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RESEARCH PAPER

Drosophila GFAT1 and GFAT2 enzymes encode obligate developmental functions

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ABSTRACT

Glutamine: fructose-6-phosphate amidotransferase (GFAT) enzymes catalyse the first committed step of the hexosamine biosynthesis pathway (HBP) using glutamine and fructose-6-phosphate to form glucosamine-6-phosphate (GlcN6P). Numerous species (e.g. mouse, rat, zebrafish, chicken) including humans and *Drosophila* encode two broadly expressed copies of this enzyme but whether these perform redundant, partially overlapping or distinct functions is not known. To address this question, we produced single gene null mutations in the fly counterparts of *gfat1* and *gfat2*. Deletions for either enzyme were fully lethal and homozygotes lacking either GFAT1 or GFAT2 died at or prior to the first instar larval stage. Therefore, when genetically eliminated, neither isoform was able to compensate for the other. Importantly, dietary supplementation with D-glucosamine-6-phosphate rescued GFAT2 deficiency and restored viability to *gfat2*$^{-/-}$ mutants. In contrast, glucosamine-6-phosphate did not rescue *gfat1*$^{-/-}$ animals.

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Introduction

Glucose-fructose amidotransferase (GFAT) enzymes carry out rate-limiting steps in the Hexosamine Biosynthetic Pathway (HBP). By converting Fruc-6-P and glutamine into GlcN-6-P (glucosamine-6-P) GFAT enzymes define critical biosynthetic activities that produce uridine 5′-diphosphate–*N*-acetylglucosamine (UDP-GlcNAc), an essential substrate for multiple glycosylation pathways, including O-linked GlcNAcylation and N-linked protein glycosylation (reviewed in [1–5]). These post-translational modifications target a widespread number of proteins and as such, they impact numerous cellular processes, including transcription, translation, metabolism and signal transduction [1,6–8]. Furthermore, perturbations in either O-linked GlcNAcylation and N-linked protein glycosylation pathways have been implicated in metabolic disorders, insulin resistance and certain cancers [6–9].

In *Drosophila*, HBP enzymes are generally well-conserved and, likewise, O-linked GlcNAc modification is clearly an important regulator of growth, metabolism, and physiology [2]. The fly genome codes for orthologs of glucosamine-phosphate N-acetyltransferase (CG1969), phosphoacetylglucosamine mutase and UDP-N-acetylglucosamine diphosphorylase. The latter two enzymes are clearly essential since mutations in the corresponding genes are lethal (genes are *nst* and *mmy*) [10–13]. In flies, the HBP is also critical to form the exoskeleton since it feeds the production of chitin, which is a polymer primarily composed of UDP-GlcNAc [11,12].

The fly genome contains two genes coding for GFAT enzymes, designated *gfat1* and *gfat2* [2,14,15]. *gfat1* mRNA expression were associated with tissues that produce chitin [14] and, in contrast to the single isoform produced from *gfat2*, the *gfat1* locus generates up to nine alternate transcript isoforms (see Flybase.org [15]). In biochemical studies, *Drosophila* GFAT1 produced enzymatic activity *in vitro* [14], but the protein coded by *gfat2* has not been similarly studied. Here we individually eliminated both genes and determined that each is essential for viability.

Results

As the rate-limiting enzyme of the HBP, glucose-fructose amidotransferase (GFAT) converts Fruc-6-P and glutamine into GlcN-6-P (glucosamine-6-P) [2]. Two GFAT genes encoding two GFAT enzymes

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are found in the genomes of humans, mouse and *Drosophila* (see Figure 1). In flies, the predicted GFAT1 and GFAT2 proteins are highly similar to each other (Figure 1). Residues involved in substrate binding [16] are conserved in both *Drosophila* GFAT1 and GFAT2 (Figure 1) and, furthermore, GFAT1 activity was shown to be regulated by UDP-N-acetylglucosamine and PKA [14]. However, the action of GFAT1 and GFAT2 in development has not been investigated and whether these genes code for distinct or redundant functions was not known. To address this question, we produced single gene null mutations at both of these genes.

As shown in Figure 2, we generated deletion mutants of both *gfat1* and *gfat2* using CRISPR technology to replace the corresponding sequences with DsRed. To edit *gfat1*, guide RNAs located at the beginning of the first intron and in the 3' UTR were chosen in order to delete most of the gene (over 10kb). Similarly, at the *gfat2* locus, guide RNAs were chosen to delete the largest exon.

For *gfat1*, five independent isolates were identified and four of these (designated B1, B2, T1, V1) were PCR verified. Similarly, we identified two independent isolates of *gfat2* (designated E1 and F1). After genomic sequencing, we found that $gfat1^{B2}$ and $gfat1^{T1}$ were identical alleles and, likewise, $gfat2^{E1}$ and $gfat2^{F1}$ were found to be identical alleles. Therefore, $gfat1^{B2}$ and $gfat2^{E1}$ flies were selected for subsequent studies and, accordingly, were crossed in the *yw* background for five generations.

The Tb marker can be reliably scored at L2 larval stage or older and, using this marker, we assessed viability. As indicated in Figure 3, only heterozygotes for either $gfat1^{B2}$ and $gfat2^{E1}$ survived to the L2 stage or later. Furthermore, as seen in Table 1, $gfat2^{E1}$ failed to complement Deficiency chromosomes that uncover *gfat2*. Likewise, $gfat1^{B2}$ failed to complement a MiMIC insertion at the *gfat1* locus. Therefore, we conclude that animals lacking either *gfat1* or *gfat2* are lethal prior to the L2 stage. To biochemically assess the effect of these mutations, we pooled 24–28 hr. animals (L1 larvae and embryos) produced from heterozygous parents and assayed these lysates for GFAT enzymatic activity. As seen in Figure 3(c), *gfat1* and *gfat2* samples were each reduced by 28.6% and 30.3% respectively compared to parental lysates, suggesting that both

Figure 1. Sequence alignment of GFAT proteins

The amino acid sequences of E. coli, human and fly GFAT1 and GFAT2 are aligned using Clustal Omega. An * (asterisk) indicates positions which have a single, fully conserved residue (red). Amino acids that are conserved among fly and human GFAT 1 and GFAT2 are yellow. A: (colon) indicates conservation between groups of strongly similar properties. A. (period) indicates conservation between groups of weakly similar properties. Residues in green frames are involved in substrate binding [16].

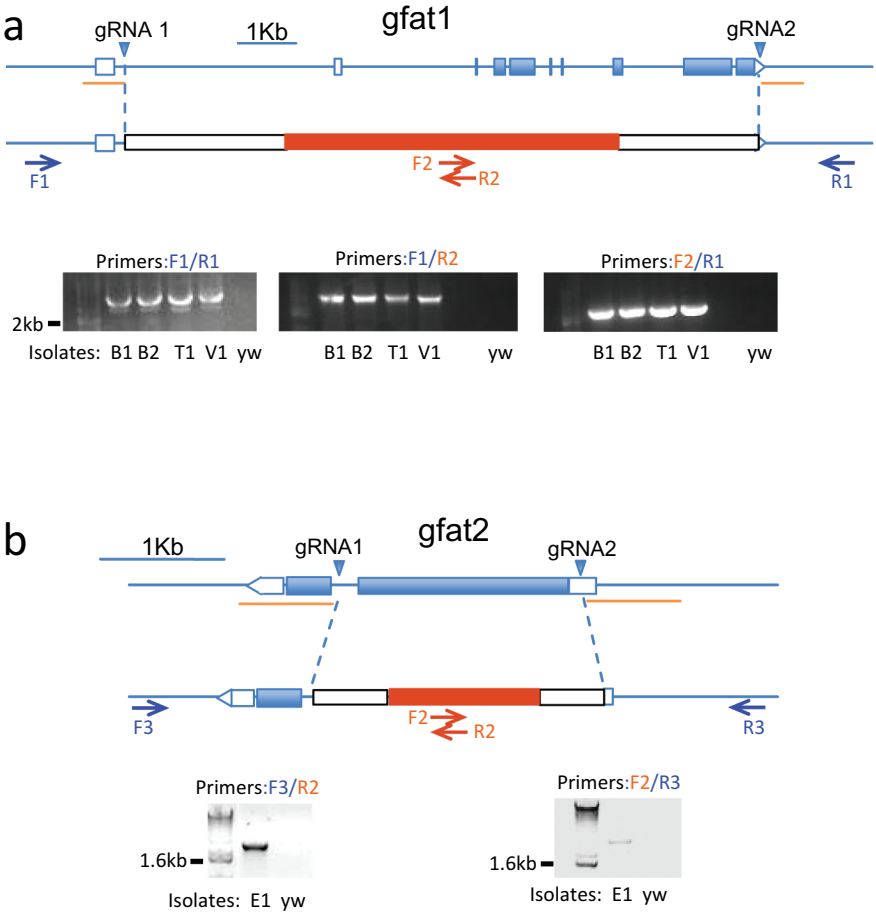

Figure 2. Crispr-mediated elimination of *Drosophila gfat* genes
In each panel, the wild type genomic structure for *gfat1* (A) and *gfat2* (B) is indicated above and edits that replace *gfat* sequences with DsRed and other sequences are below. The positions of guide RNAs (gRNAs, blue triangles) and verifying primers (arrows) for each gene are indicated. The sequences flanking the gRNAs that were cloned into the donor plasmids are labeled as light orange lines. Combinations of primers outside the homology arms (F1/R1 for gfat1 and F3/R3 for gfat2) together with DsRed specific primers (F2/R2) were used to perform PCR analyses as indicated. The diagnostic PCR products of expected sizes are shown in A (3.8kb for F1/R1, 1.9kb for F1/R2 and F2/R1) and in B (2.4kb for both F3/R2 and F2/R3).

GFAT enzymes mutually contribute to canonical activity (Figure 3(c)).

Elimination of GFAT activity, which is pivotal for HBP, should deplete GlcN-6-P in these mutants. Therefore, we tested for dietary rescue using increasing concentrations of GlcN-6-P (see Figure 3 and methods). Without dietary GlcN-6-P, all *gfat2-*^{E1} homozygotes died as early larvae or unhatched embryos (Figure 3). However, when supplemented at GlcN-6-P concentrations greater than 4 mg/ml, we observed that substantial numbers of adult *gfat2*^{E1} homozygotes eclosed. Furthermore, when supplemented with 25 mg/ml GlcN-6-P, over half of these were restored to adult viability. Importantly, as seen in Figure 3, a clear dose response was observed, as increasing concentrations of dietary GlcN-6-P permitted increasing eclosion rates. Adults homozygous for *gfat2*^{E1} that were rescued by dietary GlcN-6-P died within 3–4 days, but when crossed to wild type virgin females, we found that rescued *gfat2*^{E1} males were clearly fertile. In parallel studies, the same GlcN-6-P supplement was provided to *gfat1*^{B2} flies and, at all concentrations, *gfat1*^{B2} mutants were unaffected by dietary GlcN-6-P (Figure 3).

Discussion

We applied CRISPR editing to produce single gene deletions of both *gfat1* and *gfat2* genes in *Drosophila* and found each is required for viability. In complementation assays we further verified that *gfat1* and *gfat2* are each homozygous lethal in the 1st instar larval stage (although some fail to hatch and die as late embryos). From these observations we conclude

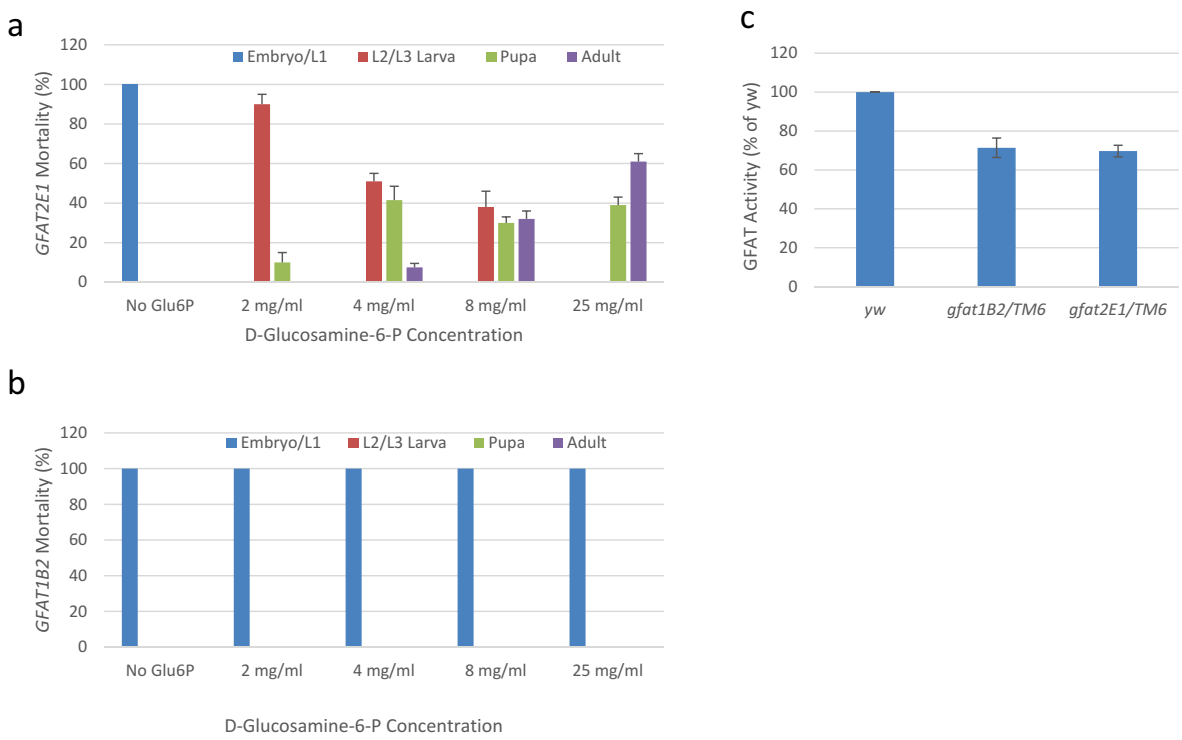

Figure 3. Dietary GlcN-6-P rescues development of animals lacking GFAT2.
In (a), with increasing dietary GlcN-6-P the lethal phase of *gfat2*^{E1} homozygotes was extended and, importantly, eclosion was restored. Note, more than 60% of homozygous *gfat2*^{E1} animals survive to adulthood when supplemented with 25mg/ml GlcN-6-P. In (b), homozygous *gfat1*^{B2} animals were similarly tested and dietary GlcN-6-P had no detectable effects. For both *gfat2*^{E1}*/TM6*,Tb and *gfat1*^{B2}*/TM6,Tb*, 50% of the embryos become heterozygous adults following the mendelian ratio with or without GlcN-6-P supplement. The number of heterozygotes were used as reference for the calculation of homozygotes survival ratios. In (c) GFAT activity was measured in lysates of pooled 24-28hr progeny generated from heterozygous parents (expected mendelian ratios are 25% homozygous and 50% heterozygous for the indicated genotypes). Specific activities were normalized to the parental yw strain. Note that minor fractions of unhatched embryos were observed in similar numbers of both mutant lines and that TM6 homozygotes should produce wild type levels of enzymatic activity. Error bars represent standard deviations. There were too few replicates to justify further statistical analysis.

Table 1. Complementation analyses for gfat1 and gfat2 crispr deletions.
gfat1^{B2} and *gfat2*^{E1} alleles were tested in trans combinations as indicated. For each cross, three vials were set up, and at least 100 flies were scored. Where crosses are labeled "yes", the expected mendelian ratio of genotypes was observed. Where crosses are labeled "no", transheterozygous mutant animals older than the L1 stage were not observed. Note that *gfat1*^{B2} did not complement *MiMIC gfat1*^{MI11277}, and *gfat2*^{E1} did not complement the two Deficiencies that uncover gfat2 (indicated). An irrelevant control Deficiency, Df(3R)Exel6209 was complemented by *gfat2*^{E1}.

Genotype	*gfat1*^{B2}	*gfat2*^{E1}	*gfat2*^{F1}
Mi{MIC}gfat1^{MI11277}	No	yes	yes
gfat1^{B2}	N/A	yes	yes
gfat2^{E1}	yes	N/A	no
gfat2^{F1}	yes	no	N/A
Df(3R)BSC460 (uncovers *gfat2*)	ND	no	no
Df(3R)BSC881 (uncovers *gfat2*)	ND	no	no
Df(3R)Exel6209 (control)	ND	yes	yes

ND: not determined.

that *gfat1* and *gfat2* are not functionally redundant. Indeed, it is noteworthy that loss of *gfat1* was not compensated by *gfat2* and, conversely, loss of *gfat2* was not compensated by *gfat1*. Hence, with regard to developmental functions the action of each must produce unique activities essential for viability.

These genetic findings could potentially be explained by non-overlapping expression patterns. For instance, *gfat1* and *gfat2* could be exclusively expressed in distinct vital tissues and, consistent with this, Dutta et al. showed that GFAT2 is the prevailing isoform in the fly midgut [17]. On the other hand, datasets available from modENCODE indicate that *gfat1* and *gfat2* share widely expressed patterns with many tissues in common [15]. Notably *gfat1* is expressed at levels lower than *gfat2* but, excluding the gonads and 3rd instar fat body (where *gfat1* RNAs are absent) inspection of these datasets showed that both are present in the 25 anatomical sites reported [15].

As expected we observed reversal of *gfat2* lethality using GlcN-6-P and, consistent with this, Mattila et al [18] found that dietary N-acetyl-D-glucosamine (GlcNAc) had a similar impact. An interesting distinction is that Mattila et al [18] delayed the lethal phase (from 1st instar to pupa) while, here, supplementation rescued viable adults that otherwise would have died as L1 larvae. Hence, although both HBP intermediates effectively restored HBP shortages in *gfat2* flies, dietary GlcN-6-P could be a more effective supplement compared to GlcNAc [18]. In contrast, 1st instar larvae homozygous for *gfat1* were surprisingly unaffected by dietary GlcN-6-P. This result might reflect barriers that prevent dietary GlcN-6-P from accessing tissue(s) where GFAT1 is essential, such as limited dispersion, inadequate absorption, compromised feeding or acute death after hatching. However, as outlined above, public datasets [15] are somewhat at odds with this prediction since they suggest that GFAT1 is often the less abundant counterpart and therefore, if anything, GFAT1 homozygotes should be easier to rescue with dietary GlcN-6-P compared to GFAT2 mutants. An alternative formal possibility is that, in addition to GlcN-6-P [14], GFAT1 might generate a non-canonical product. Though highly speculative, precedents for such promiscuous activity have been documented with other enzymes [19]. In this regard, it is worth noting that the *gfat1* locus produces nine alternatively spliced transcripts, in contrast to *gfat2*, which produces a single RNA. The precise action of *gfat1* remains to be determined and, in future studies, tissue specific analyses of intermediate HBP metabolites (as in [20]) may provide useful information.

Material and methods

Generating $gfat1^{B2}$ and $gfat2^{E1}$ using Crispr/Cas9

To generate the $gfat1^{B2}$ and $gfat2^{E1}$ alleles, guide RNAs were designed using http://tools.flycrispr.molbio.wisc.edu/targetFinder and synthesized as 5 -unphosphorylated oligonucleotides, annealed, phosphorylated and ligated into the BbsI sites of pU6-BbsI -chiRNA plasmid [21]. Homology arms upstream of the 5' gRNA sequences, and downstream of the 3' gRNA sequences (for gfat1, 919bp 5' upstream and 908bp 3' downstream, for gfat2, 923bp 5′ upstream and 922bp 3′ downstream) were synthesized as gene blocks (IDT) and cloned into pHD-dsRed-attP [22] (Addgene). The sequences of the guide RNAs are:

GFAT1 5′gRNA: CAACTTGCGGCCGTATAATAAGG,

GFAT1 3′gRNA: ATGTGGAGATTATCATGTAGAGG;

GFAT2 5′gRNA: CTGATCTTACACTTCTGAGGCGG;

GFAT2 3′gRNA: AAATTCAGCTCATGAAGCGTGGG

Guide RNAs and the donor vector were co-injected into *nosP Cas9 attP* embryos at the following concentrations: 250 ng/µl pHD-dsRed-attP donor vector and 20 ng/µl of each of the pU6-BbsI -chiRNA plasmids containing the guide RNAs (Rainbow Transgenics Inc.). Mutant flies were identified using dsRed eyes as a marker, and subsequently verified by sequencing.

Lethal phase analyses and dietary supplementation tests

Lethality in earlier stages was imputed from the numbers of heterozygous and homozygous animals scored at later stages. Specifically, embryos laid by $gfat1^{B2}$/*TM6, Tb* and $gfat2^{E1}$/*TM6, Tb* adults were collected for 4 hours, half of the collection plates were aged at 25°C for 26 hours, and embryos on the other half of the collection plates were transferred to vials at 25°C. The numbers of unhatched embryos and empty egg cases were counted. Larvae were recovered from three separate vials and scored for Tb on each day from day 2 to day 5. After pupation, the numbers of Tb+ and Tb pupae and adults were counted. A minimum of 100 heterozygous adults were

scored. For dietary supplementation, *gfat1*^{B2}*/TM6, Tb* and *gfat2*^{E1}*/TM6, Tb* flies were raised in fly food (Nutri-Fly Instant Formulation, Genesee Scientific, 66–118) containing the indicated concentrations of GlcN-6-P (Sigma-Aldrich, G5509). Homozygous larvae lacking the Tb marker on TM6 were scored. Homozygous pupae were transferred to a fresh vial, and assessed for eclosion rates. Deficiency and MiMIC strains used in the complementation testing were from Bloomington *Drosophila* Stock Centre.

GFAT activity assay

0–4 hr embryos were aged at 25°C for 24 hours. Hatched and unhatched larva, were homogenized with a pestle and lysed in enzyme assay buffer (20 mM Tris-Cl (pH 7.5), 2.5 mM $CaCl_2$, 50 mM NaCl, 10 mM $MgCl_2$,1 mM dithiothreitol, 10% glycerol and protease inhibitors) with 0.5% Triton X-100. After a 30 min incubation on ice, the lysates were spun at 18,000 RCF for 15 minutes. The resulting supernatants were transferred to a clean tube and used for the activity assay. The GFAT enzyme activity was determined by a spectrophotometric method as described [23], and adapted to a 96-well format. In 100 ul assay volumes, 30ug protein samples were incubated with 10 mM glutamine (Sigma-Aldrich, G7513), 10 mM fructose 6-phosphate (Sigma-Aldrich, F3627), 0.5 mM 3-acetylpyridine adenine dinucleotide (APAD) (Santa Cruz, sc-209519), and 2 units of glutamate dehydrogenase (Sigma-Aldrich, 10197734001) in enzyme assay buffer at 37°C for 1 h. The change in absorbance at 365 nm due to reduction of APAD to APADH was measured in separate duplicate samples over the 1 hr incubation time and standard deviations were calculated. For each lysate, 30ug of heat-inactivated protein (95°C for 10 minutes) was used as a control.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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