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Construction and Validation of U251-Cas9-IDH1 Wild-Type and R132H Mutant Stable Cell Lines

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Abstract
Background: *Mutations in isocitrate dehydrogenase 1 (IDH1), particularly the R132H variant, are defining molecular features of lower-grade gliomas and secondary glioblastomas. While IDH1 mutations confer a relatively favorable prognosis, they also create unique metabolic and epigenetic vulnerabilities that may be exploited therapeutically through synthetic lethal strategies.*
Objective: *To establish stable isogenic glioma cell models expressing wild-type IDH1 or the IDH1-R132H mutant and to perform genome-wide CRISPR/Cas9 knockout screening for the identification of synthetic lethal targets in IDH1-mutant glioma.*
Methods: *U251 glioblastoma cells were sequentially transduced with lentiviruses encoding Cas9 and either IDH1-wt or IDH1-R132H-mut. Stable cell lines were generated following antibiotic selection and monoclonal isolation, and protein expression was validated by Western blotting. Cell proliferation and clonogenic capacity were assessed using growth curve and colony formation assays. Genome-wide CRISPR/Cas9 knockout screening was performed using the human GeCKO v2 lentiviral library. Cells were harvested at baseline (T0) and after 15 days of culture (T15), and sgRNA abundance was quantified by high-throughput sequencing. Candidate dependency genes were identified by integrating screening results with DepMap database analysis.*
Results: *U251-Cas9-IDH1-wt and U251-Cas9-IDH1-mut stable cell lines were successfully established, as confirmed by Western blot detection of Cas9 (≈160 kDa), Flag-tagged exogenous IDH1, and the R132H mutation-specific band exclusively in mutant cells. IDH1-mut cells exhibited significantly reduced proliferation and colony formation ability compared with IDH1-wt cells (*p*< 0.01). Genome-wide CRISPR/Cas9 screening, combined with DepMap dependency analysis across 332 high-2-HG and 314 low-2-HG cell lines, identified selenophosphate synthetase 2 (SEPHS2) as a top candidate gene selectively essential for the survival of IDH1-mutant cells.*
Conclusion: *We have successfully constructed a robust isogenic cell model system suitable for CRISPR/Cas9-based functional genomics screening in the context of IDH1 mutation. Through unbiased genome-wide screening, SEPHS2 was identified as a promising synthetic lethal candidate warranting further mechanistic and therapeutic investigation in IDH1-mutant glioma.*
Keywords: Glioma; IDH1 mutation; CRISPR/Cas9; Synthetic lethality; Stable cell model

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I. Introduction

Glioblastoma (GBM) is the most common and aggressive primary malignant brain tumor in adults, accounting for approximately 50% of all malignant central nervous system neoplasms [1]. Despite advances in surgical resection, radiotherapy, and temozolomide chemotherapy, the median overall survival for GBM patients remains less than two years [2, 3]. The infiltrative nature and intrinsic therapeutic resistance of GBM underscore the urgent need for novel targeted therapeutic strategies.

A landmark discovery in the molecular characterization of gliomas was the identification of recurrent mutations in the isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* genes [4]. IDH1 mutations are present in the vast majority of WHO grade II and III gliomas and secondary GBM, with the *IDH1*-R132H substitution accounting for approximately 90% of all IDH1 mutations [5, 6]. Wild-type IDH1 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) within the tricarboxylic acid (TCA) cycle. In contrast, mutant IDH1 acquires a neomorphic enzymatic activity that reduces α-KG to the oncometabolite D-2-hydroxyglutarate (D-2-HG) [7]. The accumulation of D-2-HG competitively inhibits a broad spectrum of α-KG-dependent dioxygenases, leading to DNA and histone hypermethylation (the glioma CpG island methylator phenotype, G-CIMP), impaired cellular differentiation, and disrupted redox homeostasis [8, 9].

Paradoxically, while IDH1 mutations drive gliomagenesis, patients with IDH1-mutant gliomas exhibit a

significantly more favorable clinical outcome compared to those with IDH1-wildtype tumors [10]. This prognostic advantage has been attributed, at least in part, to distinct metabolic and epigenetic vulnerabilities conferred by the mutation. Consequently, identifying "synthetic lethal" partners that selectively target these vulnerabilities has emerged as a promising therapeutic avenue [11].

Synthetic lethality refers to a genetic interaction in which the concurrent perturbation of two genes results in cell death, whereas the alteration of either gene alone is compatible with viability [12]. This principle offers an elegant strategy for selectively eradicating cancer cells harboring specific driver mutations while sparing normal tissues. The successful clinical translation of this concept is exemplified by the use of poly(ADP-ribose) polymerase (PARP) inhibitors in *BRCA1/2*-mutant ovarian and breast cancers [13].

The advent of CRISPR/Cas9 technology has revolutionized functional genomics, enabling systematic and unbiased interrogation of gene function on a genome-wide scale [14, 15]. Pooled lentiviral CRISPR knockout screens provide a powerful platform for identifying context-specific essential genes in a high-throughput manner [16]. By comparing gene essentiality profiles between isogenic cell line pairs differing only by a specific oncogenic mutation, synthetic lethal candidates can be robustly nominated for subsequent validation [17].

In this study, we first established a pair of isogenic U251 glioblastoma cell lines stably expressing Cas9 along with either wild-type IDH1 or the IDH1-R132H mutant. These models were rigorously characterized for proliferation, clonogenic potential, and protein expression. Subsequently, we performed a genome-wide CRISPR/Cas9 knockout screen to systematically interrogate gene dependencies selectively associated with the IDH1-mutant state. Through integration of our screening data with publicly available cancer dependency maps, we identified selenophosphate synthetase 2 (SEPHS2) as a prominent synthetic lethal candidate. This work lays a critical foundation for the functional validation and mechanistic exploration of SEPHS2 as a potential therapeutic target in IDH1-mutant glioma.

II. Materials and Methods

2.1 Cell Culture

The human glioblastoma cell line U251 and human embryonic kidney 293T17 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; ExCell Bio) and 1% penicillin-streptomycin (BI) at 37°C in a humidified incubator with 5% CO_2.

2.2 Lentivirus Production and Stable Cell Line Generation

Lentiviruses were produced by co-transfecting 293T17 cells with the transfer plasmid (LentiCas9-Blast, pLV3-CMV-IDH1(human)-3×FLAG-Hyg, or pLV3-CMV-IDH1(human)-R132H-3×FLAG-Hyg), packaging plasmid psPAX2, and envelope plasmid pMD2.G using Lipofectamine 2000 (Thermo Fisher). Viral supernatants were collected at 48 h and 72 h post-transfection, pooled, filtered through a 0.45 μm membrane, and stored at-80°C.

U251 cells were transduced with Cas9 lentivirus in the presence of 8 μg/mL polybrene. After 24 h, cells were selected with 12 μg/mL Blasticidin S for 5 days. Surviving cells were subjected to monoclonal isolation by limiting dilution in 96-well plates. Single clones were expanded and screened for Cas9 expression by Western blotting. The clone with the highest Cas9 expression was subsequently transduced with lentiviruses encoding IDH1-wt or IDH1-R132H-mut and selected with 250 μg/mL Hygromycin B for 5 days.

2.3 Western Blotting

Cells were lysed in 1× SDS lysis buffer supplemented with protease inhibitors and boiled at 100°C for 10 min. Protein concentrations were determined using a Qubit fluorometer. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk in TBST and incubated overnight at 4°C with primary antibodies: anti-Cas9 (1:3000; Beyotime), anti-IDH1 (1:1000; Dingtong), anti-DDDDK tag (1:1000; Dingtong), anti-IDH1-R132H (1:1000; Merck), and anti-GAPDH (1:5000; Dingtong). HRP-conjugated secondary antibodies were applied for 1 h at room temperature, and signals were visualized using ECL reagent (Millipore) on a chemiluminescence imaging system.

2.4 Cell Proliferation and Colony Formation Assays

For growth curves, cells were seeded at 4,000 cells/well in 6-well plates and counted every other day for 10 days using a hemocytometer. For colony formation assays, cells were seeded at 2,000 cells/well in 6-well plates and cultured for 7 days. Colonies were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted. Colony formation rate was calculated as (number of colonies / number of seeded cells) ×100%.

2.5 Genome-Wide CRISPR/Cas9 Knockout Screening

The human GeCKO v2 CRISPR knockout pooled library (containing 123,411 sgRNAs targeting 19,050 genes) was packaged into lentiviruses. U251-Cas9-IDH1-wt and U251-Cas9-IDH1-mut cells were transduced at

a multiplicity of infection (MOI) of ~0.3 and a coverage of >300×. After 24 h, cells were selected with 2 μg/mL puromycin for 3 days. Surviving cells were harvested at baseline (T0) and after 15 days of continuous culture (T15), maintaining a coverage of >100× at each passage. Genomic DNA was extracted using the Quick-DNA Midiprep Plus Kit (Zymo Research). sgRNA sequences were amplified by PCR and subjected to high-throughput sequencing on an Illumina platform.

2.6 Bioinformatic Analysis

Sequencing reads were aligned to the GeCKO v2 library reference, and sgRNA counts were quantified using MAGeCK software. Gene-level dependency scores were calculated. Screening results were integrated with publicly available dependency data from the DepMap portal . Cell lines were stratified by 2-HG levels, and differential dependency analysis was performed to identify genes selectively essential in high-2-HG (IDH1-mutant) contexts.

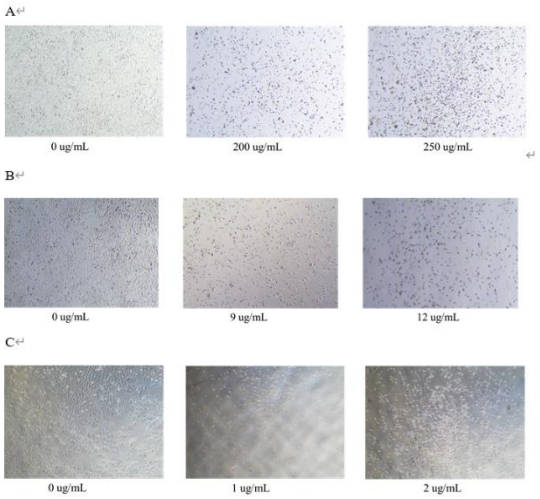
2.7 Statistical Analysis

All experiments were performed independently at least three times. Data are presented as mean ± standard deviation (SD). Statistical significance was determined using Student's *t*-test or one-way ANOVA. *p*< 0.05 was considered statistically significant.

III. Results

3.1 Determination of Optimal Antibiotic Selection Concentrations

To establish stable cell lines, we first determined the minimum lethal concentrations of Blasticidin S, Hygromycin B, and Puromycin required to eliminate non-transduced U251 cells. Dose-response experiments revealed that complete cell death was achieved at 12 μg/mL Blasticidin S (5 days), 250 μg/mL Hygromycin B (5 days), and 2 μg/mL Puromycin (3 days) (Fig. 1A-C). These concentrations were used for all subsequent selection steps.



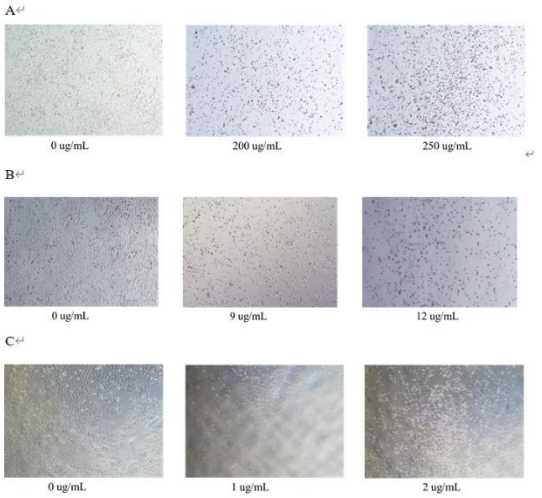


Fig. 2-1 Microscopic results of the minimum lethal concentrations of antibiotic Blasticin S S(A), hygromycin (B) and puromycin (C)（4×）

3.2 Generation and Validation of U251-Cas9 Stable Cell Lines

U251 cells were transduced with LentiCas9-Blast lentivirus and selected with Blasticidin S. Western blot analysis of the surviving polyclonal population confirmed robust expression of Cas9 protein (~160 kDa) compared to parental U251 cells (*p*< 0.001) (Fig. 2A-B).

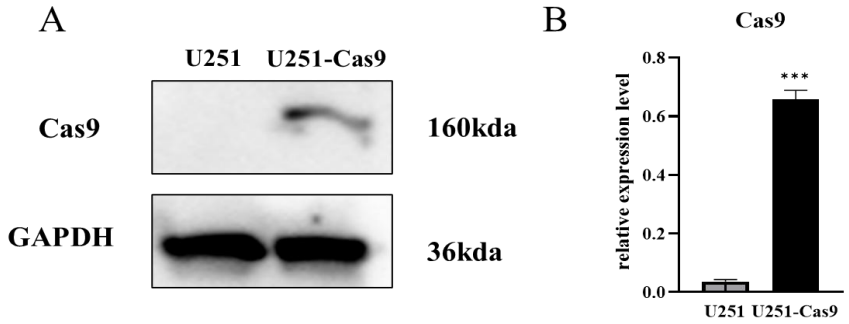


Figure 2. Verification of U251-Cas9 stable cell line.

To ensure genetic homogeneity, we performed monoclonal isolation by limiting dilution. Among several single-cell clones screened, Clone 1 exhibited the highest and most consistent Cas9 expression and was selected for downstream experiments (Fig. 3A-B).

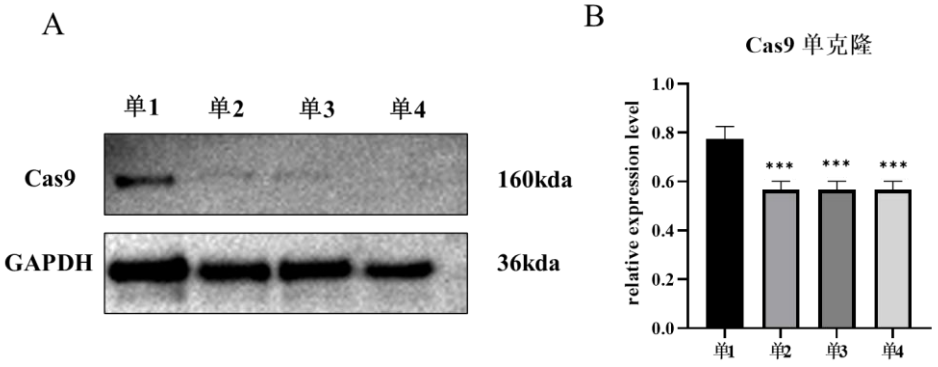


Figure 3 U251-Cas9 monoclonal WB results.

3.3 Generation and Characterization of U251-Cas9-IDH1-wt and U251-Cas9-IDH1-mut Cell Lines

The Cas9-expressing monoclonal line was subsequently transduced with lentiviruses encoding Flag-tagged IDH1-wt or IDH1-R132H-mut and selected with Hygromycin B. Western blotting using an anti-IDH1 antibody detected total IDH1 protein in all lines, with slightly varied intensities. The anti-Flag (DDDDK) antibody confirmed exogenous IDH1 expression specifically in the transduced lines. Importantly, the IDH1-R132H mutation-specific antibody detected a strong signal exclusively in the IDH1-mut line, confirming the successful integration and expression of the mutant allele (Fig. 4A). Quantitative densitometry validated these findings (*p*< 0.01) (Fig. 4B).

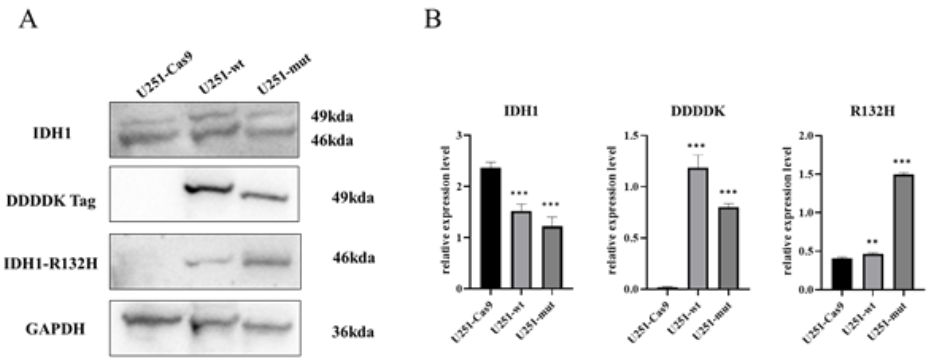


Figure 4. Identification of IDH1-wt and IDH1-mut stable cell lines by Western blot.

3.4 IDH1-R132H Mutation Suppresses Proliferation and Clonogenic Capacity

Consistent with the known biology of IDH1-mutant gliomas, growth curve analysis revealed that U251-Cas9-IDH1-mut cells proliferated at a significantly slower rate than their IDH1-wt counterparts. The difference became statistically significant from day 6 onward (*p*< 0.01) and persisted through day 10 (Fig. 5).

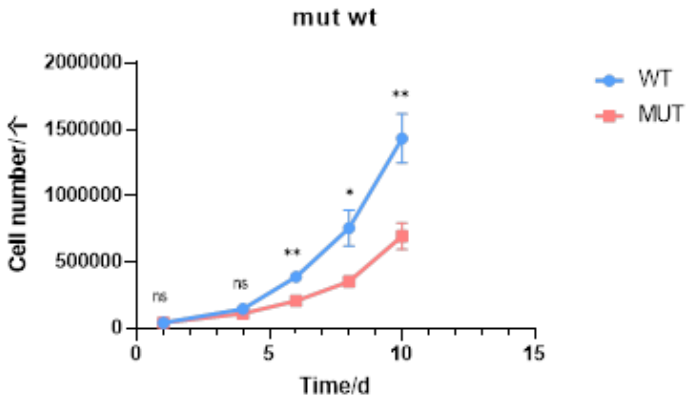


Figure 5. Growth curve of U251-Cas9 IDH1 WT/mut cells.

Colony formation assays further corroborated this finding, demonstrating a marked reduction in both colony number and colony size in IDH1-mut cells (*p*< 0.01) (Fig. 6A-B). These results confirm that the IDH1-R132H mutation confers a growth-suppressive phenotype in this isogenic model.

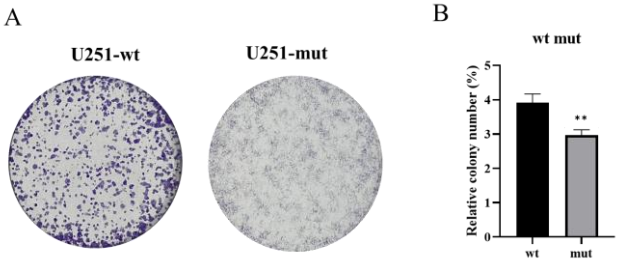


Figure 6. Colony formation assay of U251-Cas9 IDH1 wt/mut cells.

3.5 Genome-Wide CRISPR/Cas9 Screening Identifies SEPHS2 as a Synthetic Lethal Candidate

To identify genes selectively required for the survival of IDH1-mutant cells, we performed a genome-wide CRISPR/Cas9 dropout screen. High-throughput sequencing of sgRNA libraries from T0 and T15 time points yielded high-quality data, with Q30 scores exceeding 96% for all samples (Table 1).

（Table 1 Statistics on sequencing data quality）

Sample	Number	Base	AveLengthL	Q20	Q30	NonChimerasNum
mut T0	2069160	386156960	186.62	99.13%	99.13%	2069160
mut T15	1991639	371652062	186.61	99.08%	99.08%	1991639
WT T0	2066504	385507098	186.55	99.08%	99.08%	2066504
WT T15	2423226	452332966	186.67	99.09%	99.09%	2423226

We integrated our screening results with DepMap dependency data, stratifying cancer cell lines into high-2-HG (n=332) and low-2-HG (n=314) groups as a surrogate for IDH1 mutational status. Differential dependency analysis was visualized as a scatter plot, with the x-axis representing the difference in average dependency between high-2-HG and low-2-HG groups and the y-axis representing the statistical significance ($-\log_{10}$ *p*-value) (Fig. 3A). Genes located on the left side of the plot represent those preferentially essential in high-2-HG (IDH1-mutant-like) contexts. Among the top-scoring candidates, we focused on *SEPHS2* (selenophosphate synthetase 2) due to its known functional connection to GPX4 and the ferroptosis pathway. *SEPHS2* demonstrated a pronounced selective dependency in high-2-HG cell lines (Fig. 7), nominating it as a compelling synthetic lethal candidate for subsequent functional validation.

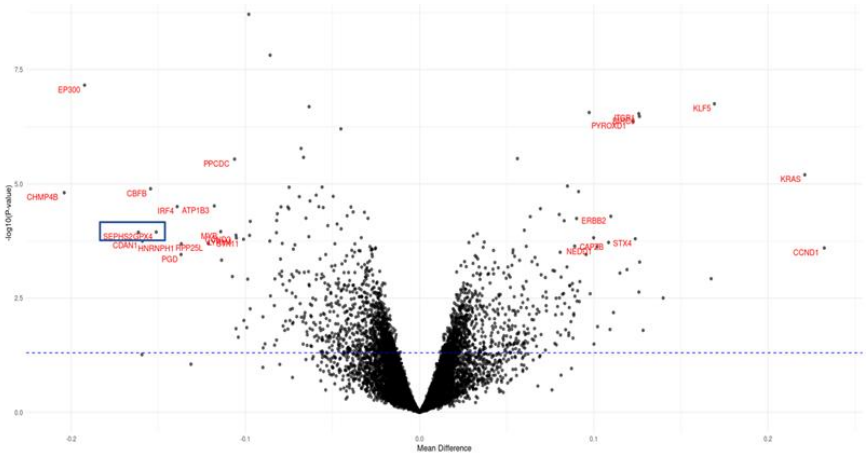


Figure 7. Scatter plot of cellular dependency on genes.

IV. Discussion

In this study, we successfully established and rigorously characterized a pair of isogenic U251 glioblastoma cell lines expressing either wild-type IDH1 or the IDH1-R132H mutant, along with the Cas9 endonuclease. These models recapitulate key phenotypic features of IDH1-mutant gliomas, including reduced proliferative capacity and impaired clonogenic growth, consistent with previous reports [18, 19]. The construction of these stable cell lines provides a robust and genetically defined platform for functional genomics studies, particularly CRISPR/Cas9-based synthetic lethality screening in the context of IDH1 mutation.

Through an unbiased genome-wide CRISPR/Cas9 knockout screen, integrated with DepMap dependency analysis, we identified *SEPHS2* as a top candidate gene selectively essential for the survival of IDH1-mutant cells. SEPHS2 encodes selenophosphate synthetase 2, the rate-limiting enzyme responsible for generating selenophosphate, the universal selenium donor required for the synthesis of all selenoproteins, including the critical antioxidant enzyme GPX4 [20, 21]. The identification of *SEPHS2* as an IDH1-mutant selective dependency aligns with emerging evidence implicating altered redox homeostasis and selenium metabolism in IDH1-mutant cancers. The oncometabolite D-2-HG produced by mutant IDH1 has been shown to deplete cellular NADPH and glutathione pools, rendering cells more vulnerable to oxidative stress [9, 22]. In this context, a heightened reliance on SEPHS2-mediated selenoprotein biosynthesis to maintain redox balance may explain the selective dependency observed in our screen.

Furthermore, SEPHS2 has recently been identified as a critical gene for cancer cell survival due to its dual role in selenium detoxification and selenoprotein production [23]. Cancer cells, including gliomas, often

exhibit "selenophilia" to support their elevated antioxidant demands, leading to the accumulation of toxic selenide intermediates that must be converted to selenophosphate by SEPHS2 [23]. The IDH1-mutant state, characterized by chronic oxidative stress, may further exacerbate this dependency, creating a synthetic lethal interaction.

The connection between SEPHS2 and ferroptosis is particularly noteworthy. GPX4, a selenoprotein whose synthesis is absolutely dependent on SEPHS2 activity, is the master suppressor of ferroptosis, a form of regulated cell death driven by iron-dependent lipid peroxidation [24, 25]. The selective dependency on SEPHS2 observed in IDH1-mutant cells suggests that these cells may be primed for ferroptotic death upon SEPHS2 inhibition. Indeed, recent studies have demonstrated that the IDH1-mutant oncometabolite D-2-HG sensitizes cells to ferroptosis [26], providing a mechanistic rationale for the synthetic lethal relationship between IDH1 mutation and SEPHS2.

While our study provides a strong foundation, several limitations should be acknowledged. First, the screen was performed in a single cell line (U251), and validation in additional IDH1-mutant glioma models, including patient-derived cells, will be essential to confirm the generalizability of this dependency. Second, the precise molecular mechanisms by which SEPHS2 loss selectively kills IDH1-mutant cells remain to be fully elucidated and will require detailed investigation of selenium metabolic flux, GPX4 activity, and ferroptosis induction. These mechanistic studies are the focus of our ongoing work.

In conclusion, we have established a robust isogenic cell model system for functional genomics in IDH1-mutant glioma and identified *SEPHS* as a promising synthetic lethal candidate through genome-wide CRISPR/Cas9 screening. These findings nominate SEPHS2 as a potential therapeutic target for IDH1-mutant gliomas and provide a compelling rationale for further mechanistic and translational investigation.

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