OPEN ACCESS
CellPress

Cell Reports
Article

Serine, but Not Glycine, Supports One-Carbon Metabolism and Proliferation of Cancer Cells

Christiaan F. Labuschagne,[1] Niels J.F. van den Broek,[1] Gillian M. Mackay,[1] Karen H. Vousden,[1,*] and Oliver D.K. Maddocks[1,*]
[1]Cancer Research UK Beatson Institute, Switchback Road, Glasgow G61 1BD, UK
*Correspondence: k.vousden@beatson.gla.ac.uk (K.H.V.), o.maddocks@beatson.gla.ac.uk (O.D.K.M.)
http://dx.doi.org/10.1016/j.celrep.2014.04.045

SUMMARY

Previous work has shown that some cancer cells are highly dependent on serine/glycine uptake for proliferation. Although serine and glycine can be interconverted and either might be used for nucleotide synthesis and one-carbon metabolism, we show that exogenous glycine cannot replace serine to support cancer cell proliferation. Cancer cells selectively consumed exogenous serine, which was converted to intracellular glycine and one-carbon units for building nucleotides. Restriction of exogenous glycine or depletion of the glycine cleavage system did not impede proliferation. In the absence of serine, uptake of exogenous glycine was unable to support nucleotide synthesis. Indeed, higher concentrations of glycine inhibited proliferation. Under these conditions, glycine was converted to serine, a reaction that would deplete the one-carbon pool. Providing one-carbon units by adding formate rescued nucleotide synthesis and growth of glycine-fed cells. We conclude that nucleotide synthesis and cancer cell proliferation are supported by serine—rather than glycine—consumption.

INTRODUCTION

Malignant development is accompanied by genetic changes in cancer cells that drive abnormal proliferation, growth, survival, and invasion (Hanahan and Weinberg, 2011). Each of these phenotypes is supported by changes in cellular metabolism, and several metabolic enzymes have been identified as oncogenes or tumor suppressors (Shaw and Cantley, 2012). Although alterations in glucose and glutamine metabolism are central to metabolic transformation (Jones and Schulze, 2012), recent studies have focused on the role of the nonessential amino acids serine and glycine in supporting tumor growth (Locasale, 2013). In addition to their role in protein synthesis, serine and glycine contribute to anabolic pathways important for the generation of glutathione, nucleotides, phospholipids, and other metabolites (Locasale, 2013) (Figure S2). The requirement for intracellular serine and glycine for the support of cell growth and division is therefore clear. However, how cancer cells obtain these nutrients (uptake versus biosynthesis) and how they metabolize them remains to be fully elucidated. Mechanistic insight into this question will significantly enhance our ability to target serine/glycine metabolism for therapeutic gain.

Amplification of PHGDH, the first enzyme of the de novo serine synthesis pathway (SSP), has been found in breast cancers and melanomas (Locasale et al., 2011; Possemato et al., 2011). Many tumor cells, however, remain highly dependent on uptake of exogenous serine (Jain et al., 2012; Maddocks et al., 2013), suggesting that in these cells, de novo serine synthesis alone cannot support the requirements for proliferation. A recent study showed that glycine uptake is correlated with rapid proliferation (Jain et al., 2012); this contrasts with other studies showing that excess dietary glycine has an inhibitory effect on tumorigenesis in multiple in vivo models (Rose et al., 1999a, 1999b). Our own work suggests that cancer cells fail to consume glycine when serine is plentiful (Maddocks et al., 2013).

The present lack of clarity is due, at least in part, to the complexity of serine and glycine metabolism, which can be carried out by mitochondrial and cytoplasmic pathways (Tibbetts and Appling, 2010), both of which are upregulated in cancer (Jain et al., 2012; Locasale et al., 2011; Possemato et al., 2011; Snell, 1985; Zhang et al., 2012). Serine can be converted to glycine by serine hydroxymethyl transferase (cytoplasmic, SHMT1; mitochondrial, SHMT2), a reaction that yields one-carbon units, which enter the tetrahydrofolate (THF) cycle and are critical for nucleotide synthesis. Glycine can also be cleaved by the mitochondrial glycine cleavage system to yield one-carbon units that are transferred to the THF cycle (Tibbetts and Appling, 2010) (Figure S2). Amplification of GLDC (a component of the glycine cleavage system) in cancers (Zhang et al., 2012) suggests that this pathway is an important source of one-carbon units. In addition to cleavage, glycine can also be converted into serine by SHMT1 and SHMT2. Taken together, these reactions allow serine and glycine to be metabolized into the same set of metabolic precursors, suggesting that serine and glycine may be used interchangeably and may be equally effective in supporting proliferation (Figure S2).

In this study we demonstrate that exogenous glycine cannot substitute for serine for the support of cancer cell proliferation. Tracking the intracellular fate of exogenous serine and glycine showed that in the absence of exogenous serine, glycine does not enter the one-carbon cycle, but is converted into serine, a process that consumes rather than produces

one-carbon units and prevents nucleotide synthesis. Consistent with this, we show that the inability of cells to grow in glycine could be rescued by addition of formate, which directly supplements the one-carbon pool for nucleotide synthesis.

RESULTS

Serine, but Not Glycine, Supports Maximal Proliferation of Cancer Cells

To test the ability of either serine or glycine to independently support cancer cell proliferation, we formulated medium analogous to Dulbecco's modified Eagle's medium (DMEM, which contains 0.4 mM serine and 0.4 mM glycine), but lacking serine and glycine (assay medium; "No Ser/Gly"). This medium could be supplemented by increasing concentrations of serine or glycine, allowing us to assess the effect of these nutrients—individually or in combination—on cell proliferation. We tested multiple cancer cell lines and found a range in requirements for exogenous serine and glycine. Some cell lines (e.g., MCF7 and MDA-MB-231) showed complete growth inhibition in the absence of serine and glycine, reflecting very low expression of enzymes for de novo serine synthesis, as reported previously (Possemato et al., 2011). However, most showed an intermediate phenotype, with reduced proliferation without serine and glycine (Figures 1A and S1A). In all seven cell lines, serine alone completely rescued proliferation at 0.4–2 mM. In contrast, 0.4 mM glycine produced a very modest improvement in proliferation (compared to no serine or glycine), and higher concentrations of glycine (1–2 mM) caused various degrees of proliferative inhibition. Glycine alone had little effect on overall cell viability, suggesting this effect was due to a proliferative defect rather than increased cell death (Figure S1B).

Cancer Cells Consume Serine in Preference to Glycine, and High Serine Uptake Corresponds with Glycine Efflux

To assess cellular uptake of exogenous serine and glycine, we measured their levels over time in culture medium. Intriguingly, under conditions where equimolar serine and glycine were present, serine was rapidly depleted from the medium, whereas glycine levels initially increased (Figures 1B and S1C). Extracellular glycine levels did not start to fall until the medium was depleted of serine (generally 0.1–0.2 mM) suggesting that these cells (which are proliferating rapidly) switch to glycine consumption when most of the available serine has been used. Glycine was also released into the medium when cells were fed serine only. However, when fed glycine only, cells showed modest glycine uptake and did not release serine. The initial rate (0–16 hr) of serine and glycine uptake and release were quantified for each cell line and normalized to the cell number (Figure 1C). Only A549 and MDA-MB-468 cell lines did not consume serine during this initial period (but did consume serine after 16 hr); all seven cell lines released glycine. MDA-MB-468 cells have *PHGDH* amplification with enhanced SSP activity (Possemato et al., 2011) and showed the highest per-cell rates of glycine release, suggesting that in cells with elevated SSP activity, glycine generated from de novo serine is effluxed.

Exogenous Serine and Glycine Concentration Influence but Do Not Dictate Their Intracellular Levels and Do Not Alter SHMT1, SHMT2, and GLDC Expression

Labeled metabolites containing heavy carbon and nitrogen atoms can be tracked by liquid chromatography-mass spectrometry (LC-MS) by virtue of their increased mass compared to natural ^{12}C and ^{14}N. After feeding cells heavy isotope ($^{13}C_3$ and $^{15}N_1$) labeled serine, we detected labeled glycine in the medium, confirming the intracellular conversion of serine to glycine followed by glycine efflux (Figure 2A). Analysis of intracellular metabolite concentrations revealed higher glycine than serine levels in fully fed (serine and glycine) cells and that glycine levels were higher in these cells than those fed 0.4 mM glycine only (Figure 2B). Appreciable glycine levels were also detected in cells fed serine only, supporting the idea that exogenous serine is converted to intracellular glycine. The higher levels of glycine seen in the cells fed serine and glycine are likely to be promoted by the higher extracellular glycine concentration under these conditions (compared to cells fed serine only), raising the intracellular concentration required for passive efflux. We assessed the effects of these changes on the major enzymes for serine and glycine interconversion and cleavage, SHMT1, SHMT2, and GLDC (Figure S2), which are all expressed by HCT116 cells. Interestingly, the changes in intracellular serine and glycine levels did not cause significant variation in the protein expression of these metabolic enzymes (Figure 2C).

Cells Fed Glycine without Serine Have Depleted Nucleotide Pools

To assess the general metabolic effects of serine/glycine consumption, we performed steady-state metabolomic analysis on HCT116 and RKO cells (Figures 3 and S3). We reported previously that serine is an allosteric activator of PKM2 and that serine deprivation causes PKM2 inhibition, resulting in accumulation of glycolytic intermediates for diversion into the SSP (Chaneton et al., 2012; Maddocks et al., 2013). Cells depleted of serine and fed only glycine had low steady-state serine levels, with elevated phosphoenolpyruvate (PEP) and decreased lactate, indicative of a low level of PKM2 activity (Figures 3A and S3A).

Serine/glycine metabolism provides essential precursors for the synthesis of reduced glutathione (GSH) and purine nucleotides, both of which are important in proliferating cells. Whereas GSH levels were maintained at relatively constant levels under all conditions, purine nucleotide metabolites (AMP and GMP) were markedly depleted in glycine- versus serine-fed cells (Figures 3A, S3A, and S3B). Further analysis of the purine biosynthesis pathway revealed an accumulation of glycinamide ribonucleotide (GAR), the metabolite generated after incorporation of glycine, but a decrease in downstream metabolites that require the addition of one-carbon units from the THF cycle (Figure 3B). In RKO cells, IMP levels were depleted in line with AMP and GMP. However, although AMP and GMP also fell in HCT116 cells, IMP levels remained relatively constant. We noted that steady-state levels of IMP were particularly low in these cells ($\sim$1 nmol/10^6 cells), suggesting that flux through IMP may be too rapid to allow accumulation of steady-state levels in the HCT116 cells. Taken together, the deficiency in nucleotide synthesis in cells fed only glycine is intriguing given

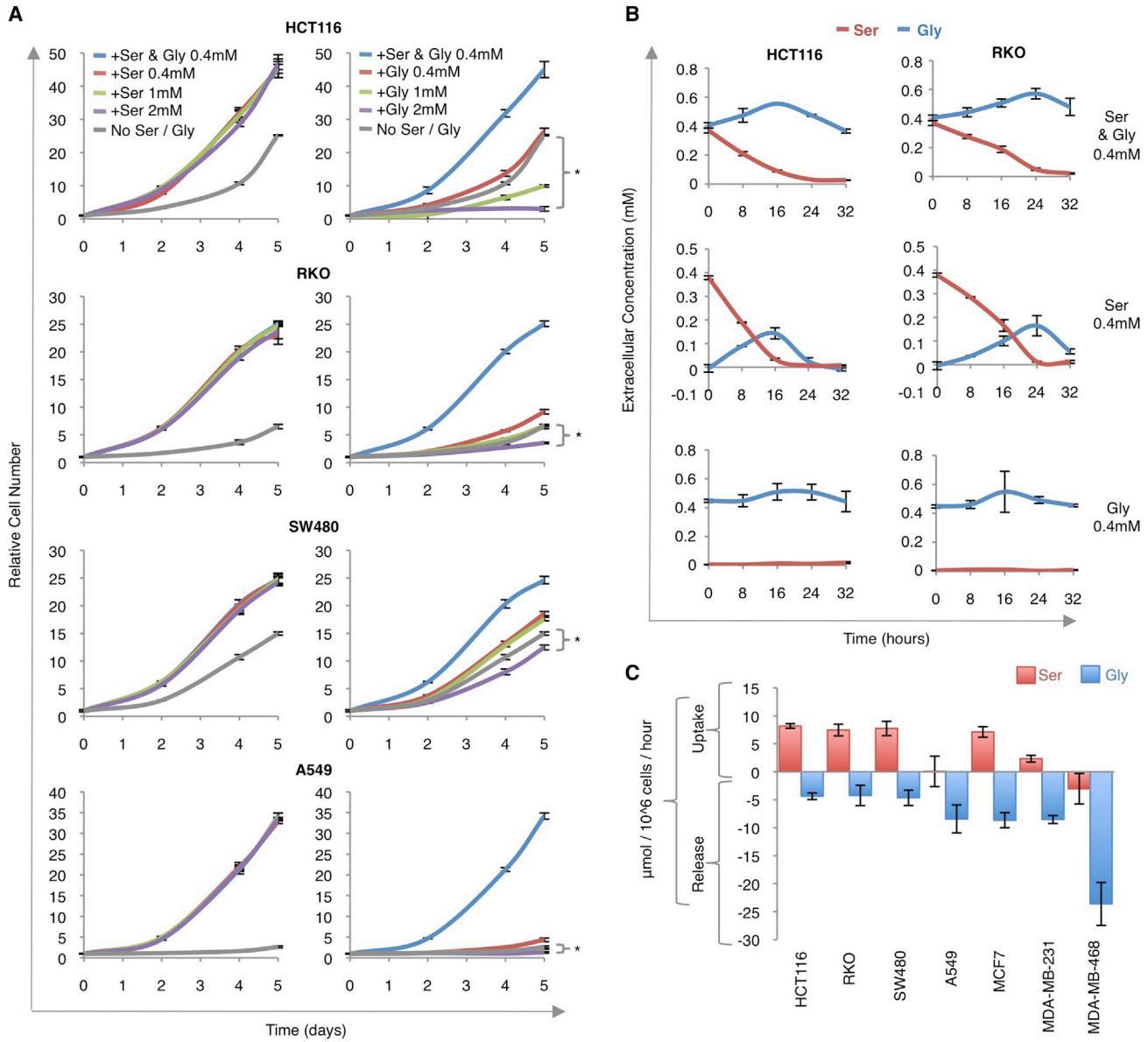


Figure 1. Serine Supports Rapid Proliferation and Cannot Be Substituted by Glycine, which Can Inhibit Proliferation

(A) Cancer cell lines were grown in the presence or absence of serine and/or glycine at the stated concentrations; cell numbers were counted over time. Data are means of triplicate wells; bars represent SD; *p < 0.05; only "No Ser/Gly" versus "+Gly 2 mM" compared.

(B) HCT116 and RKO were grown in media containing serine and/or glycine; media were sampled at the stated time points and analyzed for extracellular nutrient content by LC-MS. Data are means of triplicate wells; bars represent SD.

(C) Cells were grown in medium containing 0.4 mM serine and glycine; nutrient uptake and release was quantified for the first 16 hr using LC-MS and normalized to cell number. Data are means of triplicate wells; bars represent SD.

See also Figure S1.

that glycine alone can theoretically provide all of the precursors contributed by serine to support nucleotide synthesis (i.e., one-carbon units and glycine, Figure S2).

Excess Glycine Drives Intracellular Glycine-to-Serine Conversion and Inhibits Flux of Glycine to Purines

To assess how glycine is metabolized, we fed cells labeled $^{13}C_2{}^{15}N_1$ glycine. When fed glycine only, cells maintained incorporation of glycine into GSH at levels similar to those seen in cells fed serine and glycine. However, cells fed only glycine had no detectable incorporation of glycine into purine nucleotides at 1–3 hr (AMP and GMP, Figure 4A). Further analysis of the purine synthesis pathway confirmed that cells fed only glycine accumulated glycine-labeled GAR, but that flux through to downstream nucleotides (AMP, ADP, GMP, and GDP) was diminished (Figure S4A). These data suggest that glycine alone is unable feed

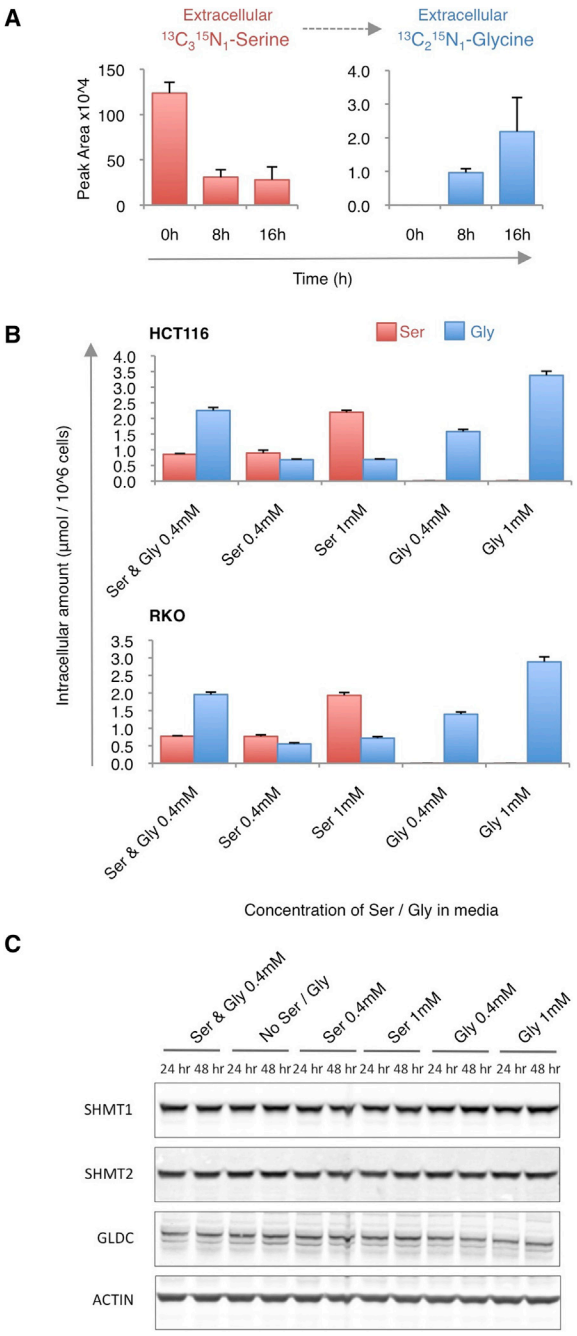


Figure 2. Extracellular Serine and Glycine Influence Intracellular Levels

(A) HCT116 cells were fed medium containing 0.4mM labeled ($^{13}C_3{}^{15}N_1$) serine (without glycine), and nutrient uptake and release was measured by analyzing media samples via LC-MS.

(B) HCT116 and RKO cells were grown in media with varying concentrations of serine and glycine for 24 hr. The media were replaced, and after 3 hr intracellular metabolites were extracted and analyzed via LC-MS. Data are means of triplicate wells; bars represent SD.

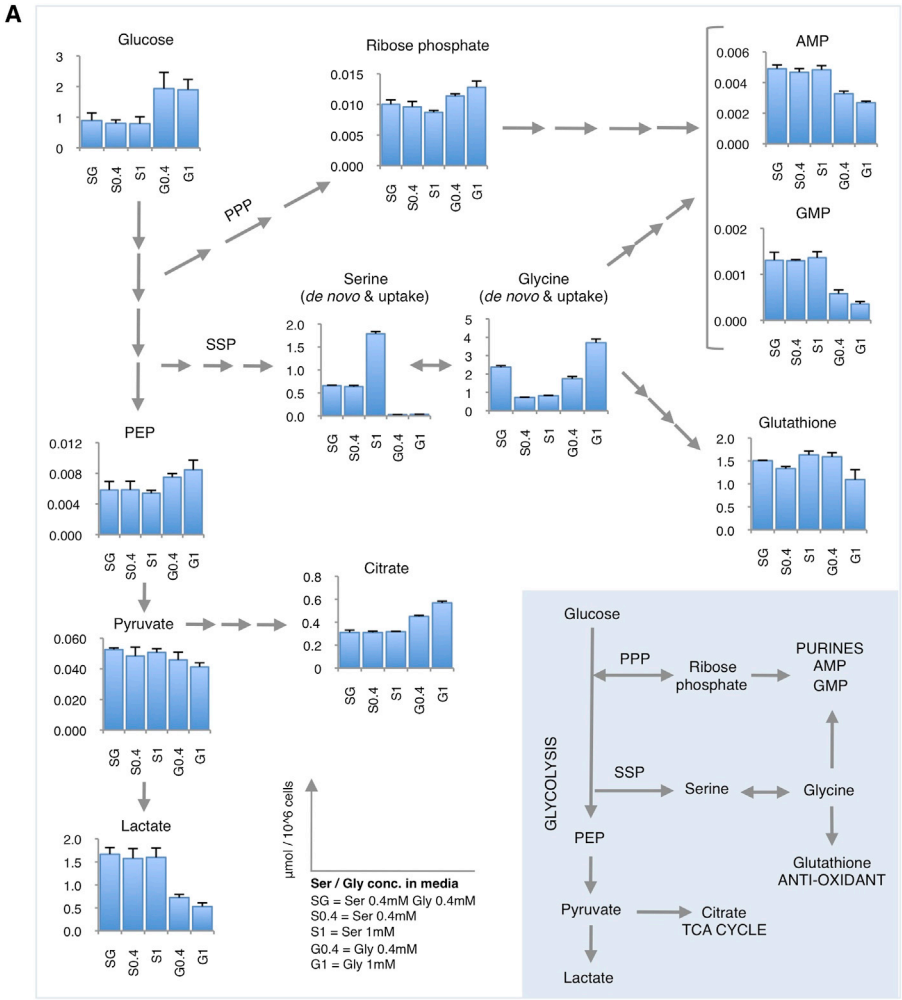
(C) HCT116 cells were grown in media with or without varying concentrations of serine and glycine for 24–48 hr. Expression of enzymes that directly metabolize serine and glycine were determined by western blot.

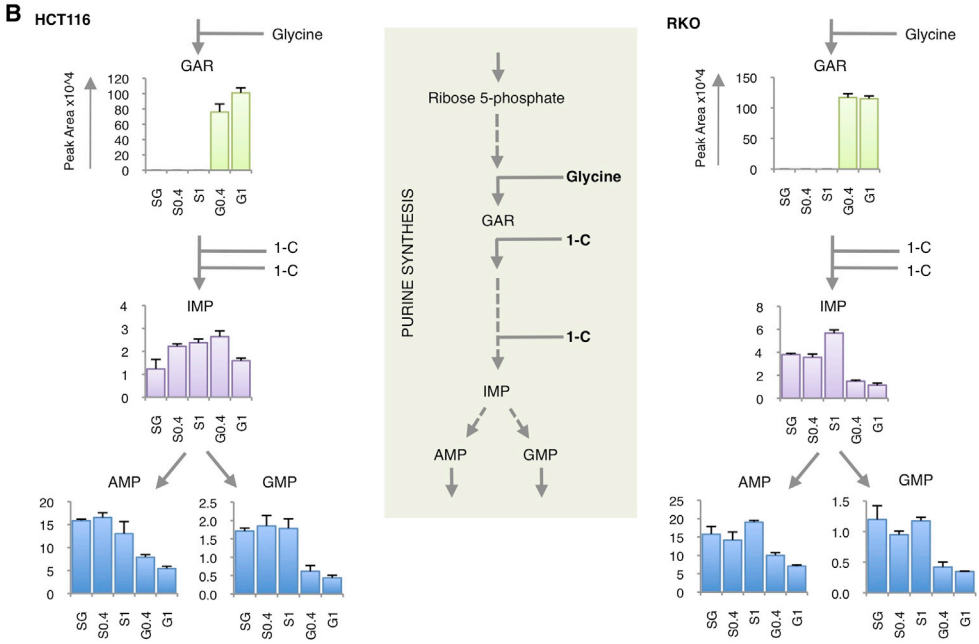
See also Figure S2.

the one-carbon cycle needed to support nucleotide (but not GSH) synthesis. This was unexpected, given that glycine can be converted to methyl THF (Me-THF) derivatives in the mitochondria by the glycine cleavage system, a pathway amplified in some cancers (Zhang et al., 2012). However, our results indicate that glycine cleavage does not support one-carbon metabolism for purine nucleotide synthesis in the cells tested here. Stable knockdown of GLDC did not impede the proliferation of HCT116 cells under fully fed, serine-fed, or glycine-fed conditions (Figure S4B), although a slight increase in intracellular glycine levels in the GLDC-depleted cells suggest that the glycine cleavage system is somewhat functional in these cells (Figure S4C). Importantly, however, GLDC depletion under fully fed conditions did not prevent normal metabolic flux of glycine into purine synthesis or deplete overall purine levels, supporting the suggestion that glycine cleavage does not make an appreciable contribution to purine synthesis in these cells. (Figure S4C).

In light of these results, we sought to understand more fully the fate of glycine taken up into cells fed only glycine. We noted that in cells fed both serine and glycine, some of the labeled glycine was converted to serine, consistent with previous analyses showing that glycine can be a significant source of serine (Kalhan and Hanson, 2012). However, glycine contributed to only a small proportion of the total serine pool, which was dominated by unlabeled serine (taken up from medium and/or synthesized de novo). By comparison, in cells fed only glycine, labeled serine accumulation was barely detectable using standard conditions with $^{13}C_2{}^{15}N_1$ glycine (Figure 4A). However, given the severely diminished steady-state levels of serine in the glycine-fed cells, we considered the possibility that our failure to detect newly synthesized serine might reflect a rapid conversion of serine to other metabolites (e.g., for protein and/or phospholipid synthesis). To test this hypothesis, we developed a new method for measuring metabolic flux that we have called "Pulse-Stop-Flux" analysis. As cellular serine uptake is extremely rapid, adding a "pulse" of (unlabeled) serine into the medium very rapidly increases the intracellular serine pool, allowing the measurable accumulation of labeled serine at short time points after addition of the pulse.

Remarkably, spiking 1 mM serine to the medium of cells previously fed only glycine caused intracellular serine levels to rise to fully fed levels in just one minute (Figure 4B). Furthermore, this pulse of unlabeled serine caused labeled serine (derived from $^{13}C_2{}^{15}N_1$ glycine) to accumulate. Quantifying labeled serine accumulation during this one-minute period allowed us to estimate the rate per minute of glycine-to-serine conversion in these cells (Figure 4C). Comparing these rates showed that increasing the exogenous concentration of glycine led to a proportional increase in the rate of glycine-to-serine conversion. These results showed that excessive intracellular glycine levels promote the conversion of glycine into serine, a process that consumes one-carbon units (thus competing with purine synthesis for one-carbon units), explaining the inhibition of purine synthesis caused by excess glycine. Furthermore, we noted here (and in all other experiments using labeled glycine) that fully labeled (i.e., $^{13}C_3{}^{15}N_1$) serine was not detected. Instead, only partially labeled ($^{13}C_2{}^{15}N_1$ including one unlabeled ^{12}C) serine was found,





(legend on next page)

indicating that the one-carbon unit used to synthesis serine from glycine was not derived from glycine cleavage. This provides further evidence that glycine cleavage does not appreciably contribute to the one-carbon pool in the cells tested here.

Formate Rescues Glycine-Induced Inhibition of Proliferation

Given that conversion of glycine to serine requires donation of a one-carbon unit from the THF cycle (Figure S2), we considered the possibility that the glycine-to-serine conversion detected in glycine-fed cells could prevent flux of one-carbon units into purine synthesis. To test this hypothesis, we replenished the one-carbon pool directly by adding formate to the media of glycine-fed cells. Formate contains a single carbon atom and reacts directly with THF, making formyl-THF a one-carbon donor for purine synthesis (Tibbetts and Appling, 2010). Although formate alone (i.e., in the absence of glycine) was unable to support proliferation, it completely rescued growth when given with either 1 mM or 2 mM glycine (Figures 5A and S5A). In glycine-fed cells, we confirmed that flux of glycine into purine synthesis was restored by formate. Whereas glycine-fed cells had low steady-state levels of purines, accumulation of GAR and low flux of glycine into purine synthesis (Figures 4A, 5B, and S4A), cells fed glycine and formate showed release of GAR accumulation, restoration of flux to purines, and steady-state levels of purines comparable to cells fed serine and glycine (Figures 5B and S5B).

Using the Pulse-Stop-Flux technique, we assessed whether the rescue seen following formate addition enhanced the rate of glycine-to-serine conversion. These analyses showed that formate did not increase the rate at which serine was synthesized from glycine, which actually showed a slight decrease (Figure 5C). This suggested that formate-induced rescue was the result of direct incorporation of formate into nucleotide synthesis via formyl-THF, and not the result of increased glycine-to-serine conversion.

To test this model and further analyze the use of serine, glycine, and one-carbon metabolites for purine synthesis, we used a range of different labeled metabolites and tracked their incorporation into GAR, AMP, ADP, GMP, and GDP using LC-MS. We were unable to reliably measure labeled IMP (which is close to the threshold of detection) in this experiment. Cells fed labeled serine ($^{13}C_3{}^{15}N_1$) accumulated purines containing predominantly m+5 and m+2 isotopomers, indicating that exogenous serine alone provides both the one-carbon and glycine components necessary for purine synthesis (Figures 6 and S6). As expected, cells fed labeled glycine ($^{13}C_2{}^{15}N_1$) showed accumulation of glycine in GAR but low levels of label incorporation in downstream metabolites, with m+3 (derived directly from a complete molecule of glycine) being the major isotopomer. This further supported the suggestion that glycine cleavage does not provide enough one-carbon units for nucleotide synthesis in these cells; if glycine cleavage had contributed significantly to purine synthesis, labeled one-carbon units would have been generated from cleaved glycine. If this were the case, m+1, m+2, and m+5 peaks would have been detected, producing a labeling pattern similar to that of serine. However, this labeling pattern was not detected. In contrast, adding unlabeled formate greatly increased incorporation of labeled glycine into purines (m+3), further confirming that a lack of one-carbon units for purine synthesis underlies the growth inhibition of glycine-fed cells. Furthermore, labeled ($^{13}C_1$) formate (when fed with unlabeled glycine) was detected in m+1 and m+2 isotopomers, confirming that the formate is incorporated as one-carbon units for nucleotide synthesis.

DISCUSSION

Although cancer cells are clearly dependent on the availability of numerous extracellular nutrients, some of these—such as essential amino acids, glucose, and glutamine—are difficult to limit in vivo. In contrast, we have shown in mice that serum serine and glycine can be significantly depleted by manipulation of diet (Maddocks et al., 2013) and that depletion of these nonessential amino acids can inhibit tumor growth without significantly impairing health. Serine and/or glycine depletion is therefore a potentially attractive therapeutic approach for cancer treatment, but before this approach can be fully realized it is critical to establish the true nature of serine, glycine, and one-carbon metabolism at a cellular level.

It is becoming clear that cancer cells use different mechanisms to achieve the metabolic changes that are required for the support of transformation (Cantor and Sabatini, 2012). Maintaining adequate flux through the one-carbon cycle and supporting nucleotide synthesis is emerging as a critical pathway in allowing malignant transformation, but this pathway can be fed by different mechanisms: some cancers amplify the endogenous SSP (Locasale et al., 2011; Possemato et al., 2011), whereas others cannot adequately activate the SSP and remain highly dependent on uptake of exogenous serine to support growth. The interconnection of serine and glycine metabolism suggests that serine and glycine may be equally effective in supporting cancer cell proliferation. In theory, all the precursors for nucleotide synthesis could be provided by serine or glycine alone. Indeed, the ability of some cancer cells to cleave glycine has been demonstrated in studies showing that overexpression of GLDC supports tumor initiation of non-small cell lung cancers (Zhang et al., 2012). This pathway is capable of generating one-carbon units for the THF cycle and, assuming that one-carbon units generated in mitochondria can be accessed for

Figure 3. Exogenous Glycine Fails to Support Nucleotide Synthesis

(A) HCT116 cells were grown in media with varying concentrations (conc.) of serine and glycine for 24 hr. The media were replaced, and after 3 hr, steady-state levels of intracellular metabolites were analyzed via LC-MS. Data are means of triplicate wells; bars represent SD. PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; SSP, serine synthesis pathway.

(B) Purine nucleotide levels in HCT116 and RKO cells were analyzed as described in (A). The data shown for AMP and GMP in RKO cells are also shown in (A). 1-C, one-carbon unit.

See also Figure S3.

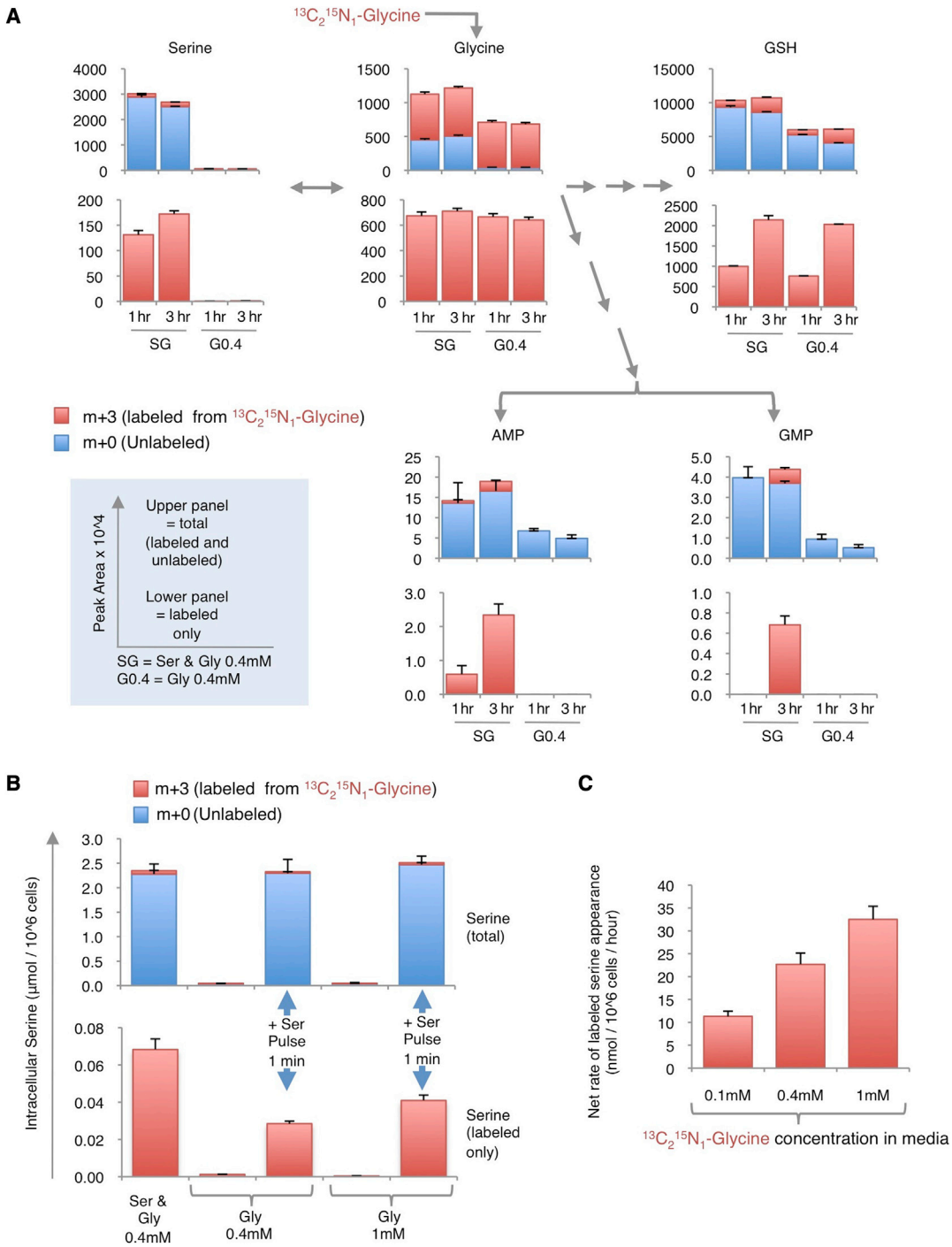


Figure 4. Cells Fed Only Glycine Have Enhanced Glycine-to-Serine Conversion

(A) HCT116 cells were grown in media with serine and glycine or glycine only for 24 hr. The media were replaced with matched media containing labeled ($^{13}C_2{}^{15}N_1$) glycine, and after 1 hr and 3 hr, intracellular metabolites were extracted and analyzed via LC-MS. Data are means of triplicate wells; bars represent SD.

(B) HCT116 cells were grown in media with varying concentrations of serine and glycine for 24 hr. The media were then replaced with media containing labeled ($^{13}C_2{}^{15}N_1$) glycine. After 1 hr intracellular metabolites were extracted and analyzed via LC-MS. To allow the accumulation and measurement of labeled serine (synthesized from labeled glycine), we added a pulse of exogenous serine (final concentration in media, 1 mM) to wells and lysed the cells after 1 min. Data are means of triplicate wells; bars represent SD.

(C) The Pulse-Stop-Flux method demonstrated in (B) was used to calculate the net rates of labeled serine appearance (i.e., glycine-to-serine conversion) in HCT116 cells under various glycine concentrations. Data are means of triplicate wells; bars represent SD.

See also Figure S4.

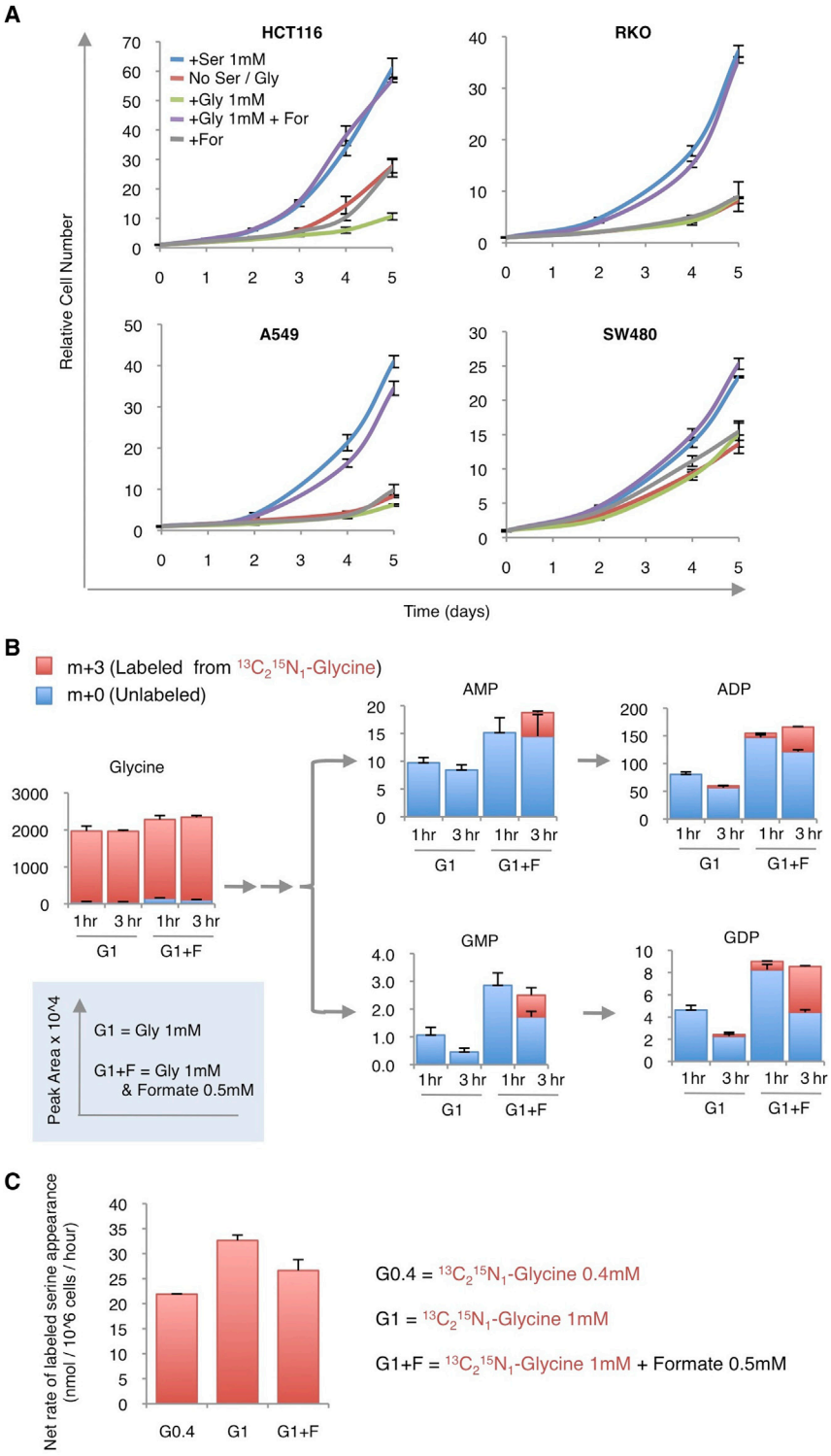


Figure 5. Formate Rescues the Growth-Inhibitory Effects of Glycine by Restoring Purine Synthesis

(A) HCT116, RKO, A549, and SW480 cells were grown in media containing 1 mM (or 2mM, Figure S4A) serine or glycine with or without formate (For); cell numbers were counted over time. Data are means of triplicate wells; bars represent SD.

(B) HCT116 cells were grown in media with glycine only or glycine and formate for 24 hr. The media were replaced with matched media containing labeled ($^{13}C_2{}^{15}N_1$) glycine; after 1 hr and 3 hr, intracellular metabolites were extracted and analyzed via LC-MS. Data are means of triplicate wells; bars represent SD.

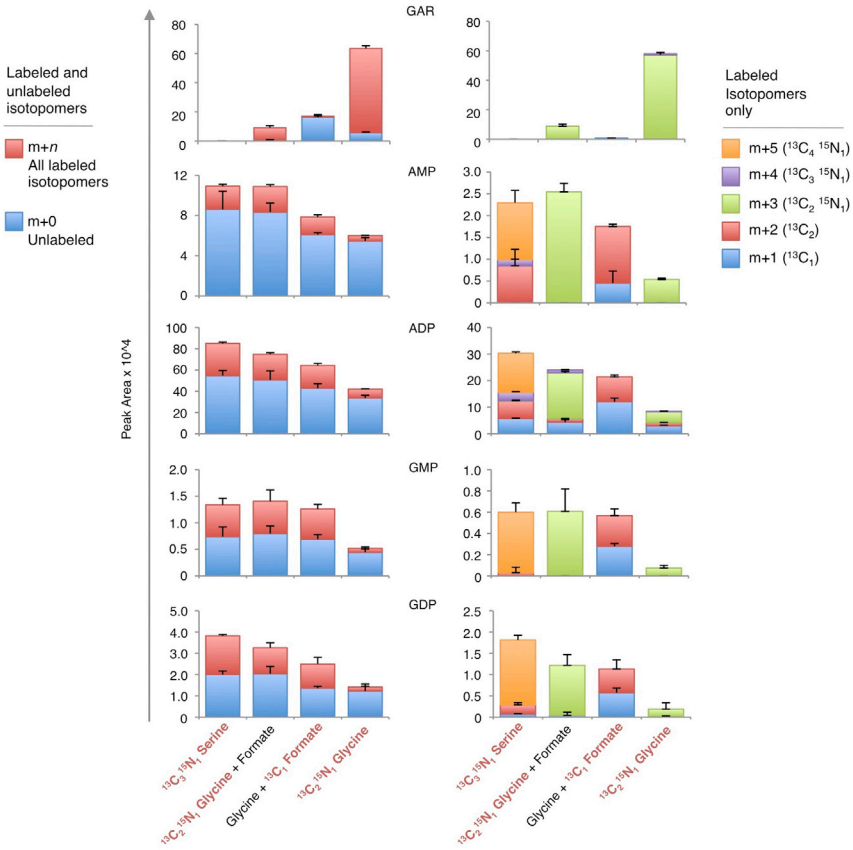
(C) The Pulse-Stop-Flux method demonstrated in Figure 4B was used to calculate the net rates of labeled serine appearance (i.e., glycine-to-serine conversion) in HCT116 cells under various glycine concentrations, with or without formate. Data are means of triplicate wells; bars represent SD.

See also Figures S2 and S5.

nucleotide synthesis, should be able to support cell growth using only glycine.

However, our data show that in a variety of cancer cell lines, the glycine cleavage system does not provide enough one-carbon units to adequately support purine synthesis and proliferation using only glycine. This is intriguing, given that global gene-expression data indicates that the cell lines used in our study all show increased GLDC expression compared to normal tissue (Barretina et al., 2012). We show in these cells that excess glycine is not used to generate Me-THF for purine synthesis, but is converted to serine through a pathway that depletes rather than replenishes Me-THF. These observations explain why increasing glycine levels progressively inhibit, rather than rescue, cell growth. Our results suggest that in a proportion of cancers, providing one-carbon units for purine synthesis is absolutely dependent on serine rather than glycine uptake. We therefore suggest that the acquisition of enhanced GLDC activity may provide an advantage during tumor initiation and/or growth in some cancers for other reasons, possibly degrading excess glycine that might otherwise accumulate and cause the growth-inhibitory effects we have described.

Another relevant previous study showed a clear correlation between glycine consumption and proliferative rate, suggesting that rapidly proliferating cancer cells preferentially take up glycine (Jain et al., 2012). Although our data also show that highly proliferative cells consume glycine, the cells only switch to glycine consumption once exogenous serine has been depleted. We suggest the previously reported correlation between glycine uptake and proliferation rate reflects the rapid depletion of exogenous serine, which



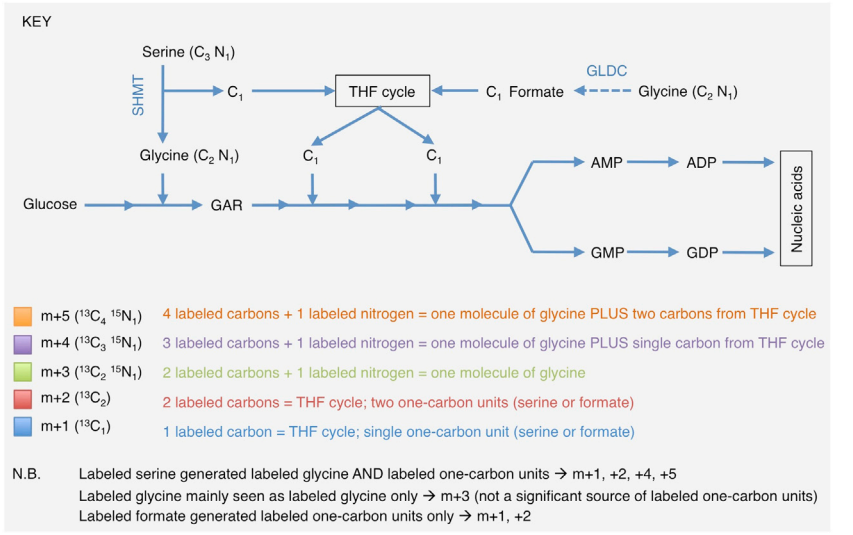


Figure 6. Nucleotide Isotopomer Distribution Confirms the Relative Contribution of Serine, Glycine, and Formate to Purine Synthesis

HCT116 cells were grown in media with serine or glycine and with or without formate (For) for 24 hr (all nutrients were unlabeled for this period). After 24 hr, media were replaced with matching media containing labeled ($^{13}C_3{}^{15}N_1$) serine only, labeled ($^{13}C_2{}^{15}N_1$) glycine only, labeled ($^{13}C_2{}^{15}N_1$) glycine with unlabeled formate, or unlabeled glycine with ($^{13}C_1$) formate. After 3 hr, intracellular metabolites were extracted and analyzed via LC-MS. Data are means of triplicate wells; bars represent SD.

is limited in a closed cell-culture system, by rapidly proliferating cells. We propose that glycine uptake is therefore a consequence of rapid proliferation, rather than its cause. Analysis of the primary data from this comprehensive study reveals that all cell lines tested consumed more serine than glycine, and it seems possible that the more-slowly proliferating cells did not take up glycine because they had not depleted their culture medium of serine over the course of the study.

Interestingly, we noted that coincident with maximal serine consumption, cells released glycine. This phenomenon can be explained by the specific precursor demands of nucleotide synthesis. Synthesizing a complete set of DNA bases (ATGC) requires (from serine/glycine metabolism) five one-carbon units and two molecules of glycine. Converting five molecules of serine to glycine yields all five one-carbon units required, but a three-molecule excess of glycine. We propose that cells release some of the excess glycine to limit intracellular glycine accumulation and thus facilitate further serine uptake and serine-to-glycine conversion to maintain maximal nucleotide synthesis and proliferation. Furthermore, as discussed above, some cells may also show enhanced glycine cleavage as an alternative (or additional) mechanism for limiting glycine accumulation.

Metabolic flux (the rate of conversion of one metabolite to another) is generally considered the critical parameter in determining the activity of a given metabolic pathway. Whereas transcriptional, proteomic, and steady-state metabolic data is now relatively easy to generate, elucidating metabolic fluxes is more challenging. Isotope labeling is an essential tool in probing metabolic fluxes, and analysis of the rate at which a nonlabeled metabolite is replaced by a labeled form allows estimation of metabolic flux. However, in highly active pathways where metabolite turnover is extremely rapid and metabolite steady-state levels are very low, the accumulation of labeled metabolite may not be detected. This is particularly relevant during nutrient starvation, wherein intracellular levels of a nutrient fall and the remaining low levels are rapidly converted into downstream metabolites. To overcome this issue, we developed a new technique involving saturation of the produced metabolite, concurrent with flux of a

$^{13}C^{15}N$-labeled substrate, which we have termed "Pulse-Stop-Flux" analysis. This technique allowed us to detect increased flux from glycine to serine under serine-starved, glycine-fed conditions and will be more widely applicable to the analysis of other pathways.

EXPERIMENTAL PROCEDURES

Cell Culture

HCT116 and RKO cells were a gift of Prof. Bert Vogelstein. SW480, A549, MDA-MB-231, MDA-MB-468, and MCF7 cells were obtained from ATCC. Cell-culture products were obtained from Gibco unless otherwise stated; catalog numbers are shown in parentheses. Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. Stock cells were maintained in McCoy's 5A medium (26600) supplemented with 10% fetal bovine serum (FBS; 10270) and penicillin-streptomycin, or in DMEM (21969) supplemented with 10% FBS (G10270), 2 mM L-glutamine, and penicillin-streptomycin. For starvation experiments, "assay medium" lacking serine and glycine was formulated with MEM (21090) supplemented with dialysed FBS (HyClone, Thermo Scientific), 2 mM L-glutamine, D-glucose (Sigma-Aldrich; final concentration 17 mM), MEM vitamins (11120), and penicillin-streptomycin.

Proliferation Assays

Cells were seeded in complete McCoy's 5A/DMEM medium at 1×10^4 – 3×10^4 cells per well in 24-well plates and allowed to adhere for 20–40 hr. Before adding assay media, cells were washed briefly in PBS. Media were formulated by adding serine/glycine/formic acid (all Sigma) at the stated concentrations to the assay medium described above. Formic acid was used at 0.5–0.25 mM (1:50,000–1:100,000 dilution in media). Triplicate wells were seeded for each experimental condition, and replicate plates were seeded for counting at the specified time points, including "time zero" so that relative cell numbers could be calculated. Cells were trypsinized, resuspended in PBS-EDTA, and counted with a CASY Model TT Cell Counter (Innovatis, Roche Applied Science). For ease of comparison, proliferation curves for fully fed ("+Ser & Gly 0.4mM" and starved "No Ser/Gly" conditions) are replicated on the left and right panels in Figure 1A; all curves displayed on the same axes were generated in the same experiment.

Cell Viability Assay

Cells were seeded in 24-well plates and grown in the stated concentrations of serine and glycine as described above. Cells were trypsinized and counted for trypan-blue exclusion using a hemocytometer.

Western Blot

Whole-cell protein lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer with complete protease inhibitors (Roche), separated using precast NuPAGE gels (Invitrogen, Life Technologies) and transferred to nitrocellulose membranes. Proteins were detected and quantified using a LI-COR Odyssey Infrared scanner and software (LI-COR Biosciences). Primary antibodies used were as follows: anti-GLDC (Sigma HPA002318), anti-SHMT1 (Sigma HPA023314), anti-SHMT2 (Santa Cruz sc-25064), and anti-Actin (Millipore MAB1501). Secondary antibodies for the relevant species were IRDye680LT and IRDye800CW conjugated (LI-COR Biosciences).

GLDC Silencing

Plasmids for stable knockdown of GLDC and control small hairpin RNA (shRNA) were purchased from QIAGEN (SureSilencing shRNA plasmid puromycin Human GLDC KH12731P, catalog no. 336314). HCT116 cells were transfected and selected according to the manufacturer's instructions. Stable clones were selected based on knockdown assessed by western blot. GLDC shRNA sequences were as follows: 1, ctagcagctatcatgattaca; 2, cgtctgaactcgcacctatca; control shRNA, ggaatctcattcgatgcatac.

Liquid Chromatography-Mass Spectrometry

LC-MS analysis was performed broadly as described previously (Maddocks et al., 2013). Cells (3–4 × 10^5/well) were seeded in complete medium in triplicate wells of 6-well plates; duplicate plates were seeded for cell counting. After 24–48 hr (if cells were grown for 48 hr prior to assay, medium was refreshed after 24 hr), cells were washed with PBS, and 2 ml assay medium (with or without serine, glycine, or formate) was added for 24 hr. After 24 hr growth in the stated conditions, media were replaced with matched media for 1–3 hr before metabolites were extracted. For isotopomer distribution assays, the fresh media contained $^{13}C_2{}^{15}N_1$ glycine/$^{13}C_3{}^{15}N_1$ serine/$^{13}C_1$ formate. Metabolites were extracted by lysing cells in ice-cold methanol/acetonitrile/H_2O (50:30:20). Samples were shaken at 4°C for 10 min, then centrifuged for 15 min at 16,000 × *g*, and the supernatant was collected and analyzed by LC-MS. Analytes were separated using hydrophilic interaction liquid chromatography with a SeQuant ZIC-pHILIC column (2.1 × 150 mm, 5 μm) (Merck) and detected with high-resolution, accurate-mass mass spectrometry using an Orbitrap Exactive in line with an Accela autosampler and an Accela 600 pump (Thermo Scientific). The elution buffers were acetonitrile for buffer A and 20 mM $(NH_4)_2CO_3$ and 0.1% NH_4OH in H_2O for buffer B. A linear gradient was programed starting from 80% buffer A and ending at 20% buffer A after 20 min, followed by wash (20% buffer A) and re-equilibration (80% buffer A) steps with a flow rate of 100 μl/min. The mass spectrometer was fitted with an electrospray-ionization probe and operated in full-scan and polar-switching mode with the positive voltage at 4.5 kV and negative voltage at 3.5 kV. Serine and glycine levels were quantified using five-point calibration curves spiked in cell lysates and media. Metabolite identification and data analysis were carried out using LCQUAN software (Thermo Scientific). For ease of comparison, specific data for some metabolite levels (e.g., AMP and GMP) are replicated in the main and supplemental figures, as stated in the figure legends.

Pulse-Stop-Flux Analysis

HCT116 cells were seeded and grown in triplicate wells of 6-well plates as described above. Cells were fed the stated media (containing 0.1 mM–1 mM glycine or 1 mM glycine plus 0.5 mM formate) for 24 hr; these media were then replaced with matched media wherein glycine was substituted with $^{13}C_2{}^{15}N_1$ glycine. After 1 hr, cells were either lysed and metabolites extracted, or they received a one-minute pulse of 80 mM serine added to the medium and mixed by gentle shaking (final concentration, 1 mM) before being lysed and analyzed by LC-MS as described above. The levels of $^{13}C_2{}^{15}N_1$ serine deriving from $^{13}C_2{}^{15}N_1$ glycine in pulsed conditions were subtracted from that in nonpulsed conditions to find the net amount of $^{13}C_2{}^{15}N_1$ serine synthesized in one minute. Data were plotted as the net serine synthesis at nmol/1 × 10^6 cells/hr.

Serine and Glycine Uptake and Release

Cells were seeded in 6-well plates (at appropriate seeding density to be ~90% confluent by the end of the assay) in complete medium and allowed to grow for 48 hr (medium was refreshed after 24 hr). At the start of the assay, cells were washed with PBS and received 1.5 ml per well of assay medium supplemented with both serine and glycine (0.4 mM) or serine or glycine only (0.4 mM). At the stated time points, 10 μl of medium was removed and added to 490 μl ice-cold methanol/acetonitrile/H_2O (50:30:20). These samples were prepared for LC-MS in the same way as cell extracts (described above). Nutrient uptake and release data were plotted on curved-line scatter plots (using the mean of triplicate wells ± SD) with Microsoft Excel (v.12.3.6). For each cell line, the initial uptake/release for serine and glycine was calculated by measuring the change in metabolite levels from 0–16 hr and normalizing for cell number. Cell number was determined by performing a cell count at 8 hr and using this as an estimate of the average cell number from 0–16 hr.

Statistical Analysis

Comparisons of proliferation rates (Figure 1A, performed for "No Ser/Gly" versus glycine 2mM only) and AMP and GMP levels (Figure S3B) were performed with TTEST (paired, two tails) using Microsoft Excel (v.12.3.6); $p < 0.05$ was considered statistically significant and is denoted with an asterisk.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.045.

AUTHOR CONTRIBUTIONS

C.F.L., K.H.V., and O.D.K.M. conceived the project and wrote the manuscript. C.F.L. and O.D.K.M. carried out the experiments with help from N.J.F.v.d.B. and G.M.M. in performing and optimizing LC-MS.

ACKNOWLEDGMENTS

This work was funded by Cancer Research UK and ERC grant 322842-METABOp53. We are grateful to Gabriela Kalna for advice on data analysis, Bert Vogelstein for cell lines, and Eyal Gottlieb for reading the manuscript.

Received: December 23, 2013
Revised: April 5, 2014
Accepted: April 29, 2014
Published: May 8, 2014

REFERENCES

Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A.A., Kim, S., Wilson, C.J., Lehár, J., Kryukov, G.V., Sonkin, D., et al. (2012). The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature *483*, 603–607.

Cantor, J.R., and Sabatini, D.M. (2012). Cancer cell metabolism: one hallmark, many faces. Cancer Discov. *2*, 881–898.

Chaneton, B., Hillmann, P., Zheng, L., Martin, A.C., Maddocks, O.D., Chokkathukalam, A., Coyle, J.E., Jankevics, A., Holding, F.P., Vousden, K.H., et al. (2012). Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature *491*, 458–462.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646–674.

Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A.L., Kafri, R., Kirschner, M.W., Clish, C.B., and Mootha, V.K. (2012). Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. Science *336*, 1040–1044.

Jones, N.P., and Schulze, A. (2012). Targeting cancer metabolism—aiming at a tumour's sweet-spot. Drug Discov. Today *17*, 232–241.

Kalhan, S.C., and Hanson, R.W. (2012). Resurgence of serine: an often neglected but indispensable amino Acid. J. Biol. Chem. *287*, 19786–19791.

Locasale, J.W. (2013). Serine, glycine and one-carbon units: cancer metabolism in full circle. Nat. Rev. Cancer *13*, 572–583.

Locasale, J.W., Grassian, A.R., Melman, T., Lyssiotis, C.A., Mattaini, K.R., Bass, A.J., Heffron, G., Metallo, C.M., Muranen, T., Sharfi, H., et al. (2011). Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. Nat. Genet. *43*, 869–874.

Maddocks, O.D., Berkers, C.R., Mason, S.M., Zheng, L., Blyth, K., Gottlieb, E., and Vousden, K.H. (2013). Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. Nature *493*, 542–546.

Possemato, R., Marks, K.M., Shaul, Y.D., Pacold, M.E., Kim, D., Birsoy, K., Sethumadhavan, S., Woo, H.K., Jang, H.G., Jha, A.K., et al. (2011). Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature *476*, 346–350.

Rose, M.L., Cattley, R.C., Dunn, C., Wong, V., Li, X., and Thurman, R.G. (1999a). Dietary glycine prevents the development of liver tumors caused by the peroxisome proliferator WY-14,643. Carcinogenesis *20*, 2075–2081.

Rose, M.L., Madren, J., Bunzendahl, H., and Thurman, R.G. (1999b). Dietary glycine inhibits the growth of B16 melanoma tumors in mice. Carcinogenesis *20*, 793–798.

Shaw, R.J., and Cantley, L.C. (2012). Decoding key nodes in the metabolism of cancer cells: sugar & spice and all things nice. F1000 Biol. Rep. *4*, 2.

Snell, K. (1985). Enzymes of serine metabolism in normal and neoplastic rat tissues. Biochim. Biophys. Acta *843*, 276–281.

Tibbetts, A.S., and Appling, D.R. (2010). Compartmentalization of Mammalian folate-mediated one-carbon metabolism. Annu. Rev. Nutr. *30*, 57–81.

Zhang, W.C., Shyh-Chang, N., Yang, H., Rai, A., Umashankar, S., Ma, S., Soh, B.S., Sun, L.L., Tai, B.C., Nga, M.E., et al. (2012). Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. Cell *148*, 259–272.