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Glucose-independent human cytomegalovirus replication is supported by metabolites that feed upper glycolytic branches

Rebekah L. Mokry[a,b] and John G. Purdy[a,b,c,1]

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Viruses with broad tissue distribution and cell tropism successfully replicate in various nutrient environments in the body. Several viruses reprogram metabolism for viral replication. However, many studies focus on metabolic reprogramming in nutrient-rich conditions that do not recapitulate physiological environments in the body. Here, we investigated how viruses may replicate when a metabolite thought to be essential for replication is limited. We use human cytomegalovirus infection in glucose-free conditions as a model to determine how glucose supports virus replication and how physiologically relevant nutrients contribute to glucose-independent virus production. We find that glucose supports viral genome synthesis, viral protein production and glycosylation, and infectious virus production. Notably, supplement of glucose-free cultures with uridine, ribose, or UDP-GlcNAc—metabolites that feed upper glycolytic branches like the pentose phosphate pathway—results in partially restored virus replication, including low levels of infectious virus production. Supplementing lower glycolysis in glucose-free cultures using pyruvate fails to restore virus replication. These results indicate that nutrients can compensate for glucose via feeding upper glycolytic branches to sustain low levels of virus production. More broadly, our findings suggest that viruses may successfully replicate in diverse metabolic niches, including those in the body with low glucose levels, through alternative nutrient usage.

human cytomegalovirus | herpesviruses | glycolysis | metabolism

Significance

The metabolic environment determines the ability of a virus to successfully replicate. Human cytomegalovirus (HCMV) has broad cell tropism and replicates in various tissues that have diverse and/or limiting metabolic environments. HCMV reprograms host central carbon metabolism to support viral replication, but there is little understanding of HCMV replication in diverse metabolic niches as most studies use high-nutrient culture media. Here, we show that glucose limitation suppresses virus production through loss of viral genome synthesis and viral protein glycosylation. However, nutrient compensation by metabolites that fuel upper glycolytic branches, such as the pentose phosphate pathway, supports low levels of glucose-independent virus production. Our work indicates that metabolite compensation may facilitate HCMV replication in nutrient-limited niches in the body.

Viruses are dependent on nutrient availability in their host environment to successfully replicate. Virus–host metabolic interactions are essential for the success of viruses since viral replication requires host metabolism. Glucose, a major central carbon metabolite, is an important determinant for a wide range of virus infections, e.g., human cytomegalovirus (HCMV) (1–3), herpes simplex virus 1 (4, 5), SARS-CoV-2 (6), Epstein–Barr virus (7, 8), adenoviruses (9), dengue virus (10), respiratory syncytial virus (11), Kaposi's sarcoma–associated herpesvirus (12–16), rhinoviruses (17), influenza A virus (18), hepatitis B virus (19, 20), human herpesvirus 6A (21), and norovirus (22). Many of these viruses are known to replicate and cause disease in several organs and tissues in the body. For example, HCMV is a prevalent herpesvirus that causes severe disease in immunocompromised individuals and neonates (23–25). Common complications include CMV pneumonitis, retinitis, hepatitis, and microcephaly (24–26). CMV diseases result from the virus's ability to infect, replicate, and spread in broad tissues via its extensive cell tropism and success in diverse nutrient environments (24). Currently, it is unknown how viruses, such as HCMV, replicate in diverse metabolic environments, including conditions where nutrients of central carbon metabolism may be low or limiting.

Investigations examining the flow of glucose carbons during virus infection using glucose-rich conditions yielded significant findings regarding virus–host metabolism interactions (4, 7, 27–31). However, a major caveat to these metabolic investigations is that some nutrients, such as glucose, are at supraphysiological levels while metabolites essential to metabolism are absent (32, 33). Use of common nutrient-rich media conditions could mask the contribution of alternative metabolites to virus replication, particularly those that provide compensating roles in conditions where nutrients may become limiting.

We sought to determine whether a virus can successfully replicate when an "essential" metabolite like glucose is limiting by focusing on HCMV replication. In glucose-rich conditions, HCMV globally reprograms cellular metabolism for its replication needs. HCMV promotes glucose uptake (2, 29, 34), which is accompanied by increased glycolytic flux during infection (4, 28, 35). Glucose is metabolized to obtain energy, and the flow of glucose carbons is redirected to support viral replication and production of infectious progeny (1–4, 27, 28, 35–38). Glucose supports key branch points in upper glycolysis such as the pentose phosphate pathway (PPP) and hexosamine pathway (4, 28, 30, 39), and glucose carbons at the end of glycolysis are shuttled to fatty acid synthesis and

Author affiliations: [a]BIO5 Institute, University of Arizona, Tucson, AZ 85719; [b]Department of Immunobiology, University of Arizona, Tucson, AZ 85724; and [c]Cancer Biology Interdisciplinary Program, University of Arizona, Tucson, AZ 85724

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[1]To whom correspondence may be addressed. Email: jgpurdy@arizona.edu.

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elongation to support lipid metabolism (4, 27, 28, 38, 40, 41). Failure to metabolize glucose results in loss of infectious virus production (29, 30, 34, 39, 42, 43).

In this study, we demonstrate that loss of virus production during glucose deprivation is the result of attenuated viral genome synthesis and subsequent viral protein production and viral protein glycosylation. In contrast to the current paradigm regarding the essentiality of glucose for HCMV replication, we determined that metabolites present in the body but absent from standard growth media support low levels of replication independent of glucose. By supplementing the metabolic environment of HCMV-infected cells with metabolites essential to metabolism in the body, our investigation revealed that alternative metabolite compensation promotes HCMV replication in glucose-limited environments. Supplementing glucose-free cultures with metabolites that feed upper glycolytic branches partially restores viral genome synthesis and, importantly, supports low levels of virus production independent of glucose. Supplementing with pyruvate, the final product of glycolysis, failed to restore viral genome levels or virus production. In the case of HCMV, nutrients that support upper glycolytic branches allow low levels of virus production in glucose-limiting environments. Overall, these studies reveal that viruses can replicate in diverse metabolic environments with little to no glucose via metabolic compensation.

Results

Glucose Deprivation Attenuates HCMV Genome Synthesis, Resulting in Decreased Viral Protein Levels. Glucose deprivation inhibits infectious virus production in HCMV-infected cells (1, 3). However, it is unknown which stage of replication is impacted by the loss of glucose. We examined multiple steps of HCMV replication during glucose deprivation to determine the stages that require glucose. Human foreskin fibroblasts (HFF) were infected with HCMV strain TB40/E encoding green fluorescent protein (TB40/E-GFP). A multiplicity of infection (MOI) of 2 infectious units per cell (IU/cell) was used at which most of the cells in the culture are infected. Experiments were performed in growth-arrested primary human fibroblasts in serum-free conditions that remove any contribution of nutrients from bovine serum to recapitulate conditions widely used in metabolic studies of HCMV (3, 4, 27–30, 34, 35, 38–41, 44–46). Glucose was removed at 1 h postinfection (hpi) and virus production, viral genome synthesis, and viral protein levels were quantified (Fig. 1). Cell-free and cell-associated viral titers were measured using 50% tissue culture infectious dose assay ($TCID_{50}$). Glucose-replete (25 mM) Dulbecco's modified Eagle Medium (DMEM) cultures had measurable levels of infectious cell-free virus production from 96 to 120 hpi and cell-associated virus production from 72 to 120 hpi with the highest titers at 120 hpi (Fig. 1*A*). Virus production was not detected in glucose-free DMEM cultures, consistent with previous studies (1, 3). To determine whether loss of glucose decreases HFF viability, we measured cytotoxicity of glucose-free conditions. Cells were infected or mock-infected as described above. At 48 and 120 hpi, culture supernatants were collected for lactate dehydrogenase cytotoxicity assay. At 48 hpi, cells maintained high viability in both mock- and HCMV-infected cultures (*SI Appendix*, Fig. S1*A*). By 120 hpi, mock-infected, glucose-free cell viability had decreased to 78%, while HCMV-infected, glucose-free cultures maintained 95% cell viability. At the later time, viability of HCMV-infected, glucose-replete cultures decreased to 89%, likely due to cell death that occurs from high levels of virus production at late stages of HCMV replication. These data indicate that HCMV offers protection against cell death induced by glucose deprivation and are consistent with prior HCMV studies in HFFs (1).

Since glucose can support nucleotide synthesis, we next examined viral genome synthesis from 4 to 120 hpi using qPCR. Viral

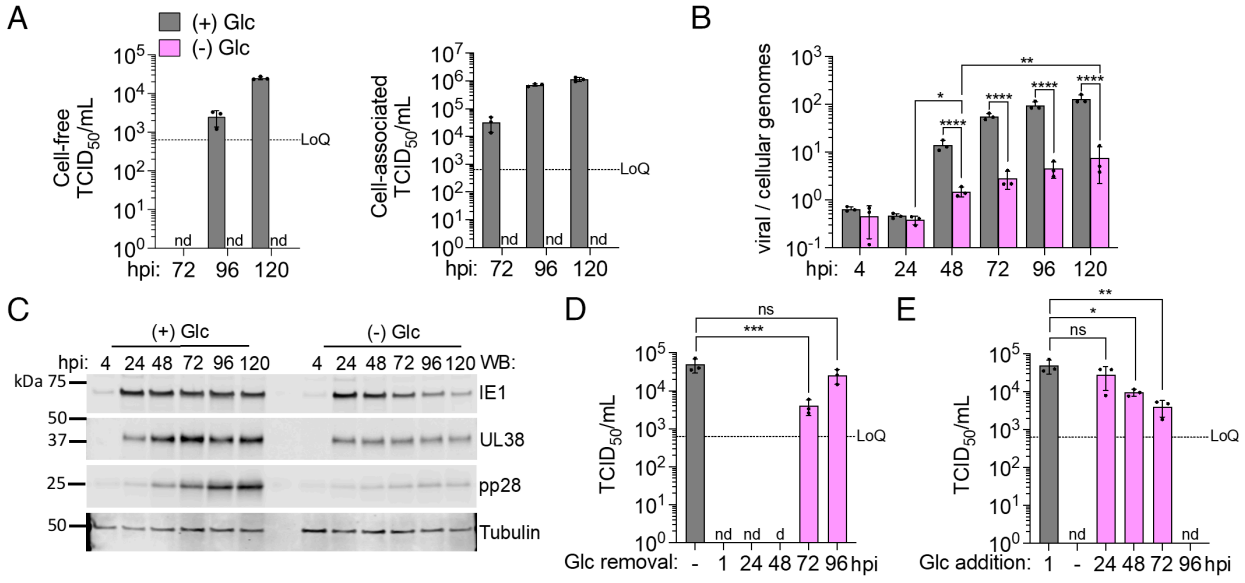

Fig. 1. Glucose supports viral genome synthesis and viral protein production and is required for infectious virus production. HFF were infected with HCMV strain TB40/E encoding free green fluorescent protein (TB40/E-GFP) at a MOI of two infectious units per cell (IU/cell). At 1 h postinfection (hpi), media were changed to glucose-replete [(+) Glc] or glucose-free [(−) Glc] DMEM. (*A*) At the indicated times, culture supernatant or cells were collected for cell-free (*Left*) and cell-associated (*Right*) virus production. Viral titers were measured by 50% tissue culture infectious dose assay ($TCID_{50}$). (*B*) Viral genomes per cell were quantified at the indicated times by qPCR. (*C*) Whole-cell lysates were collected at the indicated times and analyzed by western blot. A representative blot from three biological replicates is shown. (*D*) For glucose removal studies, glucose-replete DMEM was removed, cells were washed with phosphate-buffered saline (PBS), and media replaced with glucose-free DMEM at the indicated times. (*E*) For glucose addition studies, glucose-free DMEM was removed, cells were washed with PBS, and media replaced with glucose-replete DMEM at the indicated times. Viral titers from culture supernatant were quantified at 120 hpi by $TCID_{50}$. Limit of quantitation (LoQ) = 632 $TCID_{50}$/mL; nd, not detected; d, detected but below the LoQ. Experiments from *D* and *E* were performed concurrently using glucose-replete media (gray bar) or glucose-free media (nd) treated at 1 hpi as controls for both. Error bars represent SD. Two-way ANOVA with Tukey's (*B*) or one-way ANOVA with Dunnett's test (*D* and *E*) was used to determine significance. Statistics were performed on transformed data. ns, not significant; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***; $P < 0.0001$, ****. $n = 3$.

genome levels were attenuated by 1- to 1.3-log starting at 48 hpi (i.e., at the onset of viral genome synthesis) in glucose-free cultures compared to glucose-replete (Fig. 1*B*). Although viral genomes were significantly decreased relative to glucose-replete cultures, genomes still increased over time in glucose-free cultures, suggesting low levels of viral genome replication occur independent of glucose.

Glucose is also used to generate several amino acids needed for viral protein synthesis. We measured viral protein levels from 4 to 120 hpi to determine whether glucose is necessary to support viral protein production in each of the canonical kinetic classes of HCMV lytic replication (i.e., proteins encoded by immediate-early, early, and late viral genes). Immediate-early protein 1 (IE1) and immediate-early protein 2 (IE2) levels during glucose deprivation were similar to glucose-replete conditions up to 48 hpi, suggesting little impact on immediate-early events (Fig. 1*C* and *SI Appendix*, Fig. S1*B* and *E–F*). In contrast, early protein UL38 was significantly decreased by 48 hpi (Fig. 1*C* and *SI Appendix*, Fig. S1*C*). Another early protein UL44 was also reduced, but this decrease was only significant at 96 and 120 hpi (*SI Appendix*, Fig. S1*E* and *G*). Late protein pp28 was decreased from 48 to 120 hpi (Fig. 1*C* and *SI Appendix*, Fig. S1*D*). A second late protein, pp71, was similarly reduced at 96 and 120 hpi in glucose-free cultures (*SI Appendix*, Fig. S1 *E* and *H*). A reduction in proteins encoded by late genes is consistent with a loss in viral genome synthesis since the expression of late genes depends on viral genome replication. Taken together, these data indicate that glucose supports early viral protein production and viral genome synthesis as well as subsequent production of late viral proteins. Moreover, our findings suggest that the loss of infectious virus production caused from glucose deprivation is due to defects in early events and subsequent late steps of lytic replication.

Glucose is Essential for Infectious Virus Production but is Reversible. Based on our initial observations that glucose supports early steps in virus replication, we investigated whether the effects of glucose deprivation on HCMV are replication stage dependent. Attenuated viral genome levels suggest that glucose is required for early replication events prior to, or at the onset of, viral genome replication. We reasoned that glucose removal after initiation of viral genome synthesis would allow early events to proceed as expected while reducing activity in any glucose-dependent steps in the late stages. We infected HFFs with TB40/E-GFP in glucose-replete DMEM, then removed glucose at 1, 24, 48, 72, or 96 hpi and quantified viral titers at 120 hpi. As controls in these experiments, viral titers were determined for glucose-replete or glucose-free cultures. As we previously observed, virus production was not detected in glucose-free conditions when glucose was removed at 1 hpi (Fig. 1*D*). Glucose removal at 24 hpi also resulted in undetectable levels of infectious virus. GFP-positive infected foci below the limit of quantitation (LoQ) for $TCID_{50}$ were detected when removing glucose at 48 hpi, while glucose removal at 72 hpi resulted in measurable virus production that was reduced compared to glucose-replete. Glucose removal at 96 hpi had no effect on viral titers at 120 hpi.

We performed the reciprocal experiment (i.e., time of addition) to determine whether the impact of glucose deprivation is reversible. Cultures were glucose-starved at 1 hpi until glucose was added back at 24, 48, 72, or 96 hpi. We found glucose addition at 24 hpi supports virus production similar to glucose-replete, whereas virus produced during glucose add back at 48 or 72 hpi was reduced by ~1-log (Fig. 1*E*). Virus production at 120 hpi was not detected with glucose addition at 96 hpi, potentially due to insufficient time for recovery. These data demonstrate that defects in virus production during glucose loss are reversible with glucose addback. Further, since we observed a defect in early replicative steps and glucose dependence up to 48 hpi, our results suggest that glucose is required for early replication events and supports late replication steps.

Uridine and Ribose Supplement Support Glucose-Independent Virus Replication. Since glucose is necessary for viral genome replication, we hypothesized that metabolites that restore viral genome synthesis in the absence of glucose would promote late viral protein production, and potentially, late replicative steps. Nucleotide synthesis is supported by the conversion of glucose to ribose 5-phosphate via the PPP. Uridine can feed the PPP to restore cancer cell proliferation in glucose-limiting conditions through the production of ribose 1-phosphate and subsequent conversion to ribose 5-phosphate (47) (Fig. 2*A*). We reasoned that uridine may provide a similar restoration to HCMV genome synthesis in glucose-free cultures. Since DMEM lacks uridine, we tested this idea by feeding uridine to infected cells and measuring viral genome levels. We infected cells as previously described, supplemented with 0 to 1 mM uridine in glucose-free DMEM at 1 hpi, and measured viral genome levels and virus production at 120 hpi. As we previously observed, viral genomes were reduced in glucose-free cultures compared to glucose-repleted without uridine addition (Fig. 2*B*). Uridine supplement restored viral genomes with an ~1.5-log increase at 0.5 and 1 mM concentrations compared to glucose-free, nonsupplemented cultures. While uridine supplement in glucose-free cultures increased viral genome replication relative to the nonsupplemented conditions, viral genomes were still reduced compared to glucose-replete cultures at 120 hpi. Thus, uridine supplement provides a partial rescue of viral genome synthesis.

Next, we examined whether the partial restoration of viral genome synthesis would lead to production of infectious virus. We found that supplement of 1 mM uridine resulted in virus production above the LoQ in glucose-free conditions for one of three biological replicates (Fig. 2*C*). Moreover, in all replicates, we detected GFP-positive infected foci indicating a low but notable amount of infectious virus production independent of glucose (Fig. 2*D*). Overall, our observations demonstrate that glucose is necessary for HCMV replication in standard growth conditions and that glucose-independent replication and virus production is possible if uridine is present when glucose is limited.

Uridine restores PPP activity during glucose limitation by replacing glucose as the ribose 5-phosphate donor (47, 48). We hypothesized that if uridine restored viral genome synthesis and low-level virus production via release of ribose, then directly supplementing with ribose would result in a similar rescue (Fig. 2 *B–D*). We infected cells, supplemented 0 to 1 mM ribose in glucose-free DMEM at 1 hpi, and quantified viral genomes and virus production at 120 hpi. Similar to uridine, ribose partially restored viral genome levels with an ~1-log increase at 0.5 and 1 mM concentrations compared to glucose-free, nonsupplemented cultures (Fig. 2*E*). Supplement of 1 mM ribose in glucose-free cultures resulted in quantifiable titers, and 0.5 mM ribose supplement resulted in GFP-positive infected foci near or below the LoQ (Fig. 2*F*). In other experiments, viral titers for 1 mM ribose-supplemented, glucose-free cultures were under the LoQ with detectable GFP-positive foci formation (Fig. 2*G* and *SI Appendix*, Fig. S2*A*). We confirmed ribose restoration of virus production using a different commercially available ribose stock. Ribose supplement consistently produced infectious virus as evidenced by GFP-positive foci but were below the LoQ for three of four biological replicates, confirming that our observation is independent

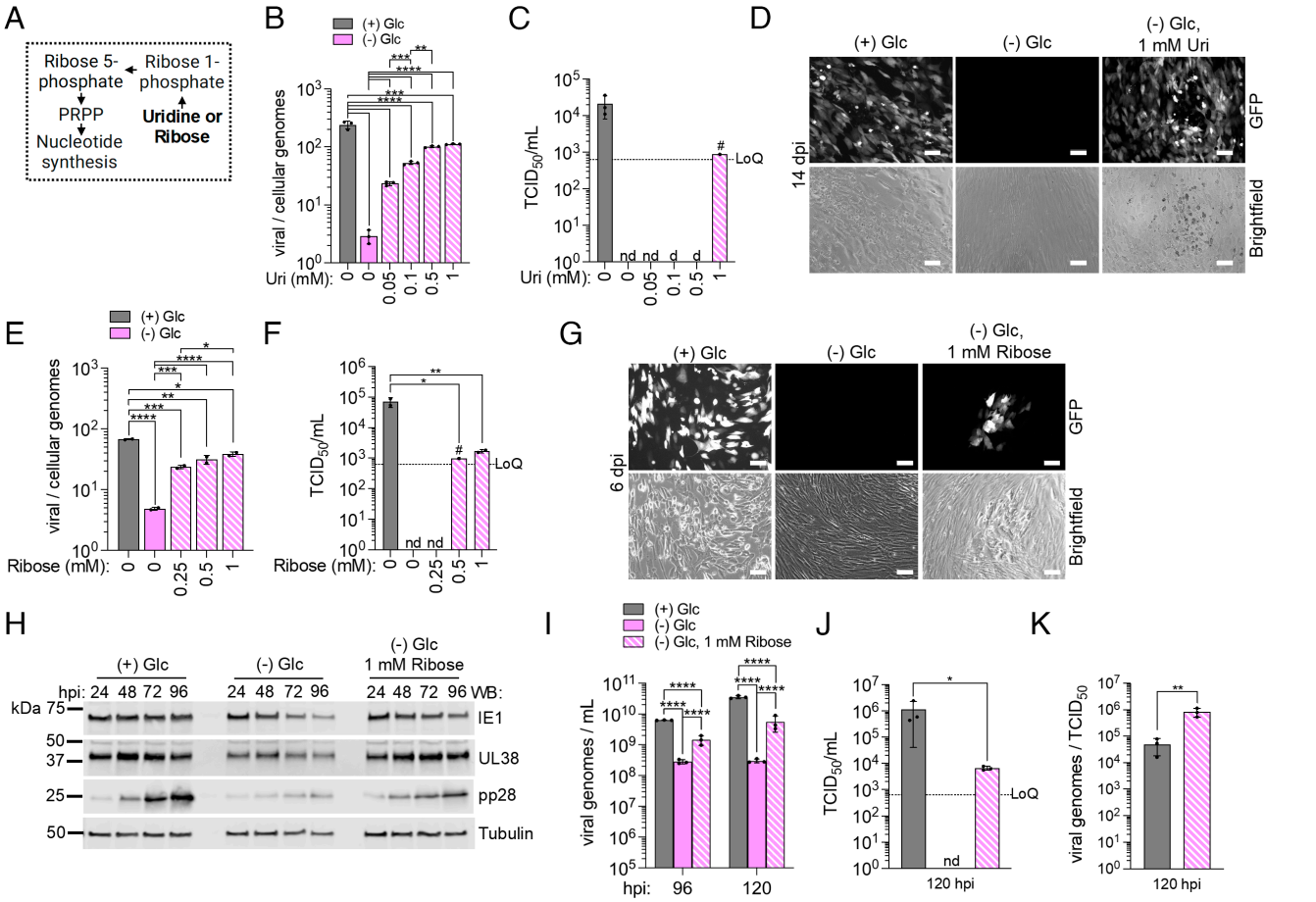

Fig. 2. Uridine and ribose support glucose-independent replication of HCMV. (*A*) Schematic of uridine or ribose feeding nucleotide synthesis, including phosphoribosyl diphosphate (PRPP). (*B–G*) HFFs were infected with TB40/E-GFP at a MOI 2. At 1 hpi, media were changed to glucose-replete or glucose-free DMEM with 0 to 1 mM uridine (Uri) (*B–D*) or 0 to 1 mM ribose (*E–G*). (*B* and *E*) Viral genomes per cell were quantified by qPCR. (*C* and *F*) Viral titers from culture supernatant at 120 hpi were quantified by $TCID_{50}$. LoQ = 632 $TCID_{50}$/mL; nd, not detected; d, detected but below the LoQ. Pound sign (#) indicates that one of three biological replicates (*C*) or two biological replicates (*F*) had measurable titers. (*D* and *G*) HFFs were infected and treated as described above. At 120 (*D*) or 144 hpi (*G*), culture supernatants were collected, diluted 1:10 in glucose-replete DMEM, and applied to uninfected, subconfluent HFFs. Cells were incubated for 6 (*G*) or 14 d (*D*) and GFP-positive infected foci were imaged. (Scale bar, 100 μm.) (*H–K*) HFFs were infected as described above. At 1 hpi, media were changed to glucose-replete, glucose-free, or glucose-free with 1 mM ribose supplement. (*H*) Whole-cell lysates were collected at the indicated times and analyzed by western blot. A representative blot from three biological replicates is shown. (*I*) At 96 and 120 hpi, 100 μL of culture supernatants was collected, treated with DNase, and released particle genomes were quantified by qPCR. (*J*) Viral titers were quantified by $TCID_{50}$ using the same culture supernatant from *I* at 120 hpi. LoQ = 632 $TCID_{50}$/mL; nd, not detected. (*K*) The ratio of total particles released to infectious particles released was determined using data from *I* and *J*. Error bars represent SD. One-way ANOVA with Tukey's (*F*) or Šídák's (*B* and *E*) test, two-way ANOVA with Tukey's test (*I*), or paired *t* test (*J–K*) was used to determine significance. Statistics were performed on transformed data for *B*, *E*, *F*, *I*, and *K*. $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***; $P < 0.0001$, ****. $n = 2$ (*E*, *F*) to 3 (*B–D* and *H–K*).

of the company supplying ribose (*SI Appendix*, Fig. S2*B*). These results demonstrate that ribose supplement restores HCMV replication at a low level that may be challenging to quantifiably measure but nonetheless consistently restores low levels of virus production.

Ribose Supplement Partially Restores Glucose-Independent Viral Genome and Protein Synthesis, Allowing for Viral Particle Production. Since ribose supplement restores viral genome synthesis during glucose deprivation, we next measured cytotoxicity to determine whether ribose supplement could rescue cell viability in mock-infected primary fibroblasts. HFFs were infected or mock-infected, and at 1 hpi, media were replaced with glucose-replete or glucose-free with 1 mM ribose. At 48 and 120 hpi, culture supernatants were collected for lactate dehydrogenase cytotoxicity assay. At 48 hpi, cells in all conditions maintained high viability in both mock- and HCMV-infected cultures (*SI Appendix*, Fig. S3*A*). At 120 hpi, mock-infected, glucose-replete, ribose-supplemented cultures maintained high cell viability but mock-infected, glucose-free, ribose-supplemented cell viability decreased to 76%, indicating ribose is not sufficient to protect uninfected HFFs from glucose deprivation-induced death. HCMV-infected, glucose-free, ribose-supplemented cultures maintained 95% cell viability while HCMV-infected, glucose-replete, ribose-supplemented cultures decreased to 86%, potentially due to cell death from HCMV replication similar to HCMV-infected, glucose-replete cultures not supplemented with ribose (*SI Appendix*, Fig. S3 *A* and S1 *A*). Our findings suggest that ribose supplement does not impact cell viability in glucose-deprived, mock- or HCMV-infected cells.

We continued to supplement with ribose to better understand how HCMV might take advantage of compensatory nutrients to successfully replicate during glucose deprivation. As ribose partially restores viral genome levels at 120 hpi, we next investigated when ribose supplement rescues viral genome synthesis. We measured viral genome levels from 4 to 96 hpi in glucose-replete or glucose-free cultures with or without 1 mM ribose. Ribose supplement significantly increased viral genome levels relative to glucose-free, nonsupplemented cultures by 48 hpi (*SI Appendix*, Fig. S3*B*). Again, viral genome levels in glucose-free, ribose

supplement were reduced compared to glucose-replete, indicating ribose partially restores viral genome synthesis in glucose-limiting conditions.

Since we found that ribose partially restores viral genome synthesis and virus production in glucose-free conditions, we reasoned that viral protein production would also be restored in supplemented cultures when glucose is limited. We measured viral protein levels from 4 to 96 hpi in glucose-replete or glucose-free DMEM with or without ribose. For immediate-early proteins, we observed that IE1 and IE2 levels remained unchanged in glucose-free, ribose-supplemented cultures compared to glucose-replete until late stages of virus replication when IE1 levels decreased (Fig. 2*H* and *SI Appendix*, Fig. S3 *C* and *F–G*). For early proteins, ribose supplement partially rescued UL38 levels by 48 hpi relative to glucose-free, nonsupplemented conditions and provided a full restoration at 96 hpi relative to glucose-replete conditions (Fig. 2*H* and *SI Appendix*, Fig. S3*D*). UL44 levels remained similar compared to glucose-free conditions until 96 hpi when levels were increased compared to glucose-free cultures yet still decreased from glucose-replete (*SI Appendix*, Fig. S3 *F* and *H*). Finally, late viral proteins pp28 and pp71 levels in ribose supplement cultures were partially restored (Fig. 2*H* and *SI Appendix*, Fig. S3 *E–F* and *I*). These findings demonstrate that ribose supports low levels of glucose-independent HCMV production via partial restoration of viral genome synthesis and viral protein levels.

Since ribose supplement in glucose-limiting conditions partially supported viral genome synthesis, early and late protein levels, and a low level of infectious virus production, we investigated whether ribose supplement was able to restore late events leading to release of virus particles. To determine whether viral particles are released during glucose deprivation with or without ribose, we infected and treated HFFs as previously described, collected and DNase-treated culture supernatant to remove any nonencapsidated viral genomes, and quantified protected viral genomes as a measurement of released viral particles. Viral particles were decreased during glucose deprivation by ~1- and 2-log at 96 and 120, respectively, and ribose supplement increased levels by ~1-log compared to glucose-free at both times (Fig. 2*I*). However, ribose-supplemented cultures still produced less viral particles than glucose-replete cultures. We quantified the ratio of total released particle levels (Fig. 2*I*) to infectious particle levels (Fig. 2*J*) as a measure of particle infectivity (Fig. 2*K*). The ratio of total released particles to infectious particles was increased by ~1-log in ribose-supplemented, glucose-free cultures compared to glucose-replete (Fig. 2*K*). Together, the data in Fig. 2 *I* and *K* demonstrate that ribose supplement increases viral particles produced by cells starved of glucose and these released particles are infectious though at a reduced level compared to glucose-replete conditions (Fig.2 *F, G,* and *J*).

Ribose Supplement Partially Restores Glycosylated gB Levels in Glucose-Free Cultures. Based on our observation that ribose supplement yields a higher ratio of released viral particles that are deficient in their ability to establish infection in cells compared to glucose-replete conditions (Fig. 2 *I* and *K*), we considered that ribose may not restore the role of glucose in the formation of functional glycoproteins. Viral glycoproteins support infectivity by enabling virus binding, fusion, and entry events into host cells. Glucose contributes to glycosylation through UDP-glucose or the synthesis of UDP-N-acetylglucosamine (UDP-GlcNAc) via the hexosamine pathway (Fig. 3*A*). Loss of UDP-sugars results in decreased viral protein glycosylation and infectious virus production (30, 39). To determine whether viral protein glycosylation is lost during glucose deprivation, we examined a representative HCMV glycoprotein, glycoprotein B (gB). In glucose-replete cultures, we observed a band just below 150 kDa that increased in intensity over time consistent with glycosylation of gB (Fig. 3*B*). The ~150 kDa band was not evident in glucose-free cultures but darkening the image revealed a faint band at 150 kDa at 24 hpi. This band was absent at later times. As the 150 kDa band is present at 24 hpi in all conditions, it is likely residual glycosylated gB from inoculating viral particles. Darkening the image revealed another band around 100 kDa that is the predominant band in glucose-free conditions from 48 to 96 hpi. In contrast, ribose-supplemented, glucose-free cultures displayed a smear from ~100 to ~150 kDa that increased over time with the strongest intensity at 96 hpi. Quantification of the ~150 kDa portion of the smear suggests that ribose partially restores some level of this band relative to glucose-free cultures (Fig. 3*C*). Next, we sought to confirm that the ~150 kDa band corresponds to glycosylated gB and the band near 100 kDa represents unglycosylated gB which has an expected size of 105 kDa (49). We collected whole-cell lysates from glucose-replete cultures at 96 hpi and hydrolyzed N-linked glycans using PNGase F enzyme. The ~150 kDa band shifted to 105 kDa following PNGase F treatment (*SI Appendix*, Fig. S3*J*), confirming that this upper band is glycosylated gB and the 105 kDa band is unglycosylated gB. These results suggest that ribose supplement partially restores glycosylated gB levels by 96 hpi during glucose deprivation.

Since glycosylation of viral receptors is required for particle infectivity (50), we hypothesized that restoration of protein glycosylation with rescue of viral genomes would restore particle infectivity in glucose-free cultures. To this end, we supplemented glucose-free cultures with ribose and/or UDP-GlcNAc, the substrate for both O- and N-linked glycosylation reactions (Fig. 3*A*). HFFs were infected as previously described. At 1 hpi, media were changed to glucose-replete, glucose-free, or glucose-free supplemented with 1 mM ribose, 100 μM UDP-GlcNAc, or both metabolites (dual supplement). Whole-cell lysates were collected at 120 and 144 hpi and protein glycosylation was evaluated by blotting for gB. In glucose-replete conditions, the ~150 kDa gB band was observed, while only a faint band representing unglycosylated gB was observed in glucose-free conditions (Fig. 3*D*). Ribose-only or dual supplement fully restored glycosylated gB levels by 120 hpi (Fig. 3 *D* and *E*). UDP-GlcNAc supplement resulted in upper and lower gB bands present at both times (Fig. 3*D*). Glycosylated gB in conditions where UDP-GlcNAc was the only supplement was lower than in glucose-replete or ribose-only supplemented, glucose-free cultures (Fig. 3 *D* and *E*). A possible explanation for the observation that ribose restores gB glycosylation to a greater extent than UDP-GlcNAc is that gB is a late protein and expression of late proteins might be higher in ribose supplement than UDP-GlcNAc supplement. We examined late protein pp28 and found that UDP-GlcNAc increased pp28 levels similar to ribose supplement alone (Fig. 3 *D* and *F*). The levels of an additional late protein, pp71, were similarly restored in both ribose supplement and UDP-GlcNAc supplement (*SI Appendix*, Fig. S4 *A* and *B*). Together, these data suggest UDP-GlcNAc supplement impacts late viral protein levels during glucose deprivation while only partially restoring glycosylation.

We next examined viral titers, total released particles, and particle infectivity during ribose and UDP-GlcNAc supplementation. Again, no infectious virus production was detected in glucose-free cultures, while ribose supplement partially restored virus production in the absence of glucose at 120 and 144 hpi (Fig. 3*G*). UDP-GlcNAc supplement alone was insufficient to restore levels of infectious virus production above the LoQ. However, we noted a

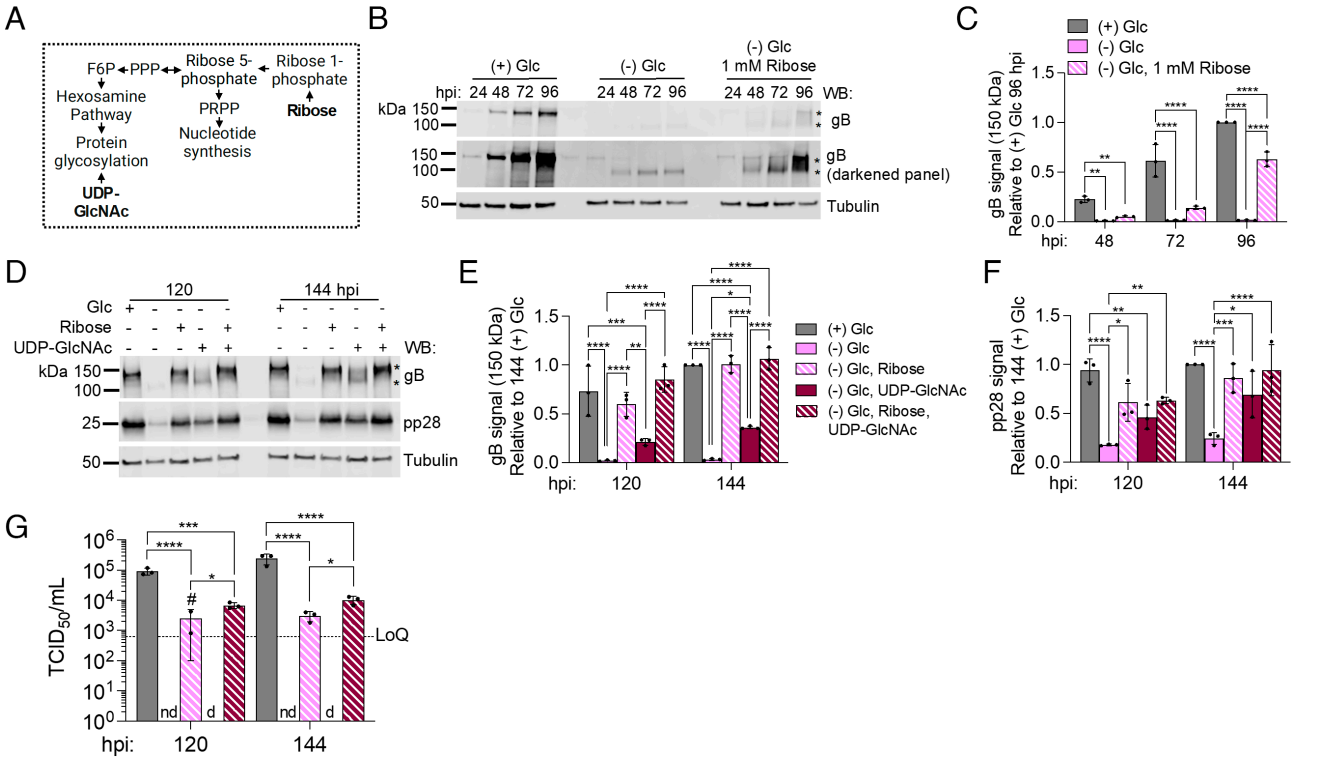

Fig. 3. Ribose and UDP-GlcNAc supplement support viral protein production, glycosylation, and infectious virus production during glucose deprivation. (*A*) Schematic of ribose feeding nucleotide synthesis, PPP, and hexosamine pathway and UDP-GlcNAc supporting glycosylation. F6P, fructose 6-phosphate; PPP, pentose phosphate pathway; PRPP, phosphoribosyl diphosphate. (*B–C*) HFFs were infected with TB40/E-GFP at a MOI 2. At 1 hpi, media were changed to glucose-replete, glucose-free, or glucose-free with 1 mM ribose supplement. (*B*) Whole-cell lysates were collected at the indicated times and analyzed by western blot using antibodies to gB and tubulin. Asterisks (*) indicate ~150 kDa (*Upper*) and ~105 kDa (*Lower*) gB bands. A representative blot from three biological replicates is shown. (*C*) The ~150 kDa gB levels were normalized to tubulin levels and quantified relative to (+) Glc at 96 hpi. (*D–G*) HFFs were infected with TB40/E-GFP at a MOI 2. At 1 hpi, media were changed to glucose-replete, glucose-free, or glucose-free DMEM supplemented with 1 mM ribose, 100 μM UDP-N-acetylglucosamine (UDP-GlcNAc), or both. (*D*) Whole-cell lysates were collected at 120 and 144 hpi and analyzed by western blot. A representative blot from three biological replicates is shown. (*E–F*) Viral protein levels were normalized to tubulin and quantified relative to (+) Glc at 144 hpi. (*G*) Viral titers were quantified from culture supernatant by $TCID_{50}$. Error bars represent SD. Two-way ANOVA with Tukey's test was used to determine significance. Statistics were performed on transformed data for G. $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***; $P < 0.0001$, ****. $n = 3$.

low level of GFP-positive infected foci on the $TCID_{50}$ plates. Adding ribose to UDP-GlcNAc supplementation further increased virus titers by ~0.5-log compared to ribose supplement alone; however, the levels in the dual supplementation were still lower than in glucose-replete conditions. UDP-GlcNAc supplemented restored released particles to a level similar to ribose supplementation, but both were lower than glucose-replete cultures (*SI Appendix*, Fig. S4*C*). Dual supplement of both ribose and UDP-GlcNAc further restored released viral particle levels compared to individual supplementation and reached a level comparable to glucose-replete conditions. Particle infectivity was calculated for conditions with measurable titers. Both ribose and dual supplement cultures had a ~1-log increase in noninfectious particles at 144 hpi compared to glucose-replete (*SI Appendix*, Fig. S4*D*), suggesting these particles still lack components required for infectivity despite restoration of viral protein glycosylation.

UDP-GlcNAc's ability to restore both late viral protein and released viral particles during glucose deprivation led us to hypothesize that it could also restore viral genome synthesis. We infected HFFs as previously described and treated the cells with glucose-replete, glucose-free, or glucose-free with 100 μM UDP-GlcNAc. We collected cells at 4, 48, and 120 hpi and quantified viral genome synthesis using qPCR. UDP-GlcNac partially restored viral genome levels at 48 hpi and 120 hpi (*SI Appendix*, Fig. S4*E*). These data suggest that the metabolism of UDP-GlcNAc supports more than protein glycosylation and that the prioritization of metabolites such as UDP-GlcNAc may be shifted when glucose is limited or starved.

Alternative Metabolites Support Low Levels of Glucose-Independent Replication. Ribose, UDP-GlcNAc, and uridine can feed anabolic pathways that branch from upper glycolysis. Our data demonstrate that these metabolites partially restore viral genome synthesis and consistently support a low level of virus production in glucose-free cultures. To better assess this consistent, low level of infectious virus production, we increased the sensitivity of our $TCID_{50}$ assay by 10-fold to quantitatively measure virus production in supplemented, glucose-free conditions. We infected HFFs and supplemented glucose-free cultures with 1 mM ribose or 1 mM uridine and measured the amount of virus present in the medium at 144 hpi. Again, we observed a lack of infectious virus production in glucose-free cultures when no metabolites were supplemented (Fig. 4*A*). In contrast, ribose or uridine supplement resulted in significantly higher levels of infectious virus produced by cells in glucose-free conditions with uridine providing the strongest rescue (Fig. 4*A*).

We further investigated the level of HCMV production in glucose-free, nutrient-supplemented conditions using an additional approach as a semiquantitative measure of virus produced. We collected the supernatant at 120 hpi from infected cells grown in supplemented and nonsupplemented conditions, diluted it 1:10, and inoculated 12 wells of uninfected cells in 96-well plates. We then counted the number of wells that contained at least one GFP-positive virus foci. As expected, in glucose-replete conditions, all 12 wells were positive in all experiments (*SI Appendix*, Fig. S4 *F–H*). For glucose-free cultures, two plates from a total of 41 plates had one and three GFP-positive wells, respectively

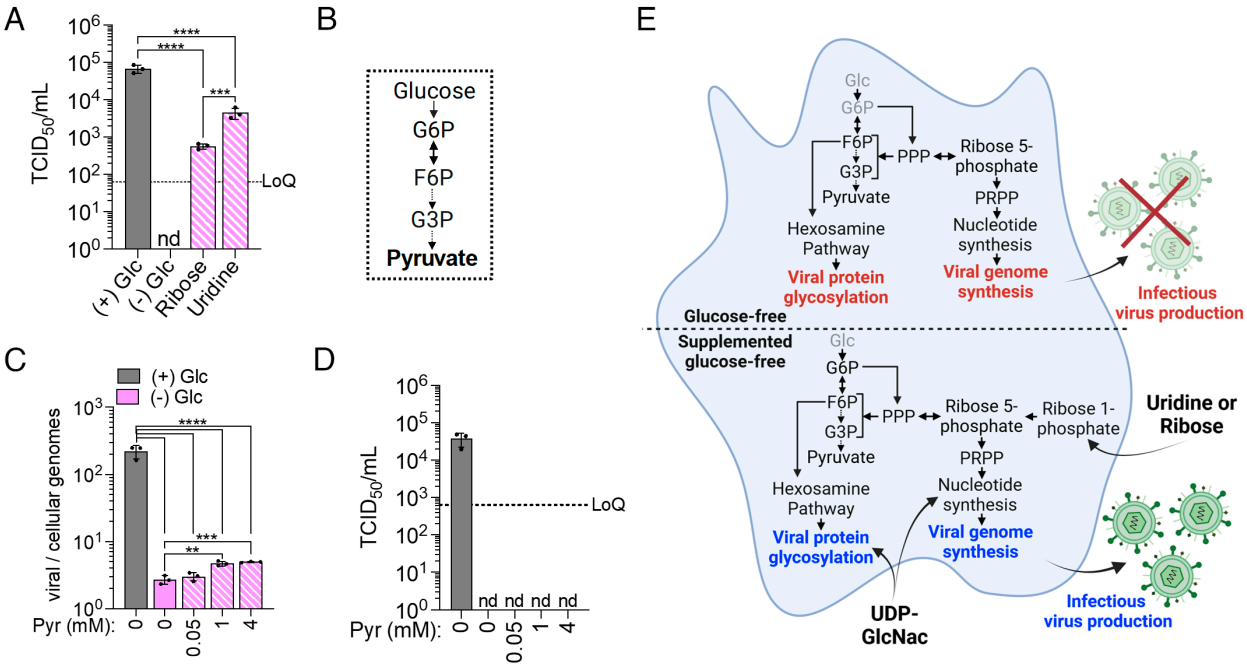

Fig. 4. Metabolites that support upper glycolytic pathways consistently restore virus production. (*A*) HFFs were infected with TB40/E-GFP at a MOI 2. At 1 hpi, media were changed to glucose-replete, glucose-free, or glucose-free DMEM with 1 mM ribose or 1 mM uridine. Viral titers from culture supernatant at 144 hpi were quantified by $TCID_{50}$. LoQ = 63.2 $TCID_{50}$/mL; nd, not detected. (*B*) Schematic of abbreviated glycolysis. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate. (*C*, *D*) HFFs were infected with TB40/E-GFP at a MOI 2. At 1 hpi, media were changed to glucose-replete, glucose-free, or glucose-free DMEM with 0-4 mM pyruvate (Pyr). (*C*) Viral genomes per cell were quantified by qPCR. (*D*) Viral titers from culture supernatant at 120 hpi were quantified by $TCID_{50}$. LoQ = 632 $TCID_{50}$/mL; nd, not detected. One-way ANOVA with Tukey's (*A*) or Šídák's (*C*) test was used to determine significance. Statistics were performed on transformed data. $P < 0.01$, **; $P < 0.001$, ***; $P < 0.0001$, ****. $n = 3$. (*E*) Model of abbreviated glycolysis and glycolytic branches during glucose deprivation and uridine, ribose, or UDP-GlcNAc supplement during glucose deprivation. Glc, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; PPP, pentose phosphate pathway; PRPP, phosphoribosyl diphosphate. Metabolites in gray indicate that metabolite is absent or predicted to be absent. Virus replication steps in red were decreased in glucose-free conditions and steps in blue indicate partial restoration of these events in supplemented, glucose-free cultures. Created with BioRender.com.

(*SI Appendix*, Fig. S4 *F–H*). In contrast, when ribose-only was supplemented, every plate (total of 36) contained at least three, and up to 12, GFP-positive wells (*SI Appendix*, Fig. S4 *F–G*). This result was consistent with an increase in low-level virus production we noted in Fig. 2*G*. For UDP-GlcNAc supplement, nine out of 12 plates had at least 2 GFP-positive wells (*SI Appendix*, Fig. S4*G*). For dual ribose and UDP-GlcNAc, we observed that all experiments resulted in enough virus production for each of the 12 wells to be positive (*SI Appendix*, Fig. S4*G*). Uridine-only supplement of glucose-free cultures consistently resulted in plates containing at least eight GFP-positive wells (*SI Appendix*, Fig. S4*H*), consistent with our previous observation that uridine supplement supported HCMV replication in glucose-free conditions (Fig. 2*D*). Our observations demonstrate that compensatory nutrients lacking in commonly used growth media can support low levels of HCMV production in glucose-free conditions.

Pyruvate Supplement Is Not Sufficient to Restore Viral Replication. We next asked whether restoration of lower glycolysis in glucose-free cultures would rescue HCMV replication. To this end, we supplemented glucose-free cultures with pyruvate, the final product of glycolysis (Fig. 4*B*). We infected HFFs, supplemented with 0 to 4 mM pyruvate in glucose-free DMEM, and measured viral genome levels and virus production at 120 hpi. Higher concentrations of pyruvate supplement in glucose-free cultures negligibly increased viral genome levels compared to glucose-free, nonsupplemented cultures by 120 hpi (Fig. 4*C*). All pyruvate concentrations tested failed to produce detectable GFP-positive foci on titer plates (Fig. 4*D*), indicating that pyruvate is insufficient to restore viral replication. Overall, our findings demonstrate that metabolites that feed upper glycolytic branches are sufficient to support low levels of glucose-independent virus production, but supplement of lower glycolysis cannot restore virus production.

Discussion

Virus–host metabolism interactions enable viruses to successfully replicate in tissues in the body with diverse metabolic environments, including environments where a nutrient may be limiting. Some latent viruses may reactivate in response to changes in a metabolic environment that would favor virus replication (51). HCMV relies on manipulation of host metabolism to replicate and does so successfully in various human tissues with different nutrient environments. Most HCMV metabolic studies are completed in cell culture media that contain supraphysiological levels of some nutrients and lack other metabolites found in human tissues and sera (1, 2, 4, 28, 29, 34, 35, 39–41, 44, 52, 53). While these studies have advanced our understanding by identifying metabolic alterations caused by virus infection, they lead to two questions: 1) do nutrients found in human tissues and sera but absent from typical cell culture media compensate and contribute to HCMV replication when some metabolites are limited? and 2) do supraphysiological nutrient concentrations mask the contribution of some nutrients to HCMV replication? We addressed these questions using a glucose-independent model of HCMV replication to demonstrate that viral genome synthesis requires glucose but metabolites that support upper glycolytic branches can compensate when glucose is limited to restore viral genome replication and low levels of virus production. Our data suggest that in glucose-limited conditions, ribose feeds nucleotide synthesis and the PPP to support downstream replication processes such as viral genome synthesis, viral protein production, and protein glycosylation, resulting in a low but notable

increase in virus production (Fig. 4*E*). Overall, our findings indicate that HCMV successfully replicates in glucose-limited environments by nutrient compensation that feeds metabolic pathways necessary for replication.

In this study, we found that uridine partially restores viral genome synthesis in glucose-free cultures (Fig. 2*B*). Uridine-derived ribose 1-phosphate could restore viral genome synthesis through its conversion to ribose 5-phosphate and then PRPP to support both purine and pyrimidine synthesis (Fig. 4*E*). In support of this possibility, ribose supplement restored viral genome synthesis to a similar level as uridine (Fig. 2*E* and *SI Appendix*, Fig. S3*B*). Similar to our observations, studies in cancer cell lines demonstrated that uridine supports glucose-independent cell proliferation and protects from glucose deprivation-induced cell death through uridine phosphorylase activity and ribose 1-phosphate release (47, 48, 54–58).

Compensation by uridine-derived ribose during glucose deprivation could be a mechanism for how HCMV successfully replicates in glucose-limiting environments. When supplementing with ribose directly, we found that in addition to restoring viral genome synthesis (Fig. 2*E* and *SI Appendix*, Fig. S3*B*), ribose also rescues glycosylated gB levels by 120 hpi (Fig. 3 *D* and *E*). This restoration could occur through anabolic reactions that contribute metabolites for UDP-GlcNAc synthesis through the hexosamine pathway (Fig. 4*E*). Ribose rescue of nucleotide synthesis would result in UTP availability for the last step of UDP-GlcNAc synthesis. Ribose could also feed the non-oxidative branch of the PPP that yields fructose 6-phosphate, which is the first substrate in the hexosamine pathway. In support of this potential mechanism, ribose 1-phosphate feeding non-oxidative PPP was found to be necessary for cancer cell proliferation during glucose starvation (47). Based on these studies, it is possible that ribose restoration of gB glycosylation results from feeding non-oxidative PPP that produces fructose 6-phosphate to support UDP-GlcNAc synthesis and glycosylation during glucose limitation (Fig. 4*E*).

Ribose supporting the non-oxidative PPP would also support lower glycolysis through the formation of glyceraldehyde 3-phosphate, or potentially, support both upper and lower glycolysis by feedback to glucose 6-phosphate via the enzyme glucose 6-phosphate isomerase that converts fructose 6-phosphate to glucose 6-phosphate (Fig. 4*E*) (47). Our data demonstrate that pyruvate supplement failed to restore viral genome levels or virus production (Fig. 4 *C* and *D*), indicating that restoring only lower glycolysis is not sufficient to restore virus production in glucose-free conditions. Thus, metabolites that support upper glycolytic branches are necessary for HCMV virus production in glucose-limiting conditions (Figs. 2 *C*, *D*, *F*, *G*, and *J*, 3*G*, and 4*A*, and *SI Appendix*, Fig. S4 *F–H*). These results indicate that a major role of glucose during HCMV replication is support of viral genome synthesis and protein glycosylation, and these processes are rate-limiting steps in glucose-independent HCMV replication.

Our data indicate that HCMV offers protection against glucose deprivation-induced cytotoxicity (*SI Appendix*, Fig. S1*A*). This observation was first reported by Chambers et al. (1). They demonstrated that HCMV increases anaplerotic glutamine consumption, leading them to suggest that HCMV infection promotes cell survival in glucose-free conditions in part by increasing glutamine utilization. Further, HCMV promotes cell survival in times of stress by encoding several proteins to inhibit cell death, including UL37x1 (vMIA) and UL38 proteins (59–63). These viral proteins may restrict the induction or signaling that leads to death in uninfected cells when glucose is not present.

In contrast to our data and the Chambers et al. study, work by Raymonda et al. showed that mock-infected MRC5 fibroblasts starved of glucose remain viable, while HCMV-infected cells have decreased viability by 48 hpi (3). There are several differences between the Chambers and Raymonda studies, such as the presence or absence of fetal bovine serum (FBS), cell type, and assay times as discussed in Raymonda et al. Our conditions of fully confluent, serum-starved cells and a brief infection period (1 to 2 h) were similar to Raymonda et al., suggesting these conditions are unlikely to be contributing to the death of HCMV-infected cells observed during glucose starvation by Raymonda et al. but not observed in our study or Chambers et al. The disparity in phenotypes observed could be due to differences in the fibroblast cells used: HFF versus MRC5 fibroblast cells. Additionally, Raymonda et al. used the lab-adapted AD169 strain, whereas Chambers et al. used Towne, and we used TB40/E. It is conceivable that there are differences between the response of primary human fibroblasts to glucose deprivation independent of infection or differences in interactions between TB40/E or Towne and fibroblasts compared to AD169 and fibroblasts in glucose-limiting conditions.

Chambers et al. and Raymonda et al. did not investigate whether physiologically relevant nutrient compensation could support glucose-independent virus replication. Importantly, our findings reveal that the presence or absence of certain nutrients impacts HCMV replication in glucose-free conditions. In glucose-rich conditions, glucose supplies nucleotide synthesis, glycosylation, and lipid synthesis (2, 4, 27–30, 35, 38–41). In this study, we demonstrate that uridine and ribose can be used as an alternative nutrient source for nucleotide synthesis and glycosylation which supports a partial restoration of infectious virus production (Fig. 2 *C*, *D*, *F*, *G*, and *J* and *SI Appendix*, Fig. S4 *F–H*). However, the lower level of infectivity in ribose-supplemented cultures compared to glucose-replete (Fig. 2*K*) suggests that other components of the viral particle (e.g., lipids found in the envelope) or the assembly process (e.g., secondary envelopment and egress) remain partially disrupted. It is unknown whether HCMV still promotes lipid synthesis in the absence of glucose. However, Raymonda et al. found that expression of HCMV UL38 protein in uninfected cells promotes fatty acid synthesis during glucose deprivation suggesting that in the absence of glucose HCMV retains the ability to reprogram host metabolism (3). Moreover, this finding would suggest that HCMV can promote the synthesis of lipids needed to build the virus envelope when glucose is limited. Since the envelope is required for infectivity, promoting the synthesis of the necessary lipids independent of glucose is consistent with the low level of virus replication we found in ribose and uridine supplementation. Our future studies will focus on the regulation of fatty acid and lipid synthesis in nutrient-limited environments to define whether HCMV can promote the use of alternative nutrients to prioritize lipid metabolism during infection when glucose is unavailable.

Our observations indicate that uridine compensates during glucose starvation, yet uridine could also contribute to HCMV replication in physiological glucose environments. Munger et al. (35) demonstrated in high glucose cultures, uridine is decreased by ~fourfold during HCMV replication compared to mock-infected cells. While the authors attributed this decrease to only nucleotide synthesis, it is plausible that uridine contributes ribose for non-oxidative PPP, and subsequently, the hexosamine pathway when glucose is at physiological concentrations in tissues or sera. However, supraphysiological glucose levels in common types of cell growth media may mask the contribution of uridine to this process.

When evaluating the reduction in glycosylation in the absence of glucose, we found that UDP-GlcNAc supplement alone only

marginally increased gB glycosylation (Fig. 3 *D* and *E*). The lack of full restoration of glycosylation by UDP-GlcNAc supplement was not due to decreased late viral protein levels as UDP-GlcNAc supplement alone partially restored these levels (Fig. 3 *D* and *F* and *SI Appendix*, Fig. S4 *A* and *B*). UDP-GlcNAc likely restores late viral protein production via rescue of viral genome synthesis (*SI Appendix*, Fig. S4*E*). UDP-GlcNAc could contribute to viral genome synthesis by producing UDP that feeds nucleotide synthesis. Ribose and UDP-GlcNAc dual supplement increased gB glycosylation more than UDP-GlcNAc supplement alone. Moreover, dual supplement consistently restored virus production greater than UDP-GlcNAc supplement alone, demonstrating that UDP-GlcNAc supports viral replication better when ribose is present in glucose-limiting conditions (Fig. 3*G* and *SI Appendix*, Fig. S4*G*). Overall, our findings suggest that when glucose is limited multiple nutrients can be reprioritized to support virus replication depending on the nutrients available in the extracellular environment.

In this study, we used HCMV replication in glucose-limiting conditions to model how a virus can be successful in an environment when an essential nutrient becomes limiting. We demonstrate that a virus previously thought to require glucose for its replication can replicate independent of glucose when cultured with other physiologically relevant metabolites. When in glucose-rich environments, HCMV-infected cells demand glucose for optimal virus production as evidenced by increased glucose uptake and glycolytic flux (2, 4, 28, 29, 34, 35). However, we found that metabolites that feed upper glycolytic branches compensate for glucose to support viral genome synthesis and promote low, consistent levels of virus production (Figs. 2 *C*, *D*, *F*, *G*, and *J*, 3*G*, and 4*A* and *SI Appendix*, Fig. S4 *F–H*). Our findings reveal the importance of considering the metabolic environment as we build better models to understand how human-tropic viruses like HCMV replicate in the natural host. Further, our finding that nutrients thought to be essential for virus replication are not required for low levels of infectious virus production when physiologically relevant metabolites are present may impact preclinical development of therapeutic strategies that target metabolism for antiviral intervention. The metabolic environment needs to be considered when performing experiments in cell culture models of infection, including those evaluating metabolic interventions. Finally, low, yet persistent, replication of HCMV in the body's metabolically diverse tissues may have importance in the virus's ability to spread and cause disease in immunosuppressed individuals or during congenital infection. Our results suggest that viruses may take advantage of alternative metabolite use in nutrient-limited conditions to support low levels of replication.

Materials and Methods

Please see *SI Appendix* for a detailed description of *Materials and Methods*.

Experimental Design. HFF (American Type Culture Collection; ATCC) were cultured in DMEM containing 10% FBS, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and penicillin/streptomycin (P/S). Viral stocks and experimental samples were titered using a 50% tissue culture infectious dose ($TCID_{50}$) assay beginning with a 1:10 dilution (LoQ 632 $TCID_{50}$/mL) or undiluted (LoQ 63.2 $TCID_{50}$/mL), as noted in the text. All titers were measured by identifying GFP-positive wells at 14 d postinfection (dpi) using technical duplicates. Wells were considered positive if they contained foci of ≥3 GFP-positive cells. If there were no GFP-positive wells in the first row of the titer plate, the sample was considered not detected (nd). If 1 to 11 GFP-positive wells were observed in the first row then the virus was considered detected (d) but below the LoQ. If all 12 wells in the first row were GFP-positive, then the virus was considered above the LoQ and titer values reported in the figures.

DMEM without glucose was either purchased (Gibco) or prepared using DMEM powder (Gibco) and adding 4 mM glutamine and 44 mM sodium bicarbonate. DMEM containing glucose was either purchased (Gibco) or prepared with a final concentration of 25 mM glucose.

DNA Quantification. qPCR was used to assess whole-cell DNA and viral particle genome levels. DNA was isolated using the Zymo Quick DNA mini-Prep kit (ThermoFisher Scientific).

Protein Analysis. Protein levels were analyzed by western blot. For hydrolysis of N-linked glycans, total protein was quantified the using Pierce BCA Protein Assay Kit (ThermoFisher Scientific), and the PNGase F assay (New England Biolabs) was completed following the manufacturer's instructions using 15 μg of protein.

Statistics. Graphs were designed, and statistical testing was performed using GraphPad Prism. The appropriate statistical tests for each experiment are noted in the figure legends.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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1. J. W. Chambers, T. G. Maguire, J. C. Alwine, Glutamine metabolism is essential for human cytomegalovirus infection. *J. Virol.* **84**, 1867–1873 (2010).
2. M. P. Landini, Early enhanced glucose uptake in human cytomegalovirus-infected cells. *J. Gen. Virol.* **65**, 1229–1232 (1984).
3. M. H. Raymonda *et al.* Cytomegalovirus-induced inactivation of TSC2 disrupts the coupling of fatty acid biosynthesis to glucose availability resulting in a vulnerability to glucose starvation. *mBio* **15**, e0303123 (2023).
4. L. Vastag, E. Koyuncu, S. L. Grady, T. E. Shenk, J. D. Rabinowitz, Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. *PLoS Pathog.* **7**, e1002124 (2011).
5. J. L. Abrantes *et al.*, Herpes simplex type 1 activates glycolysis through engagement of the enzyme 6-phosphofructo-1-kinase (PFK-1). *Biochim. Biophys. Acta* **1822**, 1198–1206 (2012).
6. A. C. Codo *et al.*, Elevated glucose levels favor SARS-CoV-2 Infection and monocyte response through a HIF-1alpha/glycolysis-dependent axis. *Cell Metab.* **32**, 437-446 (2020), 10.1016/j.cmet.2020.07.007.
7. E. N. Bonglack *et al.*, Monocarboxylate transporter antagonism reveals metabolic vulnerabilities of viral-driven lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2022495118 (2021).
8. K. McFadden *et al.*, Metabolic stress is a barrier to Epstein-Barr virus-mediated B-cell immortalization. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E782–E790 (2016).
9. M. Thai *et al.*, Adenovirus E4ORF1-induced MYC activation promotes host cell anabolic glucose metabolism and virus replication. *Cell Metab.* **19**, 694–701 (2014).
10. K. A. Fontaine, E. L. Sanchez, R. Camarda, M. Lagunoff, Dengue virus induces and requires glycolysis for optimal replication. *J. Virol.* **89**, 2358–2366 (2015).
11. L. F. Chen *et al.*, Respiratory syncytial virus co-opts hypoxia-inducible factor-1α-mediated glycolysis to favor the production of infectious virus. *mBio* **14**, e0211023 (2023).
12. T. Delgado *et al.*, Induction of the Warburg effect by Kaposi's sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 10696–10701 (2010).
13. A. P. Bhatt *et al.*, Dysregulation of fatty acid synthesis and glycolysis in non-Hodgkin lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 11818–11823 (2012).
14. E. L. Sanchez *et al.*, Glycolysis, glutaminolysis, and fatty acid Synthesis are required for distinct stages of Kaposi's Sarcoma-Associated herpesvirus lytic replication. *J. Virol.* **91**, e02237 (2017).
15. Y. Zhu *et al.*, An oncogenic virus promotes cell survival and cellular transformation by suppressing glycolysis. *PLoS Pathog.* **12**, e1005648 (2016).
16. Q. Wan *et al.*, Hijacking of nucleotide biosynthesis and deamidation-mediated glycolysis by an oncogenic herpesvirus. *Nat. Commun.* **15**, 1442 (2024).
17. G. A. Gualdoni *et al.*, Rhinovirus induces an anabolic reprogramming in host cell metabolism essential for viral replication. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E7158–E7165 (2018).
18. J. Kleinehr, J. J. Wilden, Y. Boergeling, S. Ludwig, E. R. Hrincius, Metabolic modifications by common respiratory viruses and their potential as new antiviral targets. *Viruses* **13**, 2068 (2021).
19. Y. H. Wu *et al.*, Aerobic glycolysis supports hepatitis B virus protein synthesis through interaction between viral surface antigen and pyruvate kinase isoform M2. *PLoS Pathog.* **17**, e1008866 (2021).

20. L. Zhou *et al.*, Hepatitis B virus rigs the cellular metabolome to avoid innate immune recognition. *Nat. Commun.* **12**, 98 (2021).
21. Z. Wu *et al.*, Human herpesvirus 6A promotes glycolysis in infected T cells by activation of mTOR signaling. *PLoS Pathog.* **16**, e1008568 (2020).
22. K. D. Passalacqua *et al.*, Glycolysis Is an Intrinsic factor for optimal replication of a norovirus. *mBio* **10**, e02175 (2019).
23. E. S. Mocarski, T. Shenk, P. Griffiths, R. F. Pass, "Cytomegaloviruses" in *Fields Virology*, D. M. Knipe, P. M. Howley, Eds. (Wolters Kluwer, Philadelphia, PA, 2013).
24. P. Griffiths, M. Reeves, Pathogenesis of human cytomegalovirus in the immunocompromised host. *Nat. Rev. Microbiol.* **19**, 759–773 (2021).
25. H. L. Fulkerson, M. T. Nogalski, D. Collins-McMillen, A. D. Yurochko, Overview of human cytomegalovirus pathogenesis. *Methods Mol. Biol.* **2244**, 1–18 (2021).
26. C. Sinzger, M. Digel, G. Jahn, Cytomegalovirus cell tropism. *Curr. Top. Microbiol. Immunol.* **325**, 63–83 (2008).
27. Y. Xi, S. Harwood, L. Wise, J. G. Purdy, Human cytomegalovirus pUL37x1 is important to remodeling of host lipid metabolism. *J. Virol.* **93**, 1–19 (2019).
28. J. Munger *et al.*, Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nat. Biotechnol.* **26**, 1179–1186 (2008).
29. Y. Yu, T. G. Maguire, J. C. Alwine, ChREBP, a glucose-responsive transcriptional factor, enhances glucose metabolism to support biosynthesis in human cytomegalovirus-infected cells. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 1951–1956 (2014).
30. S. R. DeVito, E. Ortiz-Riano, L. Martinez-Sobrido, J. Munger, Cytomegalovirus-mediated activation of pyrimidine biosynthesis drives UDP-sugar synthesis to support viral protein glycosylation. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 18019–18024 (2014).
31. L. W. Wang *et al.*, Epstein-barr-virus-induced one-carbon metabolism drives B cell transformation. *Cell Metab.* **30**, 539–555.e511 (2019).
32. J. R. Cantor *et al.*, Physiologic medium rewires cellular metabolism and reveals uric acid as an endogenous inhibitor of UMP synthase. *Cell* **169**, 258–272.e217 (2017).
33. J. Vande Voorde *et al.*, Improving the metabolic fidelity of cancer models with a physiological cell culture medium. *Sci. Adv.* **5**, eaau7314 (2019).
34. Y. Yu, T. G. Maguire, J. C. Alwine, Human cytomegalovirus activates glucose transporter 4 expression to increase glucose uptake during infection. *J. Virol.* **85**, 1573–1580 (2011).
35. J. Munger, S. U. Bajad, H. A. Coller, T. Shenk, J. D. Rabinowitz, Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLoS Pathog.* **2**, e132 (2006).
36. Y. Yu, A. J. Clippinger, J. C. Alwine, Viral effects on metabolism: Changes in glucose and glutamine utilization during human cytomegalovirus infection. *Trends Microbiol.* **19**, 360–367 (2011).
37. I. Rodriguez-Sanchez, J. Munger, Meal for two: Human cytomegalovirus-induced activation of cellular metabolism. *Viruses* **11**, 273 (2019).
38. Y. Xi, L. Lindenmayer, I. Kline, J. von Einem, J. G. Purdy, Human cytomegalovirus uses a host stress response to balance the elongation of saturated/monounsaturated and polyunsaturated very-long-chain fatty acids. *mBio* **12**, e00167 (2021).
39. I. Moreno Jr., I. Rodríguez-Sánchez, X. Schafer, J. Munger, Human cytomegalovirus induces neuronal enolase to support virally mediated metabolic remodeling. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2205789119 (2022).
40. E. Koyuncu, J. G. Purdy, J. D. Rabinowitz, T. Shenk, Saturated very long chain fatty acids are required for the production of infectious human cytomegalovirus progeny. *PLoS Pathog.* **9**, e1003333 (2013).
41. J. G. Purdy, T. Shenk, J. D. Rabinowitz, Fatty acid elongase 7 catalyzes lipidome remodeling essential for human cytomegalovirus replication. *Cell Rep.* **10**, 1375–1385 (2015).
42. K. D. Radsak, D. Weder, Effect of 2-deoxy-D-glucose on cytomegalovirus-induced DNA synthesis in human fibroblasts. *J. Gen. Virol.* **57**, 33–42 (1981).
43. J. McArdle, X. L. Schafer, J. Munger, Inhibition of calmodulin-dependent kinase kinase blocks human cytomegalovirus-induced glycolytic activation and severely attenuates production of viral progeny. *J. Virol.* **85**, 705–714 (2011).
44. Y. Yu, T. G. Maguire, J. C. Alwine, Human cytomegalovirus infection induces adipocyte-like lipogenesis through activation of sterol regulatory element binding protein 1. *J. Virol.* **86**, 2942–2949 (2012).
45. Y. Yu, F. J. Pierciey Jr., T. G. Maguire, J. C. Alwine, PKR-like endoplasmic reticulum kinase is necessary for lipogenic activation during HCMV infection. *PLoS Pathog.* **9**, e1003266 (2013).
46. N. J. Moorman *et al.*, Human cytomegalovirus protein UL38 inhibits host cell stress responses by antagonizing the tuberous sclerosis protein complex. *Cell Host. Microbe.* **3**, 253–262 (2008).
47. O. S. Skinner *et al.*, Salvage of ribose from uridine or RNA supports glycolysis in nutrient-limited conditions. *Nat. Metab.* **5**, 765–776 (2023).
48. Z. C. Nwosu *et al.*, Uridine-derived ribose fuels glucose-restricted pancreatic cancer. *Nature* **618**, 151-158 (2023), 10.1038/s41586-023-06073-w.
49. W. J. Britt, L. G. Vugler, Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. *J. Virol.* **63**, 403–410 (1989).
50. L. Rasmussen, M. Nelson, M. Neff, T. C. Merigan Jr., Characterization of two different human cytomegalovirus glycoproteins which are targets for virus neutralizing antibody. *Virology* **163**, 308–318 (1988).
51. E. M. Burton, R. Goldbach-Mansky, S. Bhaduri-McIntosh, A promiscuous inflammasome sparks replication of a common tumor virus. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 1722–1730 (2020).
52. I. Rodriguez-Sanchez, X. L. Schafer, M. Monaghan, J. Munger, The human cytomegalovirus UL38 protein drives mTOR-independent metabolic flux reprogramming by inhibiting TSC2. *PLoS Pathog.* **15**, e1007569 (2019).
53. R. L. Mokry, M. L. Schumacher, N. Hogg, S. S. Terhune, Nitric oxide circumvents virus-mediated metabolic regulation during human cytomegalovirus infection. *mBio* **11**, e02630 (2020).
54. B. M. Wice, L. J. Reitzer, D. Kennell, The continuous growth of vertebrate cells in the absence of sugar. *J. Biol. Chem.* **256**, 7812–7819 (1981).
55. M. Löffler, A. Wenzel, F. Schneider, Cytokinetic studies on the switch from glucose to uridine metabolism, and vice versa, of Ehrlich ascites tumour cells in vitro. *Cell Tissue Kinet.* **20**, 181–190 (1987).
56. J. W. Choi *et al.*, Uridine prevents the glucose deprivation-induced death of immunostimulated astrocytes via the action of uridine phosphorylase. *Neurosci. Res.* **56**, 111–118 (2006).
57. J. W. Choi *et al.*, Uridine protects cortical neurons from glucose deprivation-induced death: possible role of uridine phosphorylase. *J. Neurotrauma.* **25**, 695–707 (2008).
58. W. Linker, M. Löffler, F. Schneider, Uridine, but not cytidine can sustain growth of Ehrlich ascites tumor cells in glucose-deprived medium with altered proliferation kinetics. *Eur. J. Cell Biol.* **36**, 176–181 (1985).
59. S. Terhune *et al.*, Human cytomegalovirus UL38 protein blocks apoptosis. *J. Virol.* **81**, 3109–3123 (2007).
60. T. Shenk, J. C. Alwine, Human cytomegalovirus: Coordinating cellular stress, signaling, and metabolic pathways. *Annu. Rev. Virol.* **1**, 355–374 (2014).
61. V. S. Goldmacher *et al.*, A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12536–12541 (1999).
62. A. L. McCormick, Control of apoptosis by human cytomegalovirus. *Curr. Top. Microbiol. Immunol.* **325**, 281–295 (2008).
63. W. Brune, C. E. Andoniou, Die another day: Inhibition of cell death pathways by cytomegalovirus. *Viruses* **9**, 249 (2017).