mTORC2 modulates the amplitude and duration of GFAT1 Ser-243 phosphorylation to maintain flux through the hexosamine pathway during starvation

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The mechanistic target of rapamycin (mTOR) controls metabolic pathways in response to nutrients. Recently, we have shown that mTOR complex 2 (mTORC2) modulates the hexosamine biosynthetic pathway (HBP) by promoting the expression of the key enzyme of the HBP, glutamine:fructose-6-phosphate aminotransferase 1 (GFAT1). Here, we found that GFAT1 Ser-243 phosphorylation is also modulated in an mTORC2-dependent manner. In response to glutamine limitation, active mTORC2 prolongs the duration of Ser-243 phosphorylation, albeit at lower amplitude. Blocking glycolysis using 2-deoxyglucose prolongs the duration of Ser-243 phosphorylation, correlating with heightened mTORC2 activation, increased AMPK activity, and O-GlcNAcylation. However, when 2-deoxyglucose is combined with glutamine deprivation, GFAT1 Ser-243 phosphorylation and mTORC2 activation remain elevated, whereas AMPK activation and O-GlcNAcylation diminish. Phosphorylation at Ser-243 promotes GFAT1 expression and production of GFAT1-generated metabolites including ample production of the HBP end-product, UDP-GlcNAc, despite nutrient starvation. Hence, we propose that the mTORC2-mediated increase in GFAT1 Ser-243 phosphorylation promotes flux through the HBP to maintain production of UDP-GlcNAc when nutrients are limiting. Our findings provide insights on how the HBP is reprogrammed via mTORC2 in nutrient-addicted cancer cells.

Nutrients are metabolized to produce energy and synthesize macromolecules for cell growth and proliferation. Two main nutrients that the cells metabolize for bioenergetic and biosynthetic processes are glucose and glutamine (1). Glucose metabolism generates metabolites that are used by the tricarboxylic acid (TCA) cycle and other biosynthetic pathways to ultimately produce ATP and macromolecules. In highly proliferating cells, replenishing the TCA cycle intermediates, a process referred to as anaplerosis, is crucial to satisfy the increased demand for energy and building blocks. When glucose is limiting, a key nutrient for anaplerosis is glutamine, which undergoes glutaminolysis to subsequently generate the TCA cycle metabolite α-ketoglutarate (2). How cells control flux through the different metabolic pathways in coordination with nutrient availability remains to be elucidated.

mTOR is a central signaling molecule that controls cellular metabolism (3, 4). It forms two protein complexes, mTORC1 and mTORC2. Numerous studies have shown how mTORC1 promotes anabolic metabolism in response to availability of nutrients, particularly amino acids. On the other hand, the functions of mTORC2 remain poorly understood. So far, it is known to mediate growth factor signals to positively regulate mTORC1. mTORC2 consists of the evolutionarily conserved components, mTOR, rictor, SIN1, and mLST8. A conserved function of mTORC2 from yeast to human is the phosphorylation of the AGC protein kinase family members, AKT, SGK, and PKC (5–12). Most of the sites targeted by mTORC2 on these kinases are constitutively phosphorylated, underscoring the presence of basal mTORC2 activity. Phosphorylation of these targets by mTORC2 is associated with the control of protein maturation or stabilization. There is little known on other substrates that are phosphorylated by mTORC2 (13, 14). Understanding the signals that mTORC2 responds to should shed light on its cellular targets. In mammals, mTORC2 activity is further enhanced by the presence of growth factors, which stimulates the PI3K pathway (15). Activation of this pathway promotes nutrient uptake and metabolism (1). A hallmark of this increased activation is the phosphorylation of the mTORC2 substrate, AKT, at Ser-473 (16). Intriguingly, we have shown recently that mTORC2 activity is also enhanced by withdrawal of nutrients such as glucose or glutamine, suggesting that mTORC2 is responding to changes in intracellular levels of

2 The abbreviations used are: TCA, tricarboxylic acid; HBP, hexosamine biosynthesis pathway; AMPK, AMP-activated protein kinase; MEF, murine embryonic fibroblast; α-KG, α-ketoglutarate; DKG, dimethyl α-ketoglu-

tarate; 2-DG, 2-deoxyglucose.
metabolites (17). One of the biosynthetic pathways that utilize glutamine is the hexosamine biosynthesis pathway (HBP) (18, 19). We have recently shown that mTORC2 controls the HBP by modulating the expression of GFAT1 (17). During prolonged glucose starvation, mTORC2 increases GFAT1 via the transcription factor XBP1s (17, 20). In contrast, during acute glutamine starvation, mTORC2 controls maintenance of GFAT1 protein levels. This response to glutamine deprivation involves the TCA cycle and the GCN2/ATF4 pathway, but the precise mechanisms remain to be elucidated (17, 19). GFAT1 has two catalytic regions, an isomerase and an aminotransferase domain (21). It catalyzes the initial step of the HBP by isomerizing the glycolytic metabolite fructose-6-phosphate and at the same time promotes the transfer of an amino group from glutamine to produce glucosamine-6-phosphate. A series of subsequent reactions eventually produces uridine diphosphate GlcNAc (UDP-GlcNAc), a key precursor for the glycosylation of proteins and lipids (22). Glycosylation has structural, trafficking and signaling functions, highlighting the role of the HBP for normal physiological processes (23). Defects in the HBP resulting in abnormal protein glycosylation have been linked to insulin resistance, tumorigenesis, and other pathological conditions, most likely because of GFAT1 deregulation (20, 24, 25). Interestingly, certain hyperactivating mutations in Gfat1 in Caenorhabditis elegans have also been associated with increased life span in this organism (26). Thus, the HBP is likely tightly controlled via GFAT1, not just by nutrient levels but also by signaling molecules that control metabolism. Although the mechanisms remain unclear, AMPK, PKA, and GCN2, which respond to ATP, cAMP, and amino acid levels, respectively, have been linked to GFAT1 regulation (19, 27–30). How GFAT1 and the HBP are regulated during nutrient fluctuations is still poorly understood.

Based on our findings that mTORC2 associates with GFAT1 and that it modulates GFAT1 expression in response to nutrient levels (17), here we elucidated how mTORC2 could play a role in regulating GFAT1 phosphorylation. We identified Ser-243 as a phosphosite that is regulated by mTORC2. Although basal levels of phosphorylation occur under nutrient-replete conditions, a profound block in glucose metabolism, which triggers mTORC2 activation, increases Ser-243 phosphorylation. We found that mTORC2 is essential to preserve GFAT1 phosphorylation, albeit at lower amplitude during nutrient limitation to sustain flux through the HBP.

**Results**

**Phosphorylation of GFAT1 is sensitive to mTORC2 disruption or glucose deprivation**

We previously showed that GFAT1 expression depends on mTORC2 and the amount of nutrients. We also found that GFAT1 associates with mTORC2 components (17). Here, we examined whether GFAT1 could also be regulated by mTORC2 via phosphorylation. We treated WT murine embryonic fibroblasts (MEFs) with the mTOR inhibitor Torin1 for 1–24 h and analyzed the phosphorylation of GFAT1 using Phos-tag gels. Torin1 treatment led to increased mobility of GFAT1 in the Phos-tag gel, indicating decreased phosphorylation. As we have shown previously, basal GFAT1 expression in SIN1−/− MEFs is attenuated. Interestingly, GFAT1 from SIN1−/− MEFs migrated similarly as the GFAT1 from WT MEFs upon Torin1 treatment or glucose starvation (Fig. 1, A and B). These findings suggest that phosphorylation of GFAT1 requires mTORC2 and is diminished by glucose deprivation.

We next used MS to determine GFAT1 phosphosites. We found that immunoprecipitated Myc–GFAT1 that was overexpressed in WT MEFs is phosphorylated at Ser-243 (Fig. 1C). Enhanced phosphorylation of this site (Ser-243 in rat, Ser-259 in mouse, Ser-261 in humans will be referred herein as Ser-243 in keeping with the literature) was previously linked to AMPK activation (27, 28, 30). In contrast to findings from Eguchi et al. (28), we found that prolonged glucose starvation in WT MEFs led to decreased phosphorylation of Ser-243 (Fig. 1, B and D). These findings suggest that GFAT1 phosphorylation at Ser-243 is sensitive to glucose deprivation in WT MEFs.

**GFAT1 phosphorylation at Ser-243 is mediated by mTORC2**

To further examine the regulation of Ser-243, we generated antibodies against this phosphosite. The anti-phospho–Ser–243 GFAT1 antibody recognized GFAT1 phosphorylated at Ser-243, but not the extracts treated with λ phosphatase or extracts from cells overexpressing the mutant S243A (Fig. 2A and Fig. S1A). Upon quantitation, although there is a significant reduction in GFAT1 total protein, there is no apparent decrease in Ser-243 phosphorylation in SIN1−/− MEFs (Fig. 2B and Fig. S1B). However, GFAT1 phosphorylation may affect its protein expression levels. We therefore addressed whether mTORC2 can phosphorylate the Ser-243 site using in vitro translation/kinase assay (5). In vitro translated GFAT1 can be phosphorylated at Ser-243 by immunoprecipitated mTOR and this phosphorylation was prevented by incubating the reaction with Torin1 (Fig. 2C). Furthermore, reconstitution of SIN1β and thus mTORC2, in SIN1−/− MEFs increased both total GFAT1 expression and Ser-243 phosphorylation (Fig. 2D). Hence, the effect on GFAT1 phosphorylation and expression is specific to the loss of SIN1. We then treated WT MEFs with Torin1 for 1–30 h. Torin1 treatment also diminished total GFAT1 phosphorylation and expression in these cells (Fig. 2E). Hence, in MEFs, chronic mTORC2 inhibition or disruption attenuates both GFAT1 Ser-243 phosphorylation and protein expression.

We next analyzed GFAT1 phosphorylation using the cancer cell line HeLa. Our phospho-antibody also recognized the Ser–243–phosphorylated GFAT1 in these cells (Fig. 2F). This phosphorylation increased as HeLa cells were cultured in complete media for prolonged hours, correlating with increased AKT Ser-473 (marker of mTORC2 activation) but not S6 phosphorylation (marker of mTORC1 activation) (Fig. 2G). Increased Ser-243 phosphorylation was prevented upon incubation with Torin1. Together, these findings support that mTORC2 is involved in GFAT1 Ser-243 phosphorylation.

**Glutamine levels affect Ser-243 phosphorylation during glucose deprivation**

Previously, it was reported that Ser-243 phosphorylation correlated with increased glucose starvation and AMPK activation (27, 28). Because we instead observed decreased Ser-243...
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A

| SIN1<sup>−/−</sup> | WT MEF | Torin1 | Phos-tag |
|-------------------|--------|--------|----------|
| -                 | +      | +      | 100      |
| 1                 | 1      | 1      | 70       |
| 12                | 12     | 12     | 55       |
| 24                | 24     | 24     | 55       |

GFAT1 pS473 AKT α-tubulin

B

| WT MEF | SIN1<sup>−/−</sup> MEF | Glucose | Phos-tag |
|--------|------------------------|---------|----------|
| +      | +                      | +       | 100      |
| 1      | 1                      | 1       | 35       |
| 2      | 2                      | 2       | 25       |
| 3      | 3                      | 3       | 25       |

GFAT1 S6

C

241-VDpSTTCLFPVEEK-253

pSer243

241-VDSTTCLFPVEEK-253

Non-modified

D

RT:

pSer243

Relative Abundance

Non-modified

(-) Glucose

basal

AA: 21492864
BP: 862.64849

AA: 4018206
BP: 802.64949

AA: 74021946
BP: 762.686658

AA: 59612468
BP: 782.86694

basal

(-) Glucose
phosphorylation during 6 h glucose deprivation (Fig. 1D), and given our previous findings that maintenance of GFAT1 expression depends on sufficient glutamine (17), we suspected that this phosphorylation could in fact be sensitive to glutamine levels during culture. To assess contributions of glucose versus glutamine, we first resuspended growing cells in either fresh complete media or media lacking glucose. Because glutamine is unstable in media, we added fresh glutamine during resuspension of cells in media containing or lacking glucose. Under these conditions, we did not observe a decrease in GFAT1 Ser-243 phosphorylation during glucose withdrawal up to 12 h in WT MEFs (Fig. 3A). However, a robust knockdown of Sin1 in these cells diminished Ser-243 phosphorylation by 6 h of glucose deprivation (Fig. S1C). Although GFAT1 phosphorylation and expression were already dramatically reduced in Sin1−/− MEFs, no further significant decrease occurred during starvation in Sin1−/− MEFs. AMPK activation, as indicated by Thr-172 phosphorylation, increased by 12 h in WT MEFs and at all time points in Sin1−/− cells upon glucose withdrawal. Hence, the absence of glucose triggered very different kinetics of GFAT1 and AMPK phosphorylation in MEFs. As we have shown previously (17), AKT phosphorylation increased immediately upon withdrawal of glucose and stayed elevated up to 12 h of starvation in WT but was absent at all time points in Sin1−/− MEFs. The kinetics of mTORC1 activation, as shown by S6 phosphorylation, were similar in WT and Sin1−/− MEFs, slightly increasing in response to glucose withdrawal and remaining elevated up to 12 h. Levels of total cellular O-GlcNAcylation, a marker used for HBP activity, positively correlated with the amounts of GFAT1 phosphorylation (Fig. 3A). Thus, when glutamine is sufficient, glucose withdrawal alone does not diminish GFAT1 Ser-243 phosphorylation in WT MEFs. To further address this, we removed both glucose and glutamine up to 6 h. Combined deprivation of these nutrients abolished Ser-243 phosphorylation by 6 h and significantly diminished GFAT1 protein levels (Fig. 3B). mTORC2 was robustly activated upon withdrawal of both nutrients but was abolished by 6 h, coinciding with abrogation of GFAT1 phosphorylation. mTORC1 activation also diminished by 3 h and was not detectable at 6 h. Thus, in MEFs, GFAT1 Ser-243 phosphorylation can be maintained during glucose starvation as long as there is sufficient glutamine and mTORC2 activity.

Glutamine limitation lowers the amplitude but prolongs the duration of GFAT1 Ser-243 phosphorylation in an mTORC2-dependent manner

To understand how glutamine maintains GFAT1 Ser-243 phosphorylation, we first conducted prolonged (up to 30 h) glutamine withdrawal in the presence of glucose. In MEFs, GFAT1 phosphorylation and expression were diminished pronouncedly by 12 h glutamine deprivation (Fig. 4A and Fig. S2A). As we have reported previously, AKT phosphorylation was triggered transiently during prolonged culture in complete media but was robustly sustained during glutamine withdrawal (Fig. 4A). Hence, in MEFs, glutamine is required to maintain both GFAT1 phosphorylation and expression. In HeLa, there was a marked decrease in phosphorylation by 30 h of glutamine withdrawal without an obvious change in GFAT1 expression (Fig. 4B and Fig. S2B). In agreement with our previous results (17), withdrawal of glutamine also augmented AKT Ser-473 phosphorylation whereas mTORC1 activation was abolished. The decreased GFAT1 phosphorylation also corresponded to attenuated total O-GlcNAcylation after 12–24 h. Because we did not observe a marked decrease in GFAT1 expression in HeLa, we therefore used these cells to further address how glutamine is involved in maintaining Ser-243 phosphorylation. Glutamine undergoes glutaminolysis to replenish TCA cycle intermediates by the generation of α-ketoglutarate (α-KG). We therefore supplemented the glutamine-deprived cells with this metabolite 1 h before harvesting to examine if it could rescue Ser-243 phosphorylation. Strikingly, despite robust expression of GFAT1, Ser-243 phosphorylation was completely absent by 24 h upon supplementation with α-KG in glutamine-replete cells (Fig. 4C and Fig. S3A). This was accompanied by the loss of mTORC1 and mTORC2 signaling, as well as decreased O-GlcNAcylation at this time point. In contrast, under glutamine-deprived conditions, the phosphorylation of GFAT1 at Ser-243 gradually diminished during 6–24 h, whereas GFAT1 expression remained robust. Supplementation with α-KG slightly boosted Ser-243 phosphorylation during 6–24 h starvation. It is notable that whereas mTORC1 signaling was abolished during 6–24 h, AKT phosphorylation remained elevated under these conditions. AMPK phosphorylation increased during extended glutamine withdrawal and was blunted when α-KG was supplemented. Total O-GlcNAcylation was comparable between the glutamine-replete and -deplete conditions but had lower levels when GFAT1 Ser-243 phosphorylation was also severely blunted. Because the abolished GFAT1 phosphorylation and mTOR signaling occurred upon prolonged culture in the presence of α-KG, we further extended the incubation period (up to 48 h) and used dimethyl α-ketoglutarate (DKG), a derivative of α-KG. Interestingly, GFAT1 phosphorylation was abolished by 48 h in glutamine-replete media even without DKG addition (Fig. 4D and Fig. S3B). mTORC1 and mTORC2 signaling was also abolished by this time. Again, withdrawal of glutamine reduced the amplitude but sustained GFAT1 phosphorylation up to 48 h. Addition of DKG slightly elevated phos-

**Figure 1. Phosphorylation of GFAT1 is decreased in mTORC2-disrupted or glucose-deprived cells.** A, WT or Sin1−/− MEFs were treated with vehicle or Torin1 (100 nM) for the indicated hours. Cells were lysed in CHAPS buffer and lysates were fractionated using Phos-tag gel to analyze GFAT1 phosphorylation using GFAT1 antibody. Lysates were also fractionated using conventional Laemmli SDS-PAGE then immunoblotted for phospho-AKT or tubulin antibodies. B, WT or Sin1−/− MEFs were grown in complete media (+), then resuspended in complete (+) media or media lacking glucose (−) but containing glutamine, and dialyzed FBS for the indicated hours. Lysates were processed as in A and fractionated by Phos-tag gel (for GFAT1) and Laemmli SDS-PAGE for S6 as loading control. C, WT MEFs were transfected with Myc-GFAT1. Immunoprecipitated GFAT1 was separated by SDS-PAGE and the corresponding GFAT1 band was excised, digested with trypsin, and subjected to LC/MS-MS. The sequence and fragment spectra of phosphorylated versus nonphosphorylated fragments are shown. D, WT MEFs transfected with Myc-GFAT1 were resuspended in complete media or media lacking glucose and incubated for 6 h. Immunoprecipitated GFAT1 was processed as in (C). Relative abundance of phosphorylated or nonphosphorylated peptide fragments eluted over time during basal or glucose-starved conditions is plotted.
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Phosphorylation at 24–48 h. The sustained GFAT1 phosphorylation coincided with prolonged AKT phosphorylation but not AMPK or mTORC1 activation. Together, these findings reveal that glutamine limitation lowers the amplitude but increases the duration of GFAT1 phosphorylation, likely because of sustained mTORC2 signaling.

We also examined whether Ser-243 phosphorylation could be rescued during prolonged glucose and glutamine starvation.
of MEFs. Indeed, the addition of glutamine or α-KG reconstituted not only the phosphorylation but also the expression of GFAT1 at 6 h starvation (Fig. 4E). DKG modestly increased phosphorylation but restored GFAT1 protein levels. As we have shown previously, supplementation with α-KG and DKG can reduce AKT phosphorylation. Together these findings reveal that glutamine, via the glutaminolysis product α-KG, maintains Ser-243 phosphorylation of GFAT1 during nutrient limitation.

The increase in GFAT1 Ser-243 phosphorylation during glycolysis block correlates with mTORC2 activation

Previous studies have found that the glycolytic inhibitor 2-deoxyglucose (2-DG) enhances GFAT1 Ser-243 phosphorylation (28). We reasoned that blocking glycolysis could trigger mTORC2 activation and might account for increased Ser-243 phosphorylation. We therefore examined the effect of 2-DG on GFAT1 phosphorylation under nutrient-replete or -deplete conditions. When both glucose and glutamine were present, we did not observe a change in GFAT1 phosphorylation even with the addition of 2-DG (Fig. 5A). However, when 2-DG was combined with glucose withdrawal, a robust increase in Ser-243 phosphorylation occurred. This enhancement was abrogated by Torin1 treatment, suggesting the involvement of mTORC2 (Fig. 5B). The combined removal of glucose and glutamine also augmented Ser-243 phosphorylation at 3 h and this increase occurred earlier when 2-DG was added (Fig. 5A). The increase in GFAT1 Ser-243 phosphorylation correlated with the increase in mTORC2 activation but not mTORC1. On the other hand, AMPK activation only increased during glucose withdrawal, even without 2-DG. The increase in AMPK phosphorylation was abolished during combined glucose and glutamine withdrawal. Interestingly, although O-GlcNAcylation was elevated during 2-DG treatment, it was diminished when glutamine was withdrawn from the media. Hence, the increase in Ser-243 phosphorylation does not positively correlate with total O-GlcNAcylation under glucose-deprived conditions. These findings reveal that blocking glycolysis robustly enhances Ser-243 phosphorylation and that this increase occurs even under glucose-limiting conditions. These findings also further support that the increase in Ser-243 phosphorylation correlates more with mTORC2 rather than AMPK activation.

Because the above experiments revealed that the increase in GFAT1 Ser-243 phosphorylation did not always correlate with levels of O-GlcNAcylation, we further verified this by ectopic expression of WT versus the phospho-deficient mutant GFAT1 construct bearing a Ser-243 to Ala-243 mutation (S243A).

Upon expression of HA-GFAT1-WT, O-GlcNAcylation was highly robust by 6 h of 2-DG treatment in MEFs (Fig. 5C). In contrast, expression of the S243A mutant did not further increase O-GlcNAcylation at 6 h, thus suggesting that Ser-243 phosphorylation could increase GFAT1/HBP activity in MEFs during a glycolysis block. On the other hand, although total O-GlcNAcylation decreased during glutamine withdrawal, its levels remained comparable in HeLa cells overexpressing either HA-GFAT1-WT or S243A (Fig. 5D). These findings further demonstrate that the increased Ser-243 phosphorylation of GFAT1 correlates with increased O-GlcNAcylation during glycolysis block but not during glutamine limitation.

Ser-243 phosphorylation enhances GFAT1 expression and promotes flux through the HBP during nutrient limitation

The above findings suggest that the function of Ser-243 phosphorylation in promoting HBP flux may not be reflected by the levels of total cellular O-GlcNAcylation. To gain insights on how Ser-243 phosphorylation affects the HBP (Fig. 6A), we instead analyzed metabolite levels by MS. We used the same conditions as in Fig. 5A that led to a robust increase in Ser-243 phosphorylation upon 2-DG treatment under glucose-limiting or combined glucose- and glutamine-limiting conditions for 1 h. In the absence of 2-DG, glucose withdrawal decreased lactate as well as fructose-6-phosphate and α-KG levels as expected. There was not much further decrease during combined glucose and glutamine deprivation. In contrast, glutamine and glutamate levels remained normal during glucose withdrawal but declined during combined glucose and glutamine starvation. Interestingly, levels of UDP-GlcNAc, the end product of the HBP, remained normal even upon withdrawal of both nutrients. 2-DG treatment in complete media did not decrease lactate production, indicating glycolysis was not blocked under these conditions (Fig. 6B). However, 2-DG treatment of glucose-deprived cells led to a pronounced block in glycolysis as indicated by accumulation of glucose-6-phosphate and deoxyglucose-6-phosphate. We then compared metabolites produced during 2-DG treatment under glucose-starved versus combined glucose- and glutamine-withdrawal conditions wherein Ser-243 phosphorylation is enhanced. Among the metabolites that we examined, it was striking that GlcNAc-phosphate (GlcNAc-P) levels were pronouncedly elevated. This metabolite is likely accumulating because of increased GFAT1 activity and/or because of a partial block in the distal reactions. Indeed, 2-DG treatment led to a pronounced reduction in UTP, which is required for the generation of UDP-GlcNAc. Interestingly, despite low UTP, UDP-GlcNAc levels remained substantial (about 50% less than normal). As the treatment with 2-DG
**Figure 3.** Glutamine is required to maintain GFAT1 phosphorylation during glucose deprivation. A, growing WT and SIN1<sup>−/−</sup> MEFs were resuspended in glutamine-replete media containing or lacking glucose and incubated for the indicated hours. Cell lysates were fractionated by SDS-PAGE followed by immunoblotting. Quantitation of phospho–Ser-243 and total GFAT1 are shown. Amounts are expressed relative to extracts from cells incubated in complete media at 1 h; n = 3. B, growing WT MEFs were resuspended in media lacking both glucose and glutamine and processed as in (A). Amounts are expressed as in (A); error bars represent S. D. n = 3; ***, p < 0.0005; *, p < 0.05.
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coincided with increased Ser-243 phosphorylation, these findings suggest that the phosphorylation promotes flux through the HBP during nutrient limitation.

Next, we examined how phosphorylation at Ser-243 affects GFAT1 protein levels. We used the SIN1-deficient cells to express WT versus the phosphodeficient (S243A) or phospho-mimetic (E1 and E2; see Fig. S4A) mutants because these cells express reduced levels of endogenous GFAT1. Upon prolonged glutamine starvation (24 h), the phosphomimetic mutants remained more highly expressed compared with WT and S243A mutant (Fig. 6C and Fig. S4B). These findings support that phosphorylation at Ser-243 stabilizes GFAT1 and maintains its expression during nutrient starvation.

Finally, we investigated the importance of GFAT1 Ser-243 phosphorylation in an in vivo model. We used thymocytes from WT mice or mice with T-cell–specific deletion of the tumor suppressor gene Pten. PTEN normally antagonizes PI3K signaling (31). Its deletion enhances AKT phosphorylation and mTORC2 signaling. In PTEN-deficient thymocytes, mice are born with normal T-cells but acquire lymphoma at around weeks 10–20 (32) (Fig. S5). Whereas the WT thymus comprises about 85% double positive (CD4+CD8−), 10% single positive (CD4+ or CD8+), and 3% double negative (CD4−CD8−) thymocytes, the onset of lymphoma leads to about 50% CD4+ subset, which continues to increase as the lymphoma progresses. We cultured the WT versus Pten−/− thymocytes in the presence or absence of glutamine for 1–6 h. In WT (13 weeks), basal levels of GFAT1 as well as Ser-243 phosphorylation were hardly discernible (Fig. 6D). In the absence of Pten, at 6–8 weeks (no lymphoma), GFAT1 phosphorylation was more apparent correlating with discernible increase in AKT phosphorylation. Glutamine withdrawal did not have a significant effect on Ser-243 and AKT phosphorylation. During early lymphoma (20 weeks), AKT phosphorylation, GFAT1 expression, and Ser 243 phosphorylation became robust. Upon glutamine withdrawal, GFAT1 phosphorylation was diminished at 1 h. A subsequent attenuation of AKT and GFAT1 levels occurred by 3–6 h. During late lymphoma (30 weeks), AKT phosphorylation remained robust, as well as GFAT1 expression and phosphorylation. Both AKT and GFAT Ser-243 phosphorylation did not change after 1 h of glutamine withdrawal. GFAT1 phosphorylation was slightly more sustained up to 6 h compared with early lymphoma. Thus, GFAT1 Ser-243 phosphorylation and expression becomes enhanced as lymphoma develops, coinciding with augmentation of mTORC2 signaling.

Discussion

Flux through the metabolic pathways are reprogrammed depending on nutrient status to re-establish metabolic homeostasis and prevent uncontrolled proliferation or cell death. The mechanisms that allow the cell to restore metabolic homeostasis remain to be elucidated (33). In this study, we found that mTORC2 controls the amplitude and duration of phosphorylation of GFAT1 at Ser-243 in response to intracellular nutrient levels. Such mode of regulation controls flux through the hexosamine biosynthetic pathway under nutrient-limiting conditions.

The involvement of mTORC2 in modulating GFAT1 phosphorylation is supported by the following observations. First, we found that mTORC2 disruption or chronic inhibition decreased Ser-243 phosphorylation (Fig. 2, B, E, and G). In MEFs, the phosphorylation is linked to GFAT1 expression. This is reminiscent of the mTORC2-controlled constitutive phosphorylation of the AGC kinases, AKT at Thr-450 and the hydrophobic and turn motif sites in PKC (5–8). Phosphorylation of these sites occurs during their translation in an mTORC2-dependent manner. We showed here that mTORC2 can phosphorylate Ser-243 during in vitro translation of GFAT1, further supporting that it is a direct target of mTOR. In the cancer cells HeLa and T lymphoma that we examined, although Ser-243 phosphorylation did not affect GFAT1 protein levels, increased phosphorylation at this site coincided with augmented mTORC2 activation. Moreover, the abrogation of this phosphorylation when cultured in complete media for extended periods was also accompanied by a profound loss of mTORC2 signaling as even AKT expression was severely reduced in these cells (Fig. 4, C and D). Taken together, these findings are consistent with the notion that mTORC2 plays a role in mediating phosphorylation of this site. Nevertheless, it is worth noting that GFAT1 Ser-243 phosphorylation was not completely abolished in SIN1−/− cells (Figs. 2A and 3A), thus indicating that its phosphorylation could also occur in a SIN1- or mTORC2-independent fashion. Other studies using different cell types implicate AMPK in the phosphorylation of this site (27, 28, 34). However, Zibrova et al. observed that basal Ser-243 phosphorylation persisted despite depletion of AMPK (27). Hence, although our data are consistent with a role for mTORC2 in Ser-243 phosphorylation, future studies should define how this site could be co-regulated by other kinases.

Importantly, our findings reveal how Ser-243 phosphorylation responds to nutrient fluctuations. As cells proliferate in culture and nutrients become limiting (9–12 h in MEFs and 24–36 h in HeLa), mTORC2 activation increases whereas Ser-243 phosphorylation is maintained. The level of its phosphorylation depends on the amount of available glutamine and mTORC2. During glucose deprivation, there is no discernible change in Ser-243 phosphorylation unless glutamine is also withdrawn or mTORC2 is disrupted (Fig. 3 and Fig. S1C). On the other hand, sustained glutamine deprivation lowers the amplitude but prolongs the duration as long as mTORC2 is active. Glutamine serves as an important anaplerotic substrate particularly in cultured cells (2). Hence, either glutaminolysis itself or anaplerosis in general could promote Ser-243 phosphor-
ylation (Fig. 5A). In support of this notion, blocking glycolysis using 2-DG, which triggers anaplerosis, strongly increased Ser-243 phosphorylation. Indeed, 2-DG has been previously shown to rewire metabolism via mTOR partly via glutamine anaplerosis (35). Furthermore, supplementation with the glutaminolysis metabolite, α-KG or its derivative DKG, partially rescued

Figure 5. Increased Ser-243 phosphorylation during glycolysis block correlates with mTORC2 activation. A and B, HeLa cells were resuspended in media lacking glucose in the presence (+) or absence (−) of glutamine and 500 μM 2-DG or 1 μM Torin1. Cultures were allowed to incubate for the indicated hours. C and D, WT MEFs (C) or HeLa cells (D) were transfected with either HA-GFAT1-WT or HA-GFAT1-S243A. 24 h post transfection, cells were resuspended in media containing or lacking glucose and with the addition of 500 μM 2-DG (+) or lacking glutamine (−) and incubated for the indicated hours.
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(A) mTORC2 regulation of GFAT1 phosphorylation pathway.

(B) Ion counts for lactate, α-KG, glutamine, glutamate, UDP-GlcNAc, glucose-6-P, deoxy-glucose-6-P, and UTP under different conditions.

(C) SIN1−/− MEFs with (-) Glutamine.

(D) Early lymphoma and late lymphoma with PTEN−/− (no Lma) conditions.
GFAT1 Ser-243 phosphorylation even after extended glutamine deprivation (Fig. 4, C–E). Surprisingly, prolonged culture under complete media (48 h in HeLa) abolished Ser-243 phosphorylation as well as mTORC2 and mTOR1 signaling. The mechanisms behind this phenomenon are not clear at the moment. However, given that supplementation with α-KG accelerates this phenomenon whereas glutamine withdrawal prevents it, this would suggest that TCA cycle anaplerosis is tightly coupled to the HBP and mTOR signaling. Curiously, 2-DG increased Ser-243 phosphorylation despite the absence of glutamine (Fig. 5A). Whether salvage of intracellular glutamine via *de novo* biosynthesis or autophagy could feed into the TCA cycle or whether other anaplerotic substrates could account for this effect would need to be further investigated. The finding that α-KG levels are comparable during starvation conditions in the absence or presence of 2-DG (Fig. 6B) supports that there is sufficient anaplerosis under these conditions. Given the purported positive effects of AMPK activators in GFAT1 phosphorylation (27, 28, 34), whether they in fact trigger enhanced anaplerosis warrants further investigation. Taken together, our findings suggest that the induction or maintenance of GFAT1 Ser-243 phosphorylation occurs in conjunction with anaplerosis and increased mTORC2 activation.

What is the function of Ser-243 phosphorylation? Our data suggest that Ser-243 phosphorylation stabilizes GFAT1 protein during nutrient limitation and thus enables maintenance of flux through the HBP. The increased Ser-243 phosphorylation during 2-DG treatment correlated with a profound increase in GlcNAc-phosphate, a metabolite that is generated just after the GFAT1-catalyzed reaction (Figs. 5A and 6B). This increase implies elevated GFAT1 activity and/or accumulation of this metabolite because of a block in the distal reactions. Indeed, upon 2-DG treatment, the levels of UTP, a substrate required for the final step involved in UDP-GlcNAc production, decreased (Fig. 6B). Despite reduction in UTP, UDP-GlcNAc levels remained relatively high, which is likely facilitated by increased GFAT1 and most likely UAP1 (enzyme that catalyzes the last step in HBP) activity. Expression of these two enzymes has been found to be up-regulated in cancers, thus highlighting their critical role in promoting flux through the HBP (36–40). Further supporting the role of Ser-243 phosphorylation in enhancing GFAT1 activity, we found that the S243A phospho-deficient mutant blunted the increase in O-GlcNAcylation during glucose starvation/2-DG treatment (Fig. 5C). However, our findings contradict previous reports proposing that Ser-243 phosphorylation diminishes HBP activity. Those previous findings were based on decreased O-GlcNAcylation levels when cells were treated with pharmacological agents that increase Ser-243 phosphorylation (e.g., AMPK activators) (27, 28, 34). Although O-GlcNAcylation levels have been used as a marker for HBP activity (19, 20), our findings indicate that O-GlcNAcylation levels do not always correlate with the amount of cellular UDP-GlcNAc, the product of the HBP and the substrate used for O-GlcNAcylation. In particular, although blocking glycolysis indeed increased Ser-243 phosphorylation and O-GlcNAcylation, the latter was attenuated when combined with glutamine limitation (Fig. 5A). Speculatively, enzymes that are involved in O-GlcNAcylation, such as O-GlcNAc transferase, may be affected by glutamine levels (41). Hence, intracellular glutamine availability should be taken in consideration when correlating HBP activity with total O-GlcNAcylation. In another study, Li et al. (30) showed *in vitro* that phosphomimetic GFAT1 mutants have increased glucosamine-6-phosphate synthesizing activity while lowering amidohydroxyizing activity in the absence of fructose-6-phosphate and lowers the $K_m$ for fructose-6-phosphate. Thus, in addition to promoting GFAT1 expression levels as we showed here, positive modulation of GFAT1 activity would also allow the maintenance of flux through the HBP under nutrient limitation. Modulating the amplitude and duration of phosphorylation of GFAT1 in response to intracellular levels of glucose and glutamine metabolites would allow coordination of the activity of the HBP with other metabolic pathways that have input into this pathway (42). Taken together, the increase in Ser-243 phosphorylation correlates with increased GFAT1 expression and activity (increased glucosamine-6-phosphate) but not necessarily increased O-GlcNAcylation.

There is accumulating evidence that defective GFAT1 expression and activity occurs during aging, diabetes, and malignancy (24, 43). We demonstrated here using a mouse T lymphoma model that GFAT1 expression is elevated and its phosphorylation was more persistent during glutamine-limiting conditions as mTORC2 signaling was increased and as lymphoma progressed (Fig. 6D). These data further highlight how elevated mTORC2 signaling reprograms the metabolism by increasing GFAT1 phosphorylation and expression. Our findings provide rationale for targeting mTORC2 and GFAT1 regulation during malignancy and metabolic disorders that involve deregulation of the HBP.

**Experimental procedures**

**Materials**

Anti-phospho–Ser-243 GFAT1 was generated by GenScript. Anti-GFAT1 (ab125069) was from Abcam and anti-O-GlcNAc was from either Abcam (ab2739) or Sigma (O7764). HA (sc-104142) and β-tubulin (sc-47778) were from Santa Cruz Biotechnology. All other antibodies used were obtained from Cell Signaling Technology: pSer-473 AKT (4690); AKT (9272); pSer-240/244 S6 (2215); S6 (2317); pThr-

![Figure 6](https://example.com/figure6.png)

**Figure 6.** GFAT1 Ser-243 phosphorylation promotes flux through the hexosamine pathway during nutrient limiting conditions. A, the hexosamine biosynthesis pathway. B, HeLa cells were grown as in Fig. 5A and incubated for 1 h. Metabolites were extracted and analyzed by LC/MS. Complete media (C); (−) glucose (−G); (−) glucose (−G)− glutamine (−G−Q); y axis shows ion counts (arbitrary unit (au) × 100,000) of metabolites normalized to cell number (n = 3; error bars represent S.E.). C, S1N1−/− MEFs were transfected with either WT, S243A, or E2 (S243E/T244E) mutants of HA-GFAT1. 24 h post transfection, cells were resuspended in media lacking glutamine and incubated for the indicated hours. Total cell extracts were processed for immunoblotting. Arrow indicates HA-GFAT1 WT or mutant protein. Upper band is nonspecific. Amounts of WT or mutant HA-GFAT1 were quantitated and expressed relative to HA-GFAT1-WT activity. Values are mean ± S.D.; n = 2–3; **, p < 0.05; *, p < 0.01. D, thymocytes from WT mice (13 weeks) or mice with T-cell–specific deletion of Pten at 6–8 weeks (no lymphoma (Lmo)), 20 weeks (early lymphoma), or 30 weeks (late lymphoma) were incubated ex vivo in media containing (+) or lacking glutamine (−) at the indicated hours.
mTORC2 regulation of GFAT1 phosphorylation

172 AMPK (2535); AMPK (2532); and mTOR (2983). WT and SIN1−/− MEFs were described previously (44). Torin1 was purchased from Tocris. α-Ketoglutarate, 2-deoxyglucose, and dimethyl-α-ketoglutarate (DKG) were obtained from Sigma.

Plasmids

The mouse Gfpt1 cDNA was generated using the following primers: 5′ccgtccacacgggaaagacgcagcttcctttctt; E2 (S243E/T244E) forward, 5′cgacttactctactgttacagatttgg and reverse, 5′cgtggtggacgccacgacatgcctgttc and reverse, 5′gaacaggcatgtcgtggacagggaaagcggccgcttactctactgttacagatttgg. All constructs were validated by restriction enzyme digests and sequencing.

Cell culture, lysis, and immunoblotting

HeLa, WT, and SIN1−/− MEFs were cultured in complete DMEM (Sigma, D6546) with 10% FBS, 2 mM glutamine (Gibco 25030-164), and penicillin/streptomycin (Gibco 15140-122). After culturing for 24 h, reaching 70–80% confluency, cells were resuspended in either fresh complete media or starvation media (glucose starvation media, Corning 17-207-CV; glutamine, 2 mM, 106) HeLa cells were resuspended in complete or starvation media and incubated for 1 h. To harvest, medium was aspirated completely and cells resuspended in 1 ml 40:40:20 methanol:acetonitrile:water with 0.5% formic acid on ice for 5 min. 50 μl of 15% NH4HCO3 was added and cells were scraped. Supernatant was recovered by centrifugation. Samples in triplicates were analyzed using reversed-phase ion-pairing chromatography coupled by negative mode electrospray ionization to a stand-alone Orbitrap mass spectrometer (Thermo Scientific). Data were analyzed using Maven software.

Coupled in vitro translation/kinase assay

In vitro transcription/translation was performed using the PUREsystem II kit (New England Biolabs) following manufacturer’s protocol. A 25 μl reaction containing purified bacterial translation factors with 0.5 μg Gfpt1 template and immunoprecipitated mTOR from HEK293T cells, ATP, and RNase inhibitor was incubated for 2 h at 37 °C. The reaction was stopped by addition of SDS sample buffer. Mass spectrometry

Myc-GFAT1 was transfected into WT MEFs. Immunoprecipitated GFAT1 was separated by SDS-PAGE and stained using Colloidal Blue Staining Kit (Invitrogen) according to the manufacturer’s protocol. Bands of interest were cut out and submitted to the Rutgers Biological Mass Spectrometry Facility for analysis. Peptides were analyzed by Nano LC-MS/MS using Amersham Biosciences Imager 600 (GE Healthcare). 24 h post transfection, cells were resuspended in either complete or starvation media as necessary. For in vitro kinase assays, HEK293T cells grown on a 10 cm plate, reaching a confluency of 70% were lysed with immunoprecipitation lysis buffer (40 μl HEPES, 2 mM EDTA, 0.3% CHAPS, 150 mM NaCl) containing protease and phosphatase inhibitors. Lysates were precleared by adding Protein G Sepharose beads, then allowed to tumble for 1 h at 4 °C. Supernatants were recovered then incubated with mTOR antibody for 2 h at 4 °C. Protein G Sepharose beads were added then incubated for 1 h at 4 °C. Beads were washed three times with immunoprecipitation lysis buffer. A quarter of the beads were used for each kinase assay.

Copolled in vitro translation/kinase assay

In vitro transcription/translation was performed using the PUREsystem II kit (New England Biolabs) following manufacturer’s protocol. A 25 μl reaction containing purified bacterial translation factors with 0.5 μg Gfpt1 template and immunoprecipitated mTOR from HEK293T cells, ATP, and RNase inhibitor was incubated for 2 h at 37 °C. The reaction was stopped by addition of SDS sample buffer.

Mice and thymocyte analysis

Homozygous C57BL/6 Ptenfl/fl (The Jackson Laboratory) mice were crossed with C57BL/6 Lck-Cre mice (Taconic Farms), which generate T-cell specific Pten−− knockout mice. All mice were genotyped by PCR using primers described previously (17). The thymus was removed from WT or mutant mice and thymocytes were harvested in complete DMEM; then
about 5 × 10^6 cells were resuspended in media containing or lacking glutamine for each time point. Handling and experimentation protocols have been reviewed and used in accordance with the Institutional Animal Care and Use Committee regulations of Rutgers University.

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