EWS-FLI1–regulated Serine Synthesis and Exogenous Serine are Necessary for Ewing Sarcoma Cellular Proliferation and Tumor Growth

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ABSTRACT

Despite a growing body of knowledge about the genomic landscape of Ewing sarcoma, translation of basic discoveries into targeted therapies and significant clinical gains has remained elusive. Recent insights have revealed that the oncogenic transcription factor EWS-FLI1 can impact Ewing sarcoma cellular metabolism, regulating expression of 3-phosphoglycerate dehydrogenase (PHGDH), the first enzyme in *de novo* serine synthesis. Here, we have examined the importance of serine metabolism in Ewing sarcoma tumorigenesis and evaluated the therapeutic potential of targeting serine metabolism in preclinical models of Ewing sarcoma. We show that PHGDH knockdown resulted in decreased Ewing sarcoma cell proliferation, especially under serine limitation, and significantly inhibited xenograft tumorigenesis in preclinical orthotopic models of Ewing sarcoma. In addition, the PHGDH inhibitor NCT-503 caused a dose-dependent decrease in cellular proliferation. Moreover, we report a novel drug combination in which nicotinamide phosphoribosyltransferase (NAMPT) inhibition, which blocks production of the PHGDH substrate NAD^+, synergized with NCT-503 to abolish Ewing sarcoma cell proliferation and tumor growth. Furthermore, we show that serine deprivation inhibited Ewing sarcoma cell proliferation and tumorigenesis, indicating that Ewing sarcoma cells depend on exogenous serine in addition to *de novo* serine synthesis. Our findings suggest that serine metabolism is critical for Ewing sarcoma tumorigenesis, and that targeting metabolic dependencies should be further investigated as a potential therapeutic strategy for Ewing sarcoma. In addition, the combination strategy presented herein may have broader clinical applications in other PHGDH-overexpressing cancers as well.

Introduction

Ewing sarcoma is a highly aggressive bone and soft tissue malignancy that primarily affects children and adolescents in the second decade of life, and is the second most common pediatric malignant bone tumor (1–4). Despite a growing body of knowledge about the genomic landscape and molecular pathogenesis of Ewing sarcoma, translation of basic discoveries into molecularly targeted therapies and significant clinical gains has remained elusive (1, 4, 5). Genomic sequencing studies have shown that Ewing sarcoma tumors possess one of the lowest somatic mutation rates among all human cancers. Consequently, Ewing sarcoma tumors lack pharmacologically actionable recurrent mutations in classical tumor suppressors and oncogenes (4, 6–8). Rather, Ewing sarcoma tumorigenesis is driven by a chimeric transcription factor, which is the result of one of a number of well-defined chromosomal translocations (1–3). In approximately 90% of cases, the reciprocal t(11;22)(q24; q12) chromosomal translocation is observed, generating the EWS–FLI1 fusion oncoprotein. This constitutively active, aberrant transcription factor mediates global changes in gene expression to drive the malignant phenotype (1–4, 9–11). These chimeric transcription factors are extremely challenging drug targets due to a lack of intrinsic enzymatic activity and disordered protein structure (4, 7).

Reflecting the lack of molecularly targeted therapies, treatment for Ewing sarcoma includes a combination of conventional cytotoxic chemotherapeutic agents, and local control of the primary tumor with surgery and/or radiation. While this aggressive, multimodal treatment approach has improved long-term survival rates for patients with localized disease to around 70%, patients with metastatic or recurrent disease have a very poor 5-year survival rate of <20% (1–3). Furthermore, toxicities associated with current therapeutic regimens are considerable, and Ewing sarcoma survivors face a lifetime of significant treatment-related effects. Therefore, novel therapeutic strategies are critically important (3, 10, 12, 13).

Renewed interest in the metabolic properties of cancer cells has led to an exploration of targeting specific metabolic dependencies as a novel therapeutic strategy (14–16). Serine metabolism supports several critical biological processes that are crucial for the growth and survival of proliferating cells. The first committed, rate-limiting step in *de novo* serine synthesis is catalyzed by the NAD^+-dependent enzyme 3-phosphoglycerate dehydrogenase (PHGDH). PHGDH is overexpressed via focal amplification or transcription factor–mediated upregulation and plays a critical role in several cancers, including melanoma, non–small cell lung cancer, and breast cancer. Increased PHGDH expression is often correlated with higher tumor grade, more aggressive disease, and decreased overall survival (17–24). Cells with high PHGDH expression or flux through the serine synthesis pathway are exquisitely sensitive to genetic or pharmacologic inhibition of the

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pathway (17, 22, 24, 25). Conversely, cells that express little or no PHGDH are insensitive to inhibition of the pathway and rely on import of exogenous serine for optimal growth, such that serine restriction can reduce proliferation and tumor growth (22, 24–29). Recent studies have shown that EWS-FLI1 can affect cellular metabolism, including expression of PHGDH and other enzymes involved in *de novo* serine synthesis. Furthermore, Ewing sarcoma cell lines express the highest level of PHGDH mRNA among all cell lines in the Cancer Cell Line Encyclopedia, and genomic analysis of human Ewing sarcoma tumors has suggested a correlation between high PHGDH expression and poor overall survival (11, 30, 31). Despite these observations, the translational potential of targeting serine metabolism pharmacologically or with dietary interventions has not been explored in preclinical *in vivo* models of Ewing sarcoma. A deeper understanding of the dependency of Ewing sarcoma tumorigenesis on serine metabolism could represent an opportunity for development of targeted therapeutic approaches, either through the inhibition of *de novo* serine synthesis or by limiting the availability of exogenous serine.

Here, we have examined the importance of serine metabolism in Ewing sarcoma tumorigenesis and evaluated the therapeutic potential of targeting serine metabolism by investigating the effects of PHGDH inhibition and serine deprivation on cellular proliferation and tumor growth in preclinical models of Ewing sarcoma. We show that genetic, pharmacologic, and dietary modulation of serine metabolism can each significantly inhibit cellular proliferation and orthotopic xenograft tumor growth, demonstrating that serine metabolism is critical for Ewing sarcoma tumor growth and should be further investigated as a potential therapeutic target for Ewing sarcoma.

Materials and Methods

Cell culture

Human Ewing sarcoma cell lines were described previously (12). All cell lines were cultured in RPMI1640 media supplemented with 2 mmol/L L-glutamine and 10% FBS. For serine-free cell culture, 1× RPMI1640 Media without Glucose, Glycine, and Serine (Teknova) supplemented with 11 mmol/L glucose and 10% dialyzed FBS was used. All cell lines were maintained in a humidified incubator containing 5% CO_2 at 37°C.

Preparation of compounds

Stock solutions of 20 mmol/L NCT-503, 20 mmol/L PHGDH Inactive, and 10 mmol/L GNE-618 were prepared in fresh molecular biology grade DMSO. All stock solutions were aliquoted and stored at −20°C and diluted in appropriate culture media prior to use. As previously described, GNE-618 (ref. 32; originally referred to as compound 26; ref. 33), NCT-503, and PHGDH Inactive (25, 34) were synthesized and provided by the National Center for Advancing Translational Sciences (Rockville, MD).

Generation of stable cell lines

Mission shRNA Lentiviral Transduction Particles (Sigma-Aldrich) were used to generate stable cell lines according to the manufacturer's instructions. Short hairpin RNAs (shRNA) targeted human sequences and were as follows: PHGDH sh20 (TRCN0000028520, CCGGGCTTCGATGAAGGACGGCA AA CTCGAGTTTGCCGTCCTTCATCGAAGCTTTTT), PHGDH sh33 (TRCN0000233033, CCGGCCCACCCACTGTGATCAATAGCTCGAGCTATTGATCACAGTGGGTGGGTTTTTG), and shControl (SHC004V, TurboGFP shRNA Control Transduction Particles).

siRNA-mediated knockdown

FlexiTube siRNA (Qiagen) was used to knock down human EWS-FLI1 or to serve as a negative or positive control. Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) was used according to the manufacturer's instructions. siRNAs were as follows: siFLI1 #49 (SI04280549, Hs_FLI1_8 FlexiTube siRNA), siFLI1 #2 (SI00387716, Hs_FLI1_2 FlexiTube siRNA), siNegative Control (SI03650318, AllStars Negative Control siRNA), and siPositive Control (SI04381048, AllStars Hs Cell Death siRNA).

Immunoblotting

Cell lysates were prepared in RIPA buffer supplemented with a Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Tumor lysates were prepared by grinding frozen tumor tissue with a mortar and pestle and reconstituting in RIPA buffer. Clarified total cellular lysates were immunoblotted with anti-PHGDH (Cell Signaling Technology), anti-Fli1 (Abcam), and anti-actin (Abcam) antibodies using standard procedures. For studies involving siRNA-mediated EWS-FLI1 knockdown, 2×10^5 EW8, 2.5×10^5 TC32, or 4×10^5 TC71 cells/well were plated in 6-well tissue culture plates, transfected with siRNAs the following day, and harvested for immunoblotting 72 hours after transfection.

Measurement of cellular proliferation

Cells were plated at 2,000–4,000 cells/well in 96-well plates in either standard culture media or serine/glycine-free media, as indicated. Starting the day of plating, cellular proliferation was monitored in an Incucyte FLR Live Cell Analysis System (Essen BioScience) in a humidified incubator containing 5% CO_2 at 37°C. For studies involving NCT-503, PHGDH Inactive, or GNE-618, cells were treated the day after plating with the indicated concentrations of compounds in the indicated media conditions. Experiments were performed at least three times.

Isotope tracing

Cells (4×10^5 TC32 and 8×10^5 TC71) were plated in 6-cm dishes in standard culture media. The next day, cells were transfected with the indicated siRNAs. The day after siRNA transfection, media was replaced with serine/glycine-free media for overnight serine starvation. The following day media was removed, cells were washed with DPBS, and 3 mL of isotope tracer media were added, which was composed of serine/glycine-free RPMI1640 media supplemented with 10% dialyzed FBS and 11 mmol/L [U-$^{13}C_6$] D-Glucose (CLM-1396-PK, Cambridge Isotope Laboratories, Inc.). Following 6-hour incubation at 37°C, intracellular metabolites were extracted by removing isotope tracer media, rinsing cells with ice-cold saline, and scraping cells in 500 μL of ice-cold methanol. The cell–methanol mixture was snap-frozen in liquid nitrogen, subjected to multiple freeze-thaws, and spun at 14,000 rpm at 4°C for 15 minutes. The metabolite-containing supernatant was air-dried at 42°C, then resuspended in methoxyamine hydrochloride (Sigma), and heated at 42°C for 1 hour. The samples were derivatized using tert-butyldimethylsilylation (Thermo Fisher Scientific) and heated at 70°C for 1 hour. Samples were processed by gas chromatography-mass spectrometry (Agilent), and isotopologue distribution was calculated and analyzed as described previously (35).

In vivo studies

Animal studies were performed in accordance with the guidelines of the NIH Animal Care and Use Committee. Four- to 6-week-old female Fox Chase SCID-Beige mice were purchased from Charles River

Laboratories. For all studies, 2×10^6 cells were suspended in Hank's Balanced Salt Solution (Thermo Fisher Scientific, 14175095) and injected orthotopically into the gastrocnemius muscle in the left hind leg of each mouse.

For studies involving the serine-free diet, at the time of orthotopic injection, mouse diet was changed to Control AA Diet (TD.110839, Envigo) or Serine and Glycine Deficient AA Diet (TD.160752, Envigo), which differed from Control AA Diet by only the removal of serine and glycine. For studies involving NCT-503 and GNE-618, when tumors were palpable, mice were randomized into groups and treated with the indicated concentrations of either NCT-503 (200 μL/mouse, i.p.), GNE-618 (100 μL/mouse, oral gavage), combinations of NCT-503 and GNE-618, or vehicle controls (200 μL/mouse NCT-503 vehicle, i.p. + 100 μL/mouse GNE-618 vehicle, oral gavage) once daily, 5 days per week. Vehicle for NCT-503 consisted of 2% DMSO, 5% ethanol, 37% PEG-300, and 56% (2-hydroxypropyl)-beta-cyclodextrin, and vehicle for GNE-618 was 10% ethanol, 30% saline, and 60% PEG-400. All vehicle components were purchased from Sigma-Aldrich. NCT-503 was prepared daily, and aliquots of GNE-618 were prepared and stored at −20°C prior to the experiment.

All mice were maintained in a pathogen-free environment and monitored by observation of overall health and weekly body weights to determine treatment tolerability. Tumors were measured twice weekly with calipers. Tumor volume was calculated by the following formula: $V\,(\mathrm{mm}^3) = (D \times d^2)/6 \times 3.14$, where D is the longest tumor axis and d is the shortest tumor axis.

Statistical analysis

Statistical significance was determined by Student t test for comparisons between two groups, or one-way ANOVA with Dunnett's test for comparisons involving more than two groups. $P < 0.05$ was considered significant.

Results

EWS-FLI1 affects PHGDH expression and serine synthesis

To examine PHGDH protein expression in Ewing sarcoma cells, immunoblot analysis was performed on a panel of human Ewing sarcoma cell lines. The human breast cancer cell lines MDA-MB-231 (PHGDH-low) and MDA-MB-468 (*PHGDH*-amplified) were included as negative and positive controls, respectively, because PHGDH expression has been well characterized in these cell lines (20, 24). As shown in Supplementary Fig. S1, Ewing sarcoma cell lines exhibited robust expression of PHGDH protein at levels comparable with MDA-MB-468 *PHGDH*-amplified breast cancer cells.

To determine whether the Ewing sarcoma oncoprotein EWS-FLI1 may be regulating PHGDH expression in our cell lines, we knocked down EWS-FLI1 using two distinct siRNAs and examined the effect on PHGDH protein level in three different Ewing sarcoma cell lines. Sufficient knockdown of EWS-FLI1 was confirmed by immunoblot analysis (**Fig. 1A**). EWS-FLI1 knockdown by either targeting sequence caused a dramatic reduction in PHGDH protein expression in all cell lines tested (**Fig. 1A**), which is consistent with recent studies suggesting that EWS-FLI1 can regulate the expression of serine synthesis genes (11, 31). The similar results obtained with two distinct EWS-FLI1–targeting siRNAs in three different cell lines suggest that the effects seen are not off-target effects of the siRNAs.

To examine the relationship between EWS-FLI1 and *de novo* serine synthesis, we examined the effects of EWS-FLI1 knockdown on ^{13}C-labeled glucose flux through the serine synthesis pathway in two different Ewing sarcoma cell lines. Following transfection with either of two distinct EWS-FLI1–targeting sequences or a nontargeting control siRNA, cells were labeled with ^{13}C-glucose for 6 hours and then metabolites were extracted and analyzed. The schematic in **Fig. 1B** illustrates the labeling pattern of the glycolytic intermediate 3-phoshoglycerate (3-PG) and serine from ^{13}C-glucose, demonstrating the transfer of three ^{13}C-labeled carbons to each, resulting in an increase in molecular mass (M+3). As shown in **Fig. 1C**, EWS-FLI1 knockdown significantly reduced the fraction of glucose-derived, M+3–labeled serine in both cell lines tested, resulting in up to 64% reduction in TC32 and 44% reduction in TC71. Importantly, M+3–labeled 3-PG was not affected by EWS-FLI1 knockdown (**Fig. 1C**), indicating that glucose entry into glycolysis was not affected by EWS-FLI1 knockdown. Taken together, these data demonstrate that EWS-FLI1 affects serine synthesis.

PHGDH is necessary for Ewing sarcoma proliferation and tumorigenesis

To examine the biological significance of PHGDH expression in Ewing sarcoma cells, we generated stable cell lines expressing either of two distinct PHGDH-targeting shRNAs or a nontargeting negative control shRNA. Knockdown of PHGDH was confirmed by immunoblot analysis (**Fig. 2A**), and PHGDH expression in shControl cell lines was comparable with parental cells (Supplementary Fig. S2). PHGDH knockdown by either targeting sequence in all three Ewing sarcoma cell lines tested had no effect on proliferation of cells grown in the presence of serine (Supplementary Fig. S3); however, PHGDH knockdown caused an approximately 2-fold reduction in proliferation of the same cell lines grown in serine/glycine-free media, compared with shControl counterparts (**Fig. 2B**). The similar results obtained with two distinct PHGDH-targeting shRNAs in three different cell lines suggest that the results seen are not due to off-target effects of the shRNAs.

We next examined the significance of PHGDH for Ewing sarcoma tumorigenesis *in vivo*. We utilized the TC32 and TC71 stable cell lines described above. We selected TC32 and TC71 because of the well-characterized, consistent ability to rapidly form tumors when injected orthotopically into the gastrocnemius muscle adjacent to the tibia of immunocompromised mice (12, 36, 37). Following orthotopic injection of the aforementioned stable cell lines, negative control shRNA–expressing cells generated palpable tumors with a latency of approximately 1 week. As shown in **Fig. 2C**, knockdown of PHGDH in TC32 and TC71 cell lines with either of the two distinct PHGDH-targeting shRNAs caused a statistically significant decrease ($P < 0.005$) in average tumor volume over the course of the experiment, compared with shControl tumors. TC32 and TC71 shControl tumor–bearing mice reached study endpoint by day 23 or 20 after injection, at which point PHGDH shRNA–expressing tumors were 66% and 46% or 38% and 41% smaller ($P < 0.005$), respectively. These results suggest that PHGDH is necessary for Ewing sarcoma tumorigenesis.

To further evaluate the importance of PHGDH in our *in vivo* studies, we examined PHGDH protein expression in PHGDH shRNA–expressing tumors compared with shControl tumors at study endpoint. In contrast to the significant knockdown of PHGDH protein expression in preinjected cell lines (**Fig. 2A**), immunoblot analysis at study endpoint revealed that PHGDH protein expression was only minimally suppressed in TC32 and TC71 PHGDH shRNA–expressing tumors compared with shControl-expressing tumors (**Fig. 2D**). These findings demonstrate that PHGDH protein expression increased in PHGDH shRNA–expressing tumors in comparison with the preinjection PHGDH shRNA–expressing cell lines, suggesting that tumors

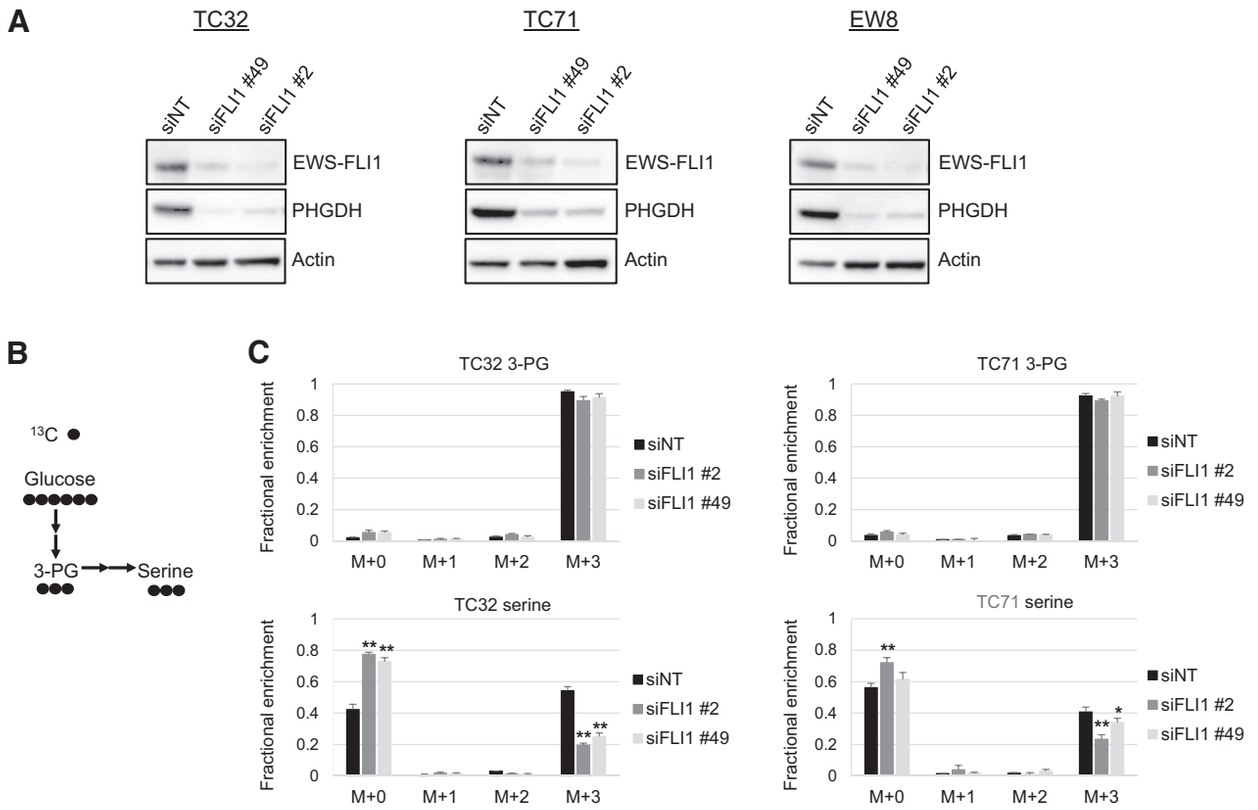

Figure 1.

EWS-FLI1 knockdown affects PHGDH expression and serine synthesis. **A,** Immunoblot analysis of EWS-FLI1 and PHGDH expression in the indicated cell lines expressing the specified FLI1 siRNAs or a nontargeting control siRNA (siNT). Actin was used as a loading control. **B,** Schematic of the incorporation of glucose-derived ^{13}C into 3-PG and serine. **C,** Detection of labeled 3-PG and serine in the indicated cell lines following transfection with the specified siRNAs. Data represent the mean + SD of three independent experiments. *, $P < 0.05$; **, $P < 0.005$ compared with siNT.

may select for higher PHGDH expression, leading to loss of PHGDH knockdown over time.

The PHGDH inhibitor NCT-503 inhibits Ewing sarcoma proliferation

Given the importance of PHGDH for Ewing sarcoma proliferation and tumorigenesis, we evaluated the sensitivity of Ewing sarcoma cells to pharmacologic inhibition of PHGDH with the previously described (25, 34) small-molecule PHGDH inhibitor NCT-503 (see schematic in **Fig. 3A**). Three different Ewing sarcoma cell lines were treated with varying concentrations of NCT-503, a structurally similar inactive control small molecule, or a vehicle control. NCT-503 treatment elicited a dose-dependent decrease in proliferation in all cell lines tested, while the inactive control molecule had no effect on proliferation compared with the vehicle control (**Fig. 3B**, top row). To further investigate the sensitivity of Ewing sarcoma cells to NCT-503 treatment, we performed the same analysis in serine/glycine-free media. The absence of serine/glycine enhanced the effects of NCT-503, increasing the sensitivity of all three cell lines tested (**Fig. 3B**, bottom row). Although individual cell lines showed slight variations in the degree of sensitivity to specific NCT-503 concentrations, these findings demonstrate that Ewing sarcoma cells are sensitive to NCT-503, and suggest that the effects seen are on-target because serine concentration altered the efficacy of NCT-503.

Nicotinamide phosphoribosyltransferase inhibition synergizes with NCT-503 to inhibit Ewing sarcoma proliferation and tumor growth

While Ewing sarcoma cells are sensitive to PHGDH inhibition by NCT-503 *in vitro*, we sought to determine whether rational combination with another inhibitor could enhance efficacy, because NCT-503 has previously shown limited efficacy *in vivo* as a single agent (25). Because PHGDH is an NAD^+-dependent enzyme, we chose to combine NCT-503 with a nicotinamide phosphoribosyltransferase (NAMPT) inhibitor, which targets the NAD^+ salvage pathway (see schematic in **Fig. 4A**). We have previously characterized the activity of NAMPT inhibitors in Ewing sarcoma cells *in vitro* and *in vivo*, and have demonstrated that NAMPT inhibitors synergistically enhance the inhibition of PARP, which is also an NAD^+-dependent enzyme (12).

Given our previous experience with the NAMPT inhibitor GNE-618 (12), we selected this inhibitor for our targeted combination studies and first confirmed the sensitivity of Ewing sarcoma cells to single-agent NAMPT inhibition. Two different Ewing sarcoma cell lines were treated with varying concentrations of GNE-618 or vehicle. As shown in Supplementary Fig. S4, GNE-618 treatment elicited a dose-dependent decrease in proliferation in both cell lines tested, consistent with our previous studies (12). On the basis of these findings, our results using NCT-503 described above, and our

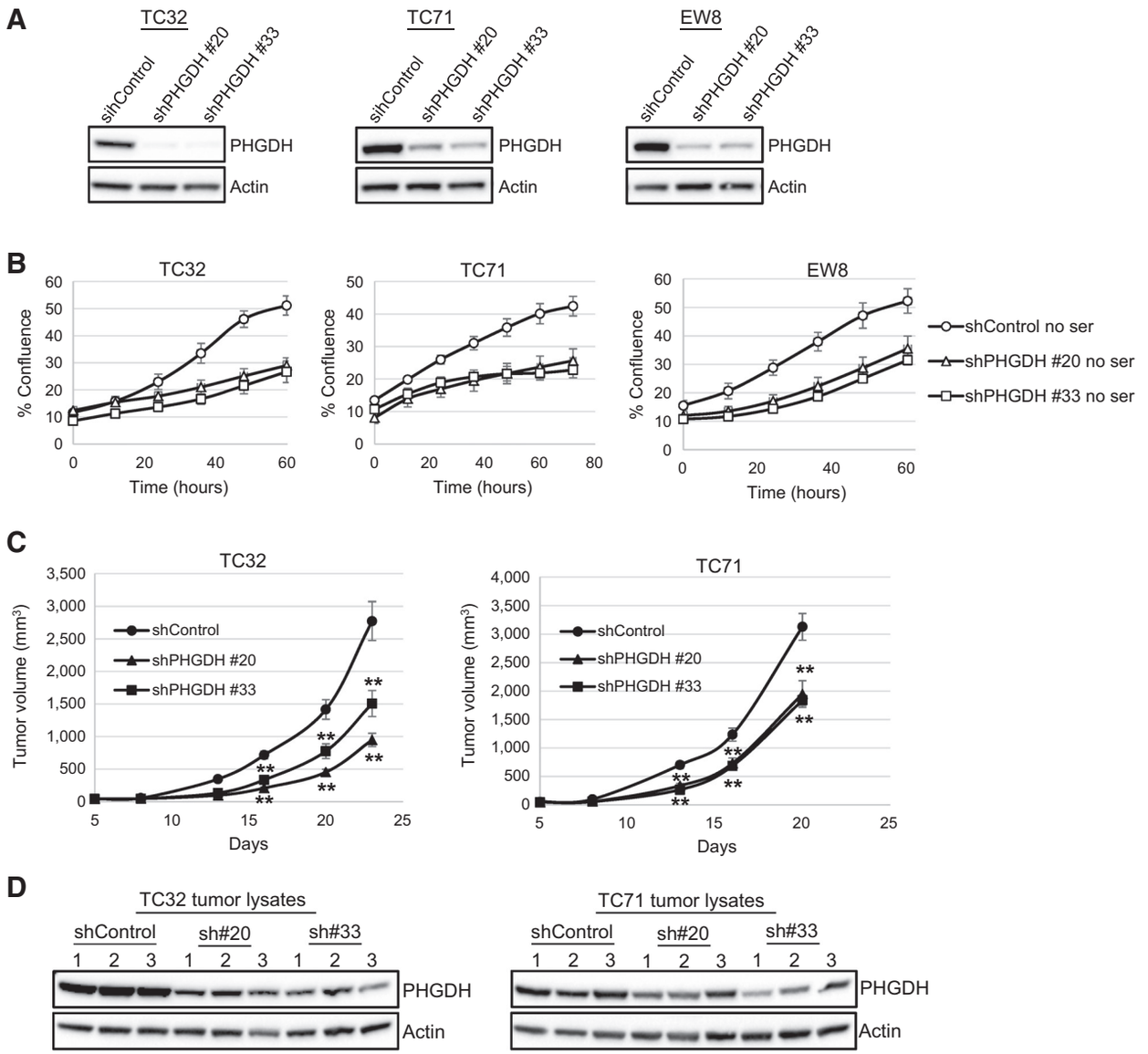

Figure 2.

PHGDH knockdown inhibits Ewing sarcoma proliferation and tumor growth. **A,** Immunoblot analysis of PHGDH expression in the indicated cell lines expressing the specified lentiviral control or PHGDH shRNAs. Actin was used as a loading control. **B,** Cellular proliferation was monitored in an Incucyte FLR. The indicated cell lines were assayed in serine/glycine-free RPMI1640 media supplemented with 10% dialyzed FBS (no ser, no serine). Data represent the mean ± SD of a representative experiment. **C,** Average tumor volume over time of mice orthotopically injected with TC32 or TC71 stable cell lines bearing the indicated shRNAs. Data represent the mean ± SEM (8 mice/group). **, $P < 0.005$ versus shControl. **D,** Immunoblot analysis of PHGDH in endpoint tumor lysates derived from the specified cell lines expressing the indicated control or PHGDH shRNAs. Actin was used as a loading control.

previous studies on GNE-618 (12), we selected concentrations of each inhibitor that had minimal impact on proliferation and evaluated whether the growth inhibitory effects of these compounds were enhanced when used in combination. As shown in **Fig. 4B**, treatment with either NCT-503 or GNE-618 as single agents at the indicated concentrations had minimal effects on proliferation of Ewing sarcoma cell lines, however combined inhibition with NCT-503 and GNE-618 completely inhibited proliferation of all cell lines tested, suggesting possible cooperativity between the compounds.

To quantify the combinatorial effects of these inhibitors and evaluate potential synergy, we performed a simultaneous evaluation of the effects of NCT-503 and GNE-618 on Ewing sarcoma cell proliferation at a series of concentrations as single agents and in combinations. The effects of each single agent (Supplementary Fig. S5) and various combinations on proliferation of three different Ewing sarcoma cell lines are summarized in Supplementary Tables S1 and S2. The CompuSyn (http://www.combosyn.com) program was utilized to quantitatively evaluate the effects using the Chou–Talalay method (38, 39) to generate combination index values for each combination

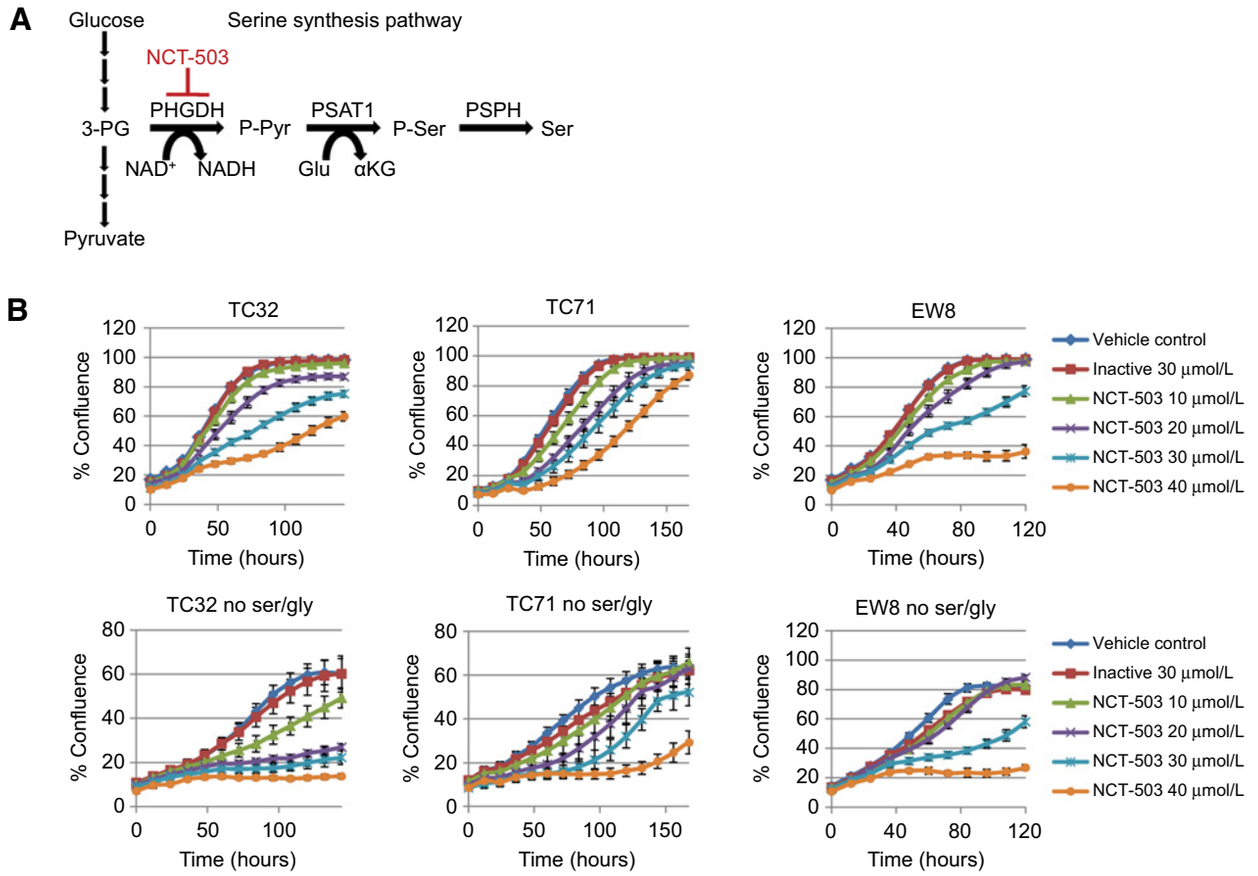

Figure 3.

The PHGDH inhibitor NCT-503 inhibits sarcoma cellular proliferation. **A,** Schematic of the serine synthesis pathway and NCT-503 inhibition of PHGDH. **B,** Cellular proliferation in response to NCT-503 or an inactive control was monitored in an Incucyte FLR. The indicated cell lines were assayed in RPMI1640 media supplemented with 10% dialyzed FBS, with or without serine and glycine (no ser/gly). Data represent the mean ± SD of a representative experiment.

(four distinct combinations in TC32 and TC71 cells and two distinct combinations in EW8 cells). In all three cell lines tested, the combination of NCT-503 and GNE-618 displayed synergy at multiple different dose combinations, as represented by a combination index of less than 1.0 (38, 39).

To evaluate the efficacy of NCT-503 and GNE-618 *in vivo*, we utilized the TC32 xenograft model described above. Following orthotopic injection, TC32 cells generated palpable tumors with a latency of approximately 10 days, at which point all mice were randomized into six treatment groups receiving either vehicle control, NCT-503 (75 mg/kg), GNE-618 (5 or 10 mg/kg), or NCT-503 (75 mg/kg) + GNE-618 (5 or 10 mg/kg). The doses of GNE-618 were chosen based on our prior *in vivo* studies (12), while a higher dose of NCT-503 was selected because of limited *in vivo* efficacy demonstrated at lower doses in previous studies (25). **Figure 4C** shows the resulting tumor growth curves for all treatment groups. Treatment with single-agent NCT-503 caused a modest, albeit statistically significant ($P < 0.05$) inhibition of tumor growth, resulting in a 29% decrease in average tumor volume at day 24 after injection, when vehicle control–treated tumors reached study endpoint volumes (**Fig. 4C**). Treatment with single-agent GNE-618 at either dose had a more dramatic effect on tumor growth rates than NCT-503 over the same period (79% or 86% reduction in average tumor volume at day24), however, tumors continued to grow while on treatment. Strikingly, combined treatment with NCT-503 and either dose of GNE-618 inhibited tumor growth more effectively than either drug alone, and while tumors treated with the combination of NCT-503 and 5 mg/kg GNE-618 grew slightly during treatment, the combination of NCT-503 and 10 mg/kg GNE-618 completely inhibited tumor growth over the approximately 3 week duration of treatment (**Fig. 4C**). Following the cessation of treatment (day 34), tumors in all remaining treatment groups displayed increased growth rates, although combination-treated tumors, especially at the higher GNE-618 dose, had persistent growth suppression. When GNE-618–treated tumors reached study endpoint volumes, the combination of NCT-503 and 5 mg/kg GNE-618 was 50% more effective ($P < 0.005$) than 5 mg/kg GNE-618 alone, and the combination of NCT-503 and 10 mg/kg GNE-618 was 86% more effective ($P < 0.005$) than 10 mg/kg GNE-618 alone, demonstrating a significant increase in efficacy of the combinations over the single agents. At day 46 after injection, combined treatment with NCT-503 and 10 mg/kg GNE-618 was resumed and, as shown in **Fig. 4C**, average tumor volume decreased, indicating that tumors were still sensitive to combined treatment. Importantly, mice tolerated all treatments well and maintained body weight over the course of the study (Supplementary Fig. S6), consistent with prior studies on the single agents (12, 25).

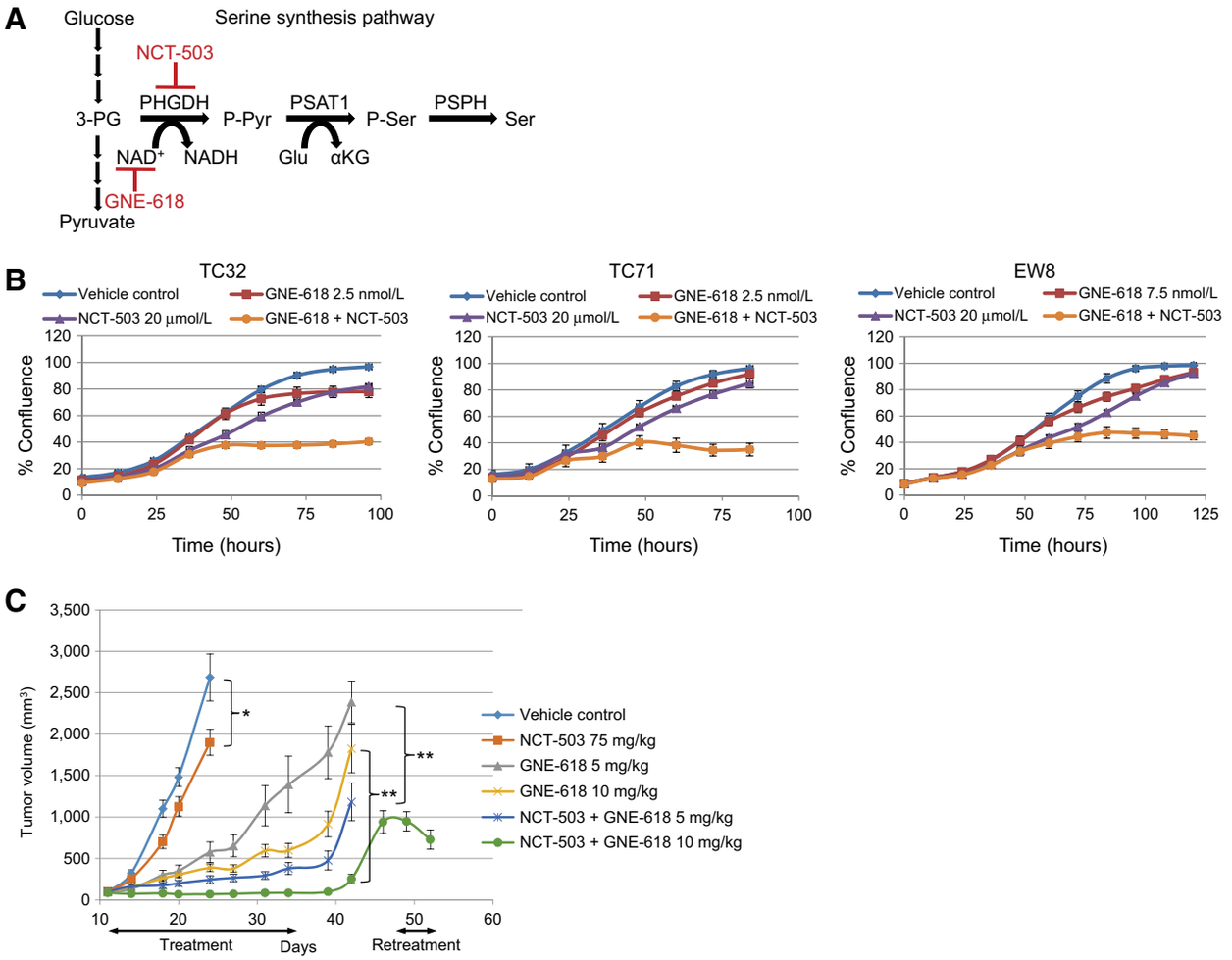

Figure 4.

Combined inhibition of PHGDH and NAMPT reduces Ewing sarcoma proliferation and tumor growth. **A,** Schematic of the serine synthesis pathway and NCT-503/GNE-618 inhibition of PHGDH. **B,** Cellular proliferation in response to NCT-503 and/or GNE-618 was monitored in an Incucyte FLR. The indicated cell lines were assayed in RPMI1640 media supplemented with 10% dialyzed FBS. Data represent the mean ± SD of a representative experiment. **C,** Average tumor volume over time of mice orthotopically injected with TC32 cells. Mice received the specified treatments, as indicated. Data represent the mean ± SEM (10 mice/group; *, $P < 0.05$; **, $P < 0.005$).

Exogenous serine is necessary for optimal Ewing sarcoma proliferation and tumor growth

While certain cancer cells upregulate PHGDH expression and *de novo* serine synthesis, others rely on exogenous serine for optimal growth. In such cases, serine deprivation can inhibit proliferation *in vitro*, while restriction of dietary serine/glycine can reduce tumor growth in xenograft, allograft, and genetically engineered mouse models (24, 26–29, 40). To evaluate the contribution of exogenous serine to Ewing sarcoma cell proliferation, we monitored the proliferation of three Ewing sarcoma cell lines grown in culture media with or without serine/glycine. As shown in **Fig. 5A**, Ewing sarcoma cell lines grown in media lacking serine/glycine displayed an approximately 2-fold decrease in growth rate compared with the same cell lines grown in standard serine/glycine-containing culture media. In contrast to cells grown under standard culture conditions, serine/glycine-restricted cells plateaued at approximately 40%–50% confluency, suggesting that Ewing sarcoma cells need exogenous serine, in addition to *de novo* synthesized serine, for maximal proliferation.

Given our results indicating that EWS-FLI1 affects PHGDH expression and serine synthesis, we investigated whether EWS-FLI1 knockdown could further sensitize Ewing sarcoma cell lines to serine deprivation. As shown in Supplementary Fig. S7, EWS-FLI1 knockdown by either of two distinct targeting sequences resulted in an approximately 2-fold or 3-fold reduction in cellular proliferation in TC32 and TC71 cells, respectively, when grown in serine/glycine-free media compared with nontargeting siRNA control–expressing cells. These findings confirm the importance of EWS-FLI1–mediated serine synthesis for optimal Ewing sarcoma cell proliferation, especially under conditions of serine deprivation.

To examine whether exogenous serine was necessary for Ewing sarcoma tumor growth, we generated orthotopic xenograft tumors using TC32 and TC71 cells and fed the mice either a control diet or an identical diet lacking serine and glycine. As shown in **Fig. 5B**, mice fed with a serine/glycine-free diet had a significant reduction in tumor growth. By day 21 after injection, when control diet–fed mouse tumors reached study endpoint volumes, TC71 and TC32 tumor–bearing mice

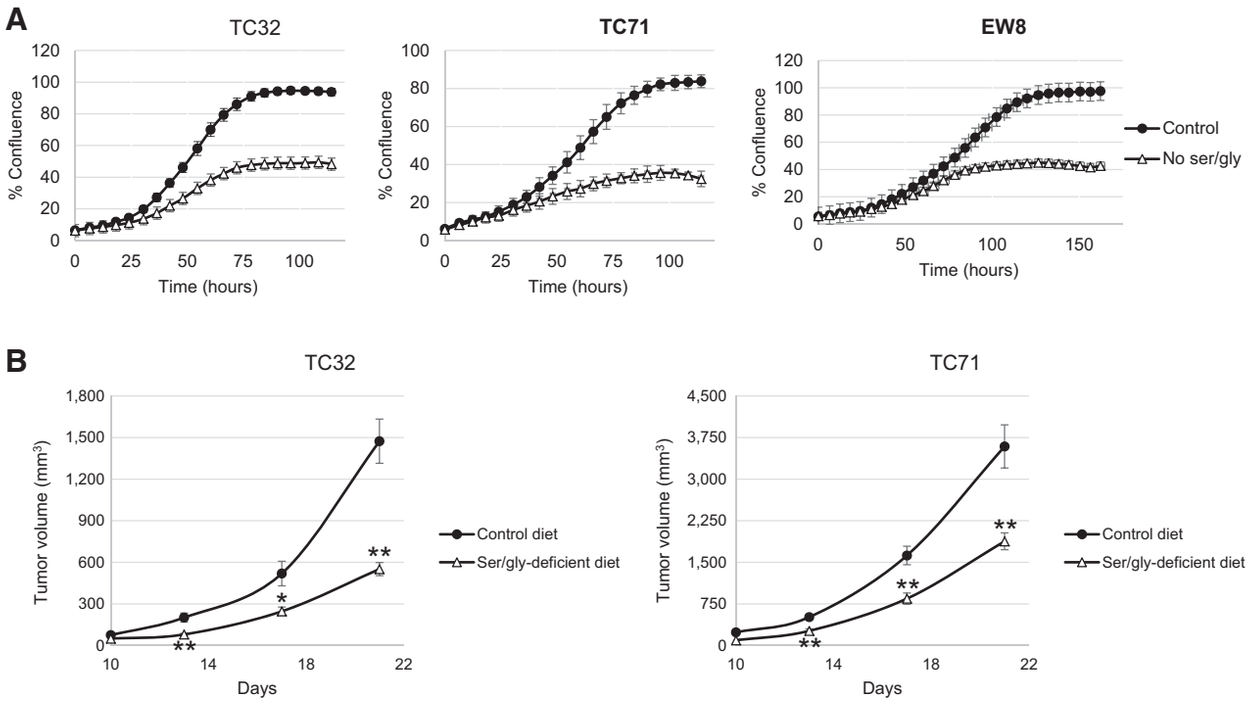

Figure 5.

Exogenous serine is required for optimal Ewing sarcoma proliferation and tumor growth. **A,** Cellular proliferation was monitored in an Incucyte FLR. Cell lines were assayed in RPMI1640 media supplemented with 10% dialyzed FBS, with (control) or without serine and glycine (no ser/gly). Data represent the mean ± SD of a representative experiment. **B,** Average tumor volume over time of the indicated orthotopically injected cell lines in mice fed with a control diet or a serine and glycine–deficient diet. Data represent the mean ± SEM (8 mice/group). *, $P < 0.05$; **, $P < 0.005$ versus control diet.

fed with the serine/glycine-free diet had a 48% or 63% lower average tumor volume, respectively ($P < 0.005$) compared with control diet–fed mice (**Fig. 5B**). Importantly, mice fed the serine/glycine-free diet tolerated it well and maintained body weight over the course of the study (Supplementary Fig. S8). These results are consistent with our *in vitro* findings using serine/glycine-free culture media, and suggest that exogenous serine is necessary for optimal Ewing sarcoma tumorigenesis.

Discussion

In this study, we examined the *in vitro* and *in vivo* dependency of Ewing sarcoma on serine metabolism by evaluating the effects of PHGDH inhibition and serine deprivation. We confirmed robust expression of PHGDH protein in a panel of Ewing sarcoma cell lines at levels comparable with MDA-MB-468 *PHGDH*-amplified breast cancer cells (20, 24). We demonstrated that EWS-FLI1 knockdown caused a decrease in PHGDH protein expression and *de novo* serine synthesis, consistent with previous large-scale genomic analyses that identified *PHGDH* as a putative EWS-FLI1 target gene (41, 42). Recent studies have further shown that EWS-FLI1 can directly regulate the transcription of several genes involved in serine/glycine synthesis (31). This is consistent with other studies showing that EWS-FLI1 drives critical metabolic pathways to support Ewing sarcoma tumorigenesis (11, 37).

While PHGDH knockdown had no effect on proliferation of cells grown in the presence of serine, it caused a significant reduction in proliferation of Ewing sarcoma cells grown in serine/glycine-free media. The reduction in proliferation seen in the absence of serine is consistent with PHGDH inhibition in other cancer types, and suggests that exogenous serine utilization may compensate for reduced PHGDH expression and/or that increased PHGDH expression may provide more of a proliferative advantage when serine concentrations are limiting (24, 40). PHGDH knockdown also significantly decreased Ewing sarcoma orthotopic xenograft tumorigenesis, and immunoblot analysis revealed that PHGDH knockdown was lost by the time tumors reached endpoint. These effects were observed even though serine was present in the diet, suggesting that serine availability may be low in the orthotopic Ewing sarcoma tumors. This is consistent with findings demonstrating that increased serine synthesis provides a growth advantage to tumors when serine levels are limiting (40).

Recently, significant efforts have been made to identify small-molecule PHGDH inhibitors (24, 25, 34, 43). In our studies we utilized NCT-503, a well-characterized PHGDH inhibitor suitable for *in vivo* studies (25, 34). NCT-503 treatment elicited a dose-dependent decrease in proliferation in multiple Ewing sarcoma cell lines, while an inactive control molecule had no effect on proliferation. Furthermore, the absence of serine/glycine increased the sensitivity of all cell lines tested, consistent with previous studies of PHGDH inhibition in breast cancer cell lines (24, 25). Although the ability of NCT-503 to effectively inhibit PHGDH has previously been well-characterized, a potential off-target effect on mitochondrial respiration has also been described previously (25). Importantly, however, this effect was also observed with the inactive control molecule, while only NCT-503 effectively inhibited serine synthesis and cellular proliferation (25), suggesting that the majority of the effects elicited by NCT-503 are likely due to on-target activity against PHGDH. Nevertheless, off-target effects cannot be ruled out, and the continued improvement and

clinical development of specific PHGDH inhibitors is critically important.

Because NCT-503 has previously shown limited efficacy *in vivo* as a single agent (25), we examined whether a rational combination strategy could enhance therapeutic potential. Since PHGDH is an NAD^+-dependent enzyme, we chose to combine NCT-503 with a NAMPT inhibitor, which targets the NAD^+ salvage pathway, leading to depletion of intracellular NAD^+ levels (12, 44). In a similar approach, we previously characterized the activity of NAMPT inhibitors in Ewing sarcoma cells *in vitro* and *in vivo*, and demonstrated that NAMPT inhibitors synergistically enhanced the inhibition of PARP, which is also an NAD^+-dependent enzyme (12). On the basis of our previous studies, we selected the well-characterized NAMPT inhibitor GNE-618 (12, 32, 33) to combine with NCT-503 and demonstrated that the two agents displayed synergy *in vitro* and inhibited tumor growth more effectively than either drug alone. We chose a higher dose of NCT-503 (75 vs. 40 mg/kg) than prior studies on the basis of the limited efficacy seen previously and the more aggressive nature of the Ewing sarcoma orthotopic xenograft model. Importantly, all treatments were well-tolerated. These findings have broad applications beyond Ewing sarcoma treatment and may represent a novel therapeutic approach for other PHGDH-overexpressing cancers like breast cancer, non–small cell lung cancer, and melanoma.

Our studies also provide important new insights into the role of exogenous serine in supporting Ewing sarcoma tumorigenesis by showing that a serine/glycine-free diet significantly inhibited Ewing sarcoma xenograft tumor growth. Our findings suggest that exogenous serine is necessary for optimal Ewing sarcoma cell proliferation and tumorigenesis, and are consistent with previous studies in other cancers showing that serine deprivation can inhibit tumor growth in xenograft, allograft, and genetically engineered mouse models (24, 26–29, 40).

In summary, we have shown that EWS-FLI1 affects PHGDH expression and *de novo* serine synthesis, and that PHGDH and exogenous serine are necessary for optimal Ewing sarcoma cellular proliferation and tumor growth. Our findings represent a potentially exciting clinical opportunity for development of targeted therapeutic approaches for Ewing sarcoma, either through inhibition of *de novo* serine synthesis or by limiting exogenous serine availability.

Disclosure of Potential Conflicts of Interest

J.M. Rohde has ownership interest (including patents) in NCT-503. R.J. DeBerardinis is a SAB member (paid consultant) at Agios Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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