fig. 1D; Supplementary Fig. S1D). These observations suggest that EGLN1 dependency is related to its canonical function in the HIF pathway. To further investigate this association with members of the HIF pathway, we calculated the correlations between dependency profiles of every pair of genes in the pathway (VHL, HIF1AN, HIF1A, HIF2A, EGLN1, EGLN2, and EGLN3). We found the strongest correlation within the pathway existed between EGLN1 dependency and VHL dependency followed by EGLN1 dependency and HIF1AN (hypoaxia inducible factor 1 alpha subunit inhibitor) dependency in CRISPR datasets (Fig. 1E).

To understand why some cell lines are more dependent on EGLN1 than others, we next searched for genomic features, including gene expression, copy number alterations, and mutations that related to EGLN1 dependency estimated from CRISPR and RNAi data (Supplementary Fig. S1E–S1I; Supplementary Tables S3 and S4). We found that higher levels of HIF1A expression were among the most significantly associated gene expression features. In addition, in the reverse analysis, EGLN1 dependency was the top correlated dependency with HIF1A expression (Supplementary Fig. S1M). In particular, we found that cell lines that express lower levels of HIF1A were not dependent on EGLN1, suggesting that high levels of expression might be required for a dependency (Fig. 1F). Finally, when we interrogated which gene sets correlated with EGLN1 dependency, we identified hypoaxia-related gene sets among the top correlated gene sets.
EGLN1 dependency is enriched in clear cell ovarian cancer and melanoma and associated with high HIF1A levels. A, Ovarian and melanoma cancer cell lines are enriched for EGLN1 dependencies in CRISPR screens. The percentage of EGLN1-dependent lines is graphed for each lineage. Red, significantly enriched lineages found by lineage enrichment analysis. Note that lung lines are also significant, but are negatively enriched for EGLN1 dependencies. B, Distribution of EGLN1 CERES scores (y-axis) in ovarian cancer cell lines. Thirteen of 33 cell lines screened in CRISPR cell lines have a greater than 50% probability of being dependent on EGLN1. CAOV3 is not identified as an EGLN1-dependency, despite having a lower CERES effect score than EGLN1-dependent line EFO27, because the screen quality and other cell line-specific differences are reflected in the CERES probabilities of dependency. C, CRISPR EGLN1 dependencies are enriched in clear cell ovarian cancer. A two-sample t-test of clear cells versus all other ovarian subtypes revealed that clear cells are significantly enriched for EGLN1 dependencies (P < 0.05). D, EGLN1-dependent cell lines (n = 13) express significantly more HIF1A (y-axis) than non-EGLN1-dependent lines (n = 20) in CRISPR. Two-sample t test, P < 0.01. E, Clear cell ovarian cancer cell lines have high HIF1A expression compared with all other ovarian lines. Two-sample t test, P < 0.05. F, ssGSEA (single sample Gene Set Enrichment Analysis) revealed that EGLN1 dependency is associated with HIF1A-related pathways. Pearson correlations of EGLN1 dependency with each profile (y-axis) were calculated, and z-score-normalized profiles are shown.
Taken together, all these observations are consistent with the hypothesis that EGLN1 regulation of HIF1A is the downstream event responsible for the dependency.

EGLN1 dependency is enriched in ovarian clear cell carcinoma

To investigate whether EGLN1 dependency was enriched in specific cancer types, we performed a lineage enrichment analysis using Fisher exact test. We found that melanoma and
Figure 4.
Inhibiting EGLN1 increases HIF1A expression and reduces proliferation in EGLN1-dependent cells. A, Immunoblot showing that pharmaceutical inhibition (FG-4592) of EGLN1 increases HIF1A levels in a dose-dependent manner. B, EGLN1 inhibitor increases HIF1A activity measured by using a HRE fused to a luciferase reporter. Luciferase activity was measured 48 hours posttreatment with increasing dose of FG-4592. Data are representative of two independent experiments. C, EGLN1 inhibitor reduces viability selectively in EGLN1-dependent cells. Cells were treated for 48 hours with increasing dose of FG-4592 (x-axis). Cell viability (y-axis) was measured using CellTiter-Glo. The data shown are representative of three independent experiments. D, EGLN1 inhibitor reduces long-term proliferation in EGLN1-dependent cells. Results are representative of three independent experiments and data are graphed as mean ± SD of three replicates. E, EGLN1 inhibitor does not affect long-term proliferation of EGLN1-insensitive cells. Results are representative of three independent experiments and data are graphed as mean ± SD of three replicates. F, EGLN1 inhibition reduces colony formation in a dose-dependent manner in EGLN1-dependent cells. Colonies were quantified as absorbance (y-axis) over drug concentration (x-axis) over 10 days of treatment normalized to DMSO. Results are representative of three independent experiments. G, EGLN1 inhibition has no significant effect on colony formation in EGLN1-insensitive cells. Colonies were quantified as absorbance (y-axis) over drug concentration (x-axis) over 10 days of treatment normalized to DMSO. Results are representative of three independent experiments. H, VHL inhibition reduces proliferation of EGLN1-dependent cells. Results are representative of three independent experiments, and data are graphed as mean ± SD of three replicates. *P < 0.05. I, VHL inhibition does not affect proliferation of EGLN1-insensitive cells. Results are representative of two independent experiments, and data are graphed as mean ± SD of biological triplicates.
ovarian cell lines were significantly enriched for EGLN1 dependencies in the CRISPR-Cas9 dataset (Supplementary Fig. S2A, FDR < 0.1). Specifically, among the ovarian cancer cell lines, we found that 13 of 33 (39%) ovarian cancer cell lines were dependent on EGLN1 (Fig. 2A and B). Similarly, we found these cell lines were also dependent on EGLN1 in RNAi dataset and cell lines in both datasets show strongly correlated EGLN1 dependencies (Supplementary Fig. S2C). Ovarian cancer represents a heterogeneous disease comprised of four major different subtypes (26–36). To determine whether EGLN1 dependency was enriched in a specific subtype, we grouped the ovarian cancer cell lines by historical pathologic, molecular, and histologic subtypes (Supplementary Table S5; adapted from refs. 31, 34, 36). We found that EGLN1 dependency was significantly enriched in ovarian clear cell carcinoma lines compared with every other ovarian subtype (Fig. 2C, P < 0.05; Supplementary Fig. S2D, P < 0.05).

We next evaluated whether the association of EGLN1 dependency with high expression levels of HIF1A remained significant in ovarian cell lines. We found that EGLN1-dependent ovarian cancer cells express HIF1A at significantly higher levels than non-EGLN1–dependent cells in the CRISPR dataset (Fig. 2D, P < 0.005). We found a similar relationship between EGLN1 dependency scores and HIF1A expression in RNAi dataset (Supplementary Fig. S2E). In particular, when subdivided by histologic subtypes, we found that ovarian clear cell lines showed the highest group expression of HIF1A in CRISPR (Fig. 2E, P < 0.05) and RNAi datasets (Supplementary Fig. S2F). These results are consistent with previous observations that HIF1A is overexpressed/activated in clear cell compared with other ovarian cancer subtypes (37, 38).

Higher levels of HIF1A and a hypoxia gene set were correlated with EGLN1 dependency within the ovarian cell lines after performing ssGSEA (Fig. 2F; Supplementary Fig. S2F), indicating that in ovarian cell lines EGLN1 dependency is also related to its known function in the HIF1A pathway.

Validation of EGLN1 dependency

To validate and characterize the observed EGLN1 dependency, we designed a series of sgRNAs to identify sgRNAs that effectively delete EGLN1. We selected an EGLN1-dependent cancer cell line, ES2 (Fig. 3A), and an EGLN1-independent cell line, TOV112D (Supplementary Fig. S3A), from the panel of cell lines screened in Project Achilles. We chose three sgRNAs (EGLN1-1, EGLN1-8, and EGLN1-9) for subsequent EGLN1 knockout experiments. We found that deleting EGLN1 led to an increase in HIF1A and subsequently phosphorylated HIF1A (39, 40). We concluded that these selected EGLN1 sgRNAs effectively targeted EGLN1.

To assess the consequences of EGLN1 inhibition, we measured cell proliferation. We found that expression of the negative control sgChr2-1 (sgRNA designed to target an intergenic region on Chromosome 2) did not affect cell proliferation. In contrast, depletion of EGLN1 using sgRNA EGLN1-9 led to a significant (P < 0.05) decrease in cell proliferation in the EGLN1-dependent cell line ES2 (Fig. 3B), but did not affect proliferation of the EGLN1-independent cell line, TOV112D (Fig. 3C).

We hypothesized that EGLN1-dependent cells would also be dependent on VHL. To test this, we generated VHL-knockout cells using VHL-specific sgRNAs and confirmed loss of VHL by immunoblotting (Supplementary Fig. S3B). We found that VHL-deficient cells showed a significant (P < 0.05) decrease in population doublings (Fig. 3D).

To assess the effects of depleting EGLN1 in a longer term assay, we performed a competition experiment in which we assessed whether cells with EGLN1 KO would be depleted over time when directly compared with cells expressing a control sgRNA (Fig. 3E). Specifically, we introduced different sgRNAs in Renilla or Firefly–Cas9–expressing cells then determined the consequences of expressing each sgRNA by assessing Firefly/Renilla ratio over time. We performed the competition assay in three EGLN1-dependent cell lines (ES2, OVTOKO, and OVISE) and two cell lines not dependent on EGLN1 (TOV112D and HEY8). As a positive control for depletion of the cell population, we used sgRNAs targeting the RNA polymerase II subunit D (sgPOLR2D) and RNA polymerase I subunit C (sgPOLR1C). As negative controls, we used sgChr2-1 and sgLaCZ. We found in ES2, OVTOKO, and OVISE (EGLN1-dependent) that cells expressing an EGLN1-targeting sgRNA were depleted over time at a rate similar to cells expressing sgRNAs targeting POIR2D or POLR1C (Fig. 3E, top). Furthermore, when we performed this same experiment in EGLN1-independent cell lines, we found no difference between cells expressing the negative control sgRNA or sgRNA targeting EGLN1 (Fig. 3E, bottom). These observations confirm that depletion of EGLN1 leads to decreased cell proliferation in a subset of ovarian cancer cell lines.

Pharmacologic inhibition of EGLN1 or VHL inhibits proliferation in a subset of cell lines

EGLN1 is a prolyl hydroxylase, and several PHD inhibitors have been described (41, 42), which prevent the degradation of HIF1A. Specifically, compound FG-4592, also known as Roxadustat, is currently being evaluated for the treatment of anemia in dialysis-dependent chronic kidney disease (CKD) and nondialysis-dependent CKD (42–45). We found that exposure of both ES2 and TOV112D to FG-4592 and the Bayer compound Molidustat (Bay 85–3934) led to increased HIF1A levels and phosphorylated HIF1A.
HIF1A in a concentration-dependent manner (Fig. 4A; Supplementary Fig. S4A–S4C). This accumulation was observed at 5–10 μmol/L and peaked at 20–40 μmol/L, consistent with the reported effective concentrations of both compounds (43–47). These concentrations (5–40 μmol/L) were used for all subsequent experiments to test the effect of pharmacologic inhibition of EGLN1 in EGLN1-dependent cells. In addition, we also tested HIF1A activity using a HRE fused to a luciferase reporter (Fig. 4B; ref. 48). Following treatment with FG-4592, we found that HIF1A activity was increased in both EGLN1-dependent cell lines and cell lines that are not EGLN1-dependent. However, when we compared HIF1A activity between both cell lines, we found that the EGLN1-dependent cell line exhibited higher HIF1A activity (Fig. 4B). This finding suggests that pharmacologic inhibition of EGLN1 in EGLN1-dependent cell line induces higher activity of HIF1A than the cell line not dependent on EGLN1. We also found that FG-4592 reduced viability in ES2 over 48 hours while no effect on TOV112D via CellTiter-Glo assay in a dose-dependent manner (Fig. 4C). To confirm these observations, we tested several other EGLN1 inhibitors FG-2216 (Fibrogen) and Daprodustat (GSK1278863 GlaxoSmithKline). We found that the EGLN1-dependent cell line ES2 showed sensitivity to all of these EGLN1 inhibitors (Supplementary Fig. S4D). These observations confirm that inhibition of EGLN1 activity recapitulates the effects observed by deleting EGLN1.

To investigate whether the observed decrease in cell number was due to apoptosis or cell-cycle arrest, we treated cells for 3 days with FG-4592 and then labeled them with proliferation agent CFSE (Invitrogen) and analyzed proliferation by flow cytometry. We found that higher doses of FG-4592 reduced proliferation of ES2, but did not have a similar effect on TOV112D (Supplementary Fig. S4E). Furthermore, we found a slight increase in apoptosis following three days of EGLN1 inhibition suggesting an initial cytotoxic effect (Supplementary Fig. S4F). Thus, we found that short-term pharmacologic inhibition of EGLN1 results in decreased proliferation. To evaluate the effect of FG-4592 on long-term proliferation, we carried out treatment of sensitive and resistant cells for up to 30 days. We found treatment with FG-4592 significantly reduced long-term proliferation in sensitive cell lines (Fig. 4D; Supplementary Fig. S4G and S4H, P < 0.01), but not insensitive cell lines (Fig. 4E). Together, these observations show that EGLN1 inhibition reduces cell proliferation.

To further understand EGLN1 dependency, we tested the effect of EGLN1 inhibition in a low-cell–density colony formation assay. We found that EGLN1-dependent cell lines were sensitive to EGLN1 inhibition (Fig. 4F; Supplementary Fig. S4I) in a dose-dependent measurement. When we focused on cell lines that do not exhibit dependence on EGLN1, we found that increasing concentrations of FG-4592 did not have the same effect (Fig. 4G; Supplementary Fig. S4J). Together, these observations suggest that these cell lines are dependent on EGLN1 for colony-forming cell viability assays.

We hypothesized that EGLN1 sensitivity to small-molecule inhibition would persist in the context of hypoxia. To test this hypothesis, we performed colony-forming assays in the context of normal culture conditions (normoxia) or in a hypoxic incubator at 5% oxygen (hypoxia). We found that EGLN1-dependent cell lines were sensitive to EGLN1 small-molecule inhibitor FG-4592 in a dose-dependent manner in hypoxia and normoxia (Supplementary Fig. S4J). We did not observe an increased sensitivity to EGLN1 inhibition in 5% oxygen with the EGLN1-insensitive cell line TOV112D, suggesting that EGLN1 dependency persists under reduced oxygen concentrations in vitro.

Several inhibitors developed for clinical trials have the ability to target EGLN2 and EGLN3. To confirm that the effect that we observed was related to EGLN1 inhibition, we used the more selective EGLN1 inhibitor IOX2. We found in a short-term viability assay that IOX2-mediated inhibition of EGLN1 resembled FG-4592 inhibition of EGLN1 (Supplementary Fig. S5A and S5B). Furthermore, when we looked at colony growth, we found EGLN1-dependent cell lines exhibited reduced dose-dependent colony growth compared with cell lines that were not dependent on EGLN1 (Supplementary Fig. S5C). These findings suggest that the effects observed with FG-4592 and other EGLN clinical inhibitors are predominantly through inhibition of EGLN1, not EGLN2 or EGLN3.

We also hypothesized that pharmacologic inhibition of VHL should mimic pharmacologic inhibition of EGLN1. We used VH298 (Tocris), a novel chemical probe that is reported to block the interaction of VHL and HIF1A downstream of hydroxylation of HIF1A by EGLN1 (42), leading to HIF1A stabilization (Supplementary Fig. S5G) and activation of HIF target genes. We found that treatment with VH298 substantially decreased cell proliferation in ES2 (Fig. 4H) and slightly decreased proliferation in TOV112D (Fig. 4I). We observed this effect in several other EGLN1-dependent cell lines (Supplementary Fig. S5H and S5I). These findings suggested that EGLN1-dependent cells were more sensitive to VHL inhibition than EGLN1-independent cells.

Deletion of HIF1A rescues EGLN1 and VHL dependency

On the basis of our observation that EGLN1 dependency was associated with higher expression of HIF1A, we hypothesized that deleting EGLN1 would affect HIF1A targets and pathways. We therefore performed RNA sequencing in the EGLN1-dependent cell lines ES2 and OVISE with and without EGLN1. We found that among the genes that were differentially expressed (Supplementary Table S6; Supplementary Fig. S6A), several genes act downstream of the EGLN1–HIF1A pathway. We next performed GSEA to identify significant changes in the EGLN1 knockout population. Within the top scoring pathways, we found enrichment in hypoxia and HIF1A-related pathways (Fig. 5A; Supplementary Fig. S6B). Together, these observations suggest that loss of EGLN1 strongly affects HIF1A-related pathways.

As HIF1A pathways were significantly affected by EGLN1 inhibition, we hypothesized that knockout of HIF1A would rescue EGLN1 dependency. We designed several sgRNAs targeting HIF1A and selected three that efficiently depleted HIF1A in EGLN1-sensitive cell line ES2 (Fig. 5B). We found that deletion of HIF1A failed to affect the proliferation of EGLN1-insensitive cell lines. However, we found that deletion of HIF1A rescued EGLN1 sensitivity in ES2 (Fig. 5C) and OVISE (Fig. 5D) to FG-4592 (P < 0.05). These observations suggest that the observed dependence of EGLN1 requires HIF1A.

Because HIF1A knockout rescued EGLN1 dependency, we hypothesized that HIF1A knockout may rescue VHL dependency. When compared with DMSO-treated control, we found that
knockout of HIF1A rescued cell proliferation blocked by VHL inhibitor VH298 (Fig. 5E, P < 0.05), suggesting that VHL dependency functioned through the regulation of HIF1A.

Genetic and pharmacologic inhibition of EGLN1 reduces tumor growth

We then tested whether depletion of EGLN1 affected tumor growth in vivo. Specifically, we used cells expressing sgRNAs against EGLN1 or Chr2-1 (negative control) and subcutaneously implanted these into recipient mice (Fig. 6A). We implanted control and knockout cells into opposite flanks of the same mouse to control for any tumor variation between mice. We found that EGLN1 knockout significantly reduced tumor size over time in ES2 (Fig. 6B). Furthermore, we found that OVISE tumor cells with deleted EGLN1 grew slower than OVISE tumors in which EGLN1 was expressed (Fig. 6C). When we looked at the RNA expression of a subset of the tumors that had grown in ES2 cells with inhibited EGLN1, we found a correlation with EGLN1 expression and tumor size (Fig. 6D), suggesting that inactivation of the EGLN1 gene significantly inhibits tumor growth.

To determine whether pharmacologic inhibition of EGLN1 also inhibited tumor growth, we used an implantable microdevice to directly deliver multiple compounds to different parts of the tumor (23). By inserting this microdevice directly into the tumor, we assessed the effect of FG-4592, and VH298 in tumors in vivo. Specifically, we implanted ES2 cells with intact HIF1A (ES2) or HIF1A knockout (ES2 HIF1A KO) in immunodeficient mice and allowed the tumors to grow to 1 cm² before implanting a microdevice containing the EGLN1 inhibitor FG-4592 and VHL inhibitor VH298. Following implantation, we collected the tumors after 48 hours and sectioned and stained the tumors for proliferation with an antibody against Ki-67, a cellular marker for proliferating cells, or cleaved caspase-3, a cellular marker for apoptosis. As expected, local delivery of PEG (polyethylene glycol, the vehicle used for drug formulation) had no effect on cell proliferation or apoptosis, demonstrated by clear

Figure 6.
EGLN1 deletion or inhibition reduces tumor formation induced by EGLN1-dependent cells. A, Schematic of tumor formation experiments. Each recipient mouse receives control (sgChr2-1) and EGLN1-KO cells. B, EGLN1 knockout impairs tumor formation induced by ES2 cells. Control and EGLN1 KO ES2 cells (100,000) were injected subcutaneously in parallel flanks and tumor size was measured. Data are represented as mean ± SD (n = 15) and represent two experimental repeats. *, P < 0.01. C, EGLN1 knockout impairs tumor formation induced by OVISE cells. Control and EGLN1 KO OVISE cells (250,000) were injected subcutaneously in parallel flanks and tumor size was measured. Data are represented as mean ± SD (n = 6). *, P < 0.05. D, EGLN1 expression levels in the knockout cells correlate with tumor size. RNA was harvested from tumors and expression was quantified using qRT-PCR. Data are represented as the average of four biological replicates and represent two experimental repeats. E, Microdevice delivery of EGLN1 inhibitors and VHL inhibitors to ovarian tumor with HIF1A expression reduces proliferation. Microdevices were loaded with PEG-formulated compounds and implanted into 1-cm² tumors formed from ES2 or from ES2 with HIF1A KO and grown to 1 cm². At 48 hours postmicrodevice implantation, tumors were harvested and serially sectioned and stained. The control for drug formulation (PEG control) has no effect on the proliferation of ES2 tumor cells with or without HIF1A (top). Doxorubicin treatment reduces tumor cell proliferation (top middle). Inhibition of EGLN1 with FG-4592 blocks proliferation of tumor cells, while knockout of HIF1A rescues EGLN1 inhibition (bottom middle). Inhibition of VHL with VH298 blocks proliferation of tumor cells while knockout of HIF1A rescues VHL inhibition. Figure is representative of three independent experiments. Arrow, area where drug is released. Black line, 1 mmol/L.
immunostaining of Ki-67 (Fig. 6E; Supplementary Fig. S7, top) in both HIF1A WT or HIF1A KO tumors. We found that addition of doxorubicin reduced proliferation and increases apoptosis in both HIF1A WT and HIF1A KO cells (Fig. 6E; Supplementary Fig. S7, top middle). Consistent with our previous in vitro data (Fig. 4), we found that pharmacologic inhibition of EGLN1 causes a dramatic reduction in proliferating cells and increased apoptosis in the region of tumor exposed to the local microdevice delivery of FG-4592 (Fig. 6E; Supplementary Fig. S7, bottom middle), while knockout of HIF1A exhibited no change in proliferation or apoptosis upon local delivery of FG-4592. Similarly, we found addition of VH298 resulted in the reduction of proliferating cells and increased apoptosis, which was also dependent on intact HIF1A (Fig. 6E; Supplementary Fig. S7, bottom). These data collectively demonstrate that genetic and pharmacologic perturbation of EGLN1 reduces tumor burden, cell proliferation, and increase apoptosis in vivo and that this effect requires HIF1A.

**Discussion**

Under normal conditions, EGLN1 functions to hydroxylate HIF1A and target it for ubiquitination and subsequent degradation by VHL (Fig. 7A). Here we show that in a subset of clear cell ovarian cancer and melanomas, genetic knockout or pharmacologic inhibition of EGLN1 stabilizes HIF1A and inhibits proliferation (Fig. 7B). Similarly, genetic knockout or pharmacologic inhibition of VHL prevents HIF1A ubiquitination and degradation that results in HIF1A stabilization and, subsequently, a decrease in proliferation (Fig. 7C). Deletion of HIF1A rescues both EGLN1 dependency and VHL dependency (Fig. 7D). Pharmacologic inhibition of EGLN1 recapitulated the effects of genetic suppression of EGLN1, both in vitro and in vivo, suggesting that EGLN1 inhibition is a potential therapeutic strategy in these tumors.

As expected, genetic deletion of EGLN1 led to HIF1A accumulation (Fig. 3A), which in turn led to a decrease in proliferation (Fig. 3B) and reduced fitness in our competition assay compared with EGLN1 functional cells (Fig. 3E). After pharmacological inhibition of EGLN1, we observed similar accumulation of HIF1A and increased HIF1A activity (Fig. 4A, B). While EGLN1 has been described both as an oncogene and a tumor suppressor (39, 45, 49–53), a specific dependency of a subset of cancers on EGLN1 has not been reported. Jokilehto and colleagues (39) found that EGLN1 expression was strongly associated with highly proliferative head and neck squamous cell carcinomas (HNSCC). Furthermore, Klotzsche-von Ameln and colleagues found EGLN1 inhibition led to a decrease in tumor growth using mouse lung cancer cell line LLC and osteosarcoma line LM8 (51). Conversely, Chan and colleagues found that low PHD2 expression in colorectal cancers correlated with poorer survival, and experiments in cell line models showed that low PHD2 expression correlated with increased tumor vasculature (52). Bordoli and colleagues observed decreased EGLN1 was associated with increased tumor growth through increased VEGF, Amphiregulin, and IL8 (53).

Thus, EGLN1 is another of a growing number of genes that can act both as an oncogene and tumor suppressor gene depending on the context (54).

Our findings suggest that increased HIF1A decreases the fitness of EGLN1-dependent cells. Specifically, we found that inactivation of EGLN1, using either genetic or pharmacologic means, resulted in a pronounced decrease in proliferation. We observed that this decrease in proliferation coincided with stabilization of HIF1A. Thus, we hypothesized that HIF1A loss could rescue EGLN1-mediated death. Indeed, we found that...
EGLN1-dependent cell lines were rendered insensitive to EGLN1 inhibition by loss of HIF1A (Fig. 5C and D). Together, these observations demonstrate that EGLN1 dependency requires HIF1A expression.

We found a large number of cell lines dependent on VHL (Supplementary Fig. S1A). We also found that cell lines dependent on EGLN1 were also dependent on VHL (Figs. 3D and 4H; Supplementary Fig. S5H and S5I). While all the EGLN1-dependent cell lines were also dependent on VHL in the CRISPR dataset (Fig. 1D and E), we were surprised to observe VHL scored as an essential gene (Supplementary Fig. S1A). As VHL is downstream of EGLN1 and directly responsible for the ubiquitination and degradation of HIF1A (16, 17, 40–42), one possible hypothesis for this observation is that loss of VHL can drive stabilization and accumulation of HIF1A in cell lines. We found that dependency on VHL in EGLN1-dependent cell lines was dependent on functional HIF1A. Similar to our previous observations with EGLN1 dependency, knockout of HIF1A rescues VHL dependency (Fig. 5E). This finding suggests VHL also functions as a cancer dependency in EGLN1-dependent cells through negative regulation of HIF1A.

Previous reports found that overexpression of HIF1A has been associated with multiple tumor types, and tumors in hypoxia are known to require HIF1A to survive (14–17). However, other reports have also implicated HIF1A as a negative regulator of proliferation (49–51). There are a several hypotheses that could explain these observations. First, the observed effects of activating HIF1A may be tumor type–specific. In our initial analysis, we found significant enrichment in of EGLN1 dependency in melanoma and clear cell ovarian cancer. Indeed, these clear cell ovarian cancer cell lines died when HIF1A expression was stabilized.

Alternatively, it was possible that the observed difference in sensitivity involved whether the cells experienced hypoxia. However, we found under conditions of 5% oxygen that EGLN1-dependent cells were still susceptible to EGLN1 inhibition. Furthermore, when we examined in vivo growth of tumors under presumably hypoxic conditions, we found that genetic knockout and small-molecule inhibition of EGLN1 reduced tumor growth and suppressed proliferation. These findings suggest that cell line dependency on EGLN1 is independent of hypoxia.

The standard therapeutic procedure to clear cell ovarian cancer remains surgical cytoreduction and subsequent chemotherapy using platinum agents and taxane (55). Clear cell ovarian cancers, compared with other histologic ovarian subtypes, are resistant to chemotherapy (55). Thus, targeted therapy has been proposed, and the need for potential targets and therapy is pressing. EGLN1 inhibitors are in clinical trials for anemia in patients with CKD (43–45). Our observations suggest that these inhibitors may also be useful in EGLN1-dependent cancers. Our data suggest that cancers with low HIF1A expression would not respond to EGLN1 therapy, while a subset of patients with high HIF1A expression would respond. This association was stronger in melanoma and extremely strong in clear cell ovarian cancer cells. Previous work has shown that patients with clear cell ovarian cancer have higher expression of HIF1A (37, 38). Thus, these patients would be the ideal patient population to assess the efficacy of EGLN1-targeted therapy in cancers.

Disclosure of Potential Conflicts of Interest
A. Tsherniak is a consultant/advisory board member for Tango Therapeutics. O. Jonas reports receiving a commercial research grant from Novartis and is a consultant/advisory board member for Kibbr Medical. W.C. Hahn reports receiving a commercial research grant from Deerfield, has ownership interest (including stock, patents, etc.) in KQ5 Therapeutics, is a consultant/advisory board member for Thermo Fisher Scientific, KQ5 Therapeutics, Ajbil, MPM, and Parexel. No potential conflicts of interest were disclosed by the other authors.

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