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Protein kinase A controls the hexosamine pathway by tuning the feedback inhibition of GFAT-1

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1 **Abstract**

2 The hexosamine pathway (HP) is a key anabolic pathway whose product
3 uridine 5'-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc) is an essential
4 precursor for all glycosylation processes in mammals. It modulates the ER
5 stress response and HP activation extends lifespan in *Caenorhabditis elegans*.
6 The highly conserved glutamine fructose-6-phosphate amidotransferase 1
7 (GFAT-1) is the rate-limiting HP enzyme. GFAT-1 activity is modulated by UDP-
8 GlcNAc feedback inhibition and through phosphorylation by protein kinase A
9 (PKA). Molecular consequences of GFAT-1 phosphorylation, however, remain
10 poorly understood. Here, we identify the GFAT-1 R203H substitution that
11 elevates UDP-GlcNAc levels in *C. elegans*. In human GFAT-1, the R203H
12 substitution interfered with both UDP-GlcNAc inhibition and with PKA-mediated
13 Ser205 phosphorylation. Our data indicate that phosphorylation affects the
14 relative positioning of the two GFAT-1 domains to control its activity. Of note,
15 Ser205 phosphorylation had two discernible effects: It lowered baseline
16 GFAT-1 activity and abolished UDP-GlcNAc feedback inhibition. Thus, PKA
17 controls the HP by uncoupling the metabolic feedback loop of GFAT-1.

18 **Introduction**

19 The hexosamine pathway (HP) converts fructose-6-phosphate (Frc6P) to
20 uridine 5'-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc) (Fig. 1a)¹. 2 to
21 3 % of cellular glucose enter the HP, as well as L-glutamine (L-Gln), acetyl-
22 coenzyme A, and uridine². Hence, the HP integrates sugar, amino acid, fatty
23 acid, and nucleotide metabolism and is considered an important nutrient
24 sensing pathway. The GlcNAc moiety of UDP-GlcNAc is used as a building
25 block for several macromolecules, including peptidoglycans in bacteria, chitin
26 in fungi and insects, or glycosaminoglycans such as hyaluronic acid in
27 vertebrates³⁻⁸. Moreover, UDP-GlcNAc is an essential precursor for
28 glycosylation reactions in eukaryotes. N-linked glycosylation takes place in the
29 endoplasmic reticulum (ER) and is crucial for proper protein folding and the
30 solubility of proteins⁹. Mucin-type O-linked glycosylation occurs in the Golgi
31 apparatus and is found on many cell surface and secreted proteins¹⁰. In protein
32 O-GlcNAcylation that occurs in cytoplasm and nucleus, UDP-GlcNAc is the
33 donor for the attachment of a single GlcNAc moiety to serine or threonine
34 residues¹¹. This process can fine tune a protein's function akin to
35 phosphorylation and often O-GlcNAcylation and phosphorylation compete for
36 modification of the same sites^{12,13}.

37 HP activity is regulated by its first and rate-limiting enzyme glutamine fructose-
38 6-phosphate amidotransferase (GFAT, EC 2.6.1.16)². Two GFAT paralogs
39 exist that primarily differ in their tissue-specific expression patterns¹⁴. GFAT is
40 a modular enzyme composed of a glutaminase domain responsible for
41 hydrolysis of L-Gln into L-glutamate (L-Glu) and an isomerase/transferase
42 domain that catalyzes the isomerization of Fru6P to glucose-6-phosphate
43 (Glc6P) as well as the transfer of ammonia to Glc6P to build glucosamine-6-
44 phosphate (GlcN6P)¹⁵. The orientation of the two domains with their respective
45 active sites is relevant for catalysis and affected by substrate binding. In the
46 absence of Frc6P, the glutaminase domain is very flexible (Fig. 1b, 1) and no
47 corresponding electron density is visible in the *E. coli* GFAT structure, although
48 SDS-PAGE analyses of dissolved crystals indicate the presence of the full-
49 length protein^{16,17}. The reaction is initiated by binding of Frc6P to the isomerase
50 domain, which triggers the glutaminase domain to adopt a specific position

51 relative to the isomerase domain¹⁶ (Fig. 1b, 2). Subsequently, L-Gln binds to
52 the glutaminase domains, closing the pocket of the glutaminase active site¹⁸.
53 This is accompanied by a rotation of the glutaminase domain by 21° relative to
54 the isomerase domain¹⁹ (Fig. 1b, 2). Finally, a solvent-inaccessible channel is
55 formed that links the two active sites to allow the diffusion of ammonia¹⁹⁻²¹.
56 A disturbed function of GFAT-1 due to mutations within *gfat-1* cause limb-girdle
57 congenital myasthenic syndrome with tubular aggregates^{22,23}. This inherited
58 disorder is characterized by defective neuromuscular transmission through
59 impaired neuromuscular junctions, which transmit impulses from motor neurons
60 to skeletal muscle fibers²². Moreover, many studies suggest a role of the HP
61 and GFAT in the development and progression of diabetes or cancer²⁴⁻²⁶. Both
62 disorders are characterized by an aberrant glucose metabolism, and the HP
63 links altered metabolism with aberrant glycosylation. Especially elevated O-
64 GlcNAcylation contributes to the pathogenesis of diabetes and cancer. Altered
65 O-GlcNAcylation was also reported to play a critical role in neurodegenerative
66 diseases, heart disease, and inflammation^{27,28}.
67 Given that GFAT drives the metabolic flux of the HP, its regulation is of great
68 physiological importance and in eukaryotes it occurs through UDP-GlcNAc
69 feedback inhibition²⁹⁻³¹ and through phosphorylation events. In human GFAT-1,
70 Ser205 and Ser235 are phosphorylated by cAMP-dependent protein kinase
71 (PKA)³²⁻³⁴. Ser205 phosphorylation has reported effects on GFAT-1 activity,
72 although the published data are contradictory: On the one hand,
73 phosphorylation of Ser205, and the corresponding Ser202 in human GFAT-2,
74 was reported to increase activity^{32,34}. On the other hand, it was reported that
75 Ser205 phosphorylation leads to decreased GFAT-1 activity³³. Phosphorylation
76 of the second PKA site Ser235 in GFAT-1, which is not conserved in all
77 eukaryotes and is absent in GFAT-2, seems not to affect the enzymatic
78 activity^{33,35}. Ser243 was shown to be phosphorylated by adenosine
79 monophosphate (AMP)-activated protein kinase (AMPK) and
80 calcium/calmodulin-dependent kinase II, but their effect on GFAT activity
81 remains elusive. Phosphorylation at this site is reported to be activating or
82 inhibiting³⁶⁻³⁸.
83 Previously, in a *C. elegans* forward genetic mutagenesis screen we identified
84 gain-of-function mutations in *gfat-1* that suppressed tunicamycin-induced

85 proteotoxic stress³⁹. These mutations increased UDP-GlcNAc concentrations
86 in the worms that also showed improved protein quality control as well as a
87 significant lifespan extension³⁹. Recently, we showed that loss of regulation by
88 UDP-GlcNAc feedback inhibition constitutes a gain-of-function mechanism⁴⁰.
89 Although UDP-GlcNAc binds to the isomerase domain of GFAT-1, this binding
90 inhibits the glutaminase function and consequently the GlcN6P production of
91 the whole enzyme⁴¹. We identified a critical role of the interdomain linker during
92 UDP-GlcNAc inhibition and proposed that UDP-GlcNAc disturbs the tight
93 coupling of the active sites by interfering with the relative orientation of the two
94 domains⁴⁰ (Fig. 1b, 3).
95 Here, we identify the R203H GFAT-1 gain-of-function mutation that interferes
96 with UDP-GlcNAc inhibition and with PKA-dependent phosphorylation at
97 Ser205. Analyses of the phospho-mimic S205D substitution resolved the
98 controversially discussed effect of Ser205 phosphorylation. Our data
99 demonstrate that PKA phosphorylation at Ser205 lowers GFAT-1 activity and
100 simultaneously blocks UDP-GlcNAc inhibition. We propose a model how
101 Ser205 phosphorylation might modulate the activity and feedback inhibition of
102 GFAT-1 through phosphorylation-induced domain movement.

103 **Results**

104 **A new GFAT-1 gain-of-function point mutant is resistant to ER stress**

105 Previously, we performed a forward genetic screen in *C. elegans* for mutants
106 that are resistant to tunicamycin-induced proteotoxic stress³⁹. From this screen,
107 we obtained the mutant allele *dh783*, which was not characterized until now.
108 While 10 mg/ml tunicamycin was toxic for N2 wild type worms, the *dh783* allele
109 conferred strong tunicamycin resistance (Fig. 1c, Supplementary Fig. 1a). To
110 identify the causal mutation, we performed Hawaiian single nucleotide
111 polymorphism (SNP) mapping⁴². The normalized linkage score identified SNPs
112 in exons of five candidate genes on chromosome II including *gfat-1* (Fig. 1d).
113 Given that tunicamycin toxicity can be suppressed by elevated UDP-GlcNAc
114 levels through gain-of-function mutations in *gfat-1*³⁹, we quantified UDP-
115 GlcNAc and its epimer UDP-GalNAc by mass spectrometry. Indeed, we found
116 significantly elevated UDP-GlcNAc and UDP-GalNAc levels in whole worm
117 lysates in mutant carriers of the *dh783* allele (Fig. 1e, Supplementary Fig. 1b).
118 We conclude that tunicamycin resistance was caused by the *gfat-1(dh783)*
119 allele due to the GFAT-1 R203H gain-of-function substitution.

120 **The GFAT-1 R203H substitution interferes with UDP-GlcNAc inhibition** 121 **leading to gain-of-function**

122 GFAT-1 is well-conserved from *C. elegans* to humans (Supplementary Fig. 2a).
123 To decipher the effect of the R203H substitution, we crystallized human
124 GFAT-1 R203H and characterized it in activity assays. GFAT-1 R203H crystals
125 diffracted to a resolution limit of 2.77 Å (Table 1). Overall, compared to wild type
126 GFAT-1, the R203H substitution does not cause major structural changes
127 (Fig. 2a, b, Supplementary Fig. 2b). While Arg203 interacts with the backbone
128 residues of Ile178 and Leu181, as well as with Asp279 in wild type GFAT-1,
129 these interactions are abolished in the R203H GFAT-1 (Fig. 2b). In the mutant
130 enzyme, however, the side chain of His203 protrudes between Asp278 and
131 Asp279, thereby stabilizing the orientation of the neighboring loop (residues
132 277-280) in a similar position as in the wild type (Fig. 2b). Subsequently, we
133 analyzed the R203H substitution of human GFAT-1 in activity assays and found
134 similar kinetics for L-Glu and GlcN6P production as in wild type GFAT-1

135 (Fig. 2c, d, Table 2). In contrast, UDP-GlcNAc dose response assays revealed
136 an approximately 6-fold higher IC₅₀ value of R203H GFAT-1 compared to wild
137 type GFAT-1 (R203H: 231.7 -15.2/+16.3 μM; wild type: 41.4 -3.5/+3.9 μM)
138 (Fig. 2e). This finding suggests that a lower sensitivity to UDP-GlcNAc feedback
139 inhibition leads to the gain-of-function caused by the R203H substitution *in vivo*.

140 **The GFAT-1 R203H substitution interferes with PKA phosphorylation at** 141 **Ser205**

142 In addition to the observed gain-of-function caused by reduced feedback
143 inhibition, the R203H substitution alters the PKA consensus sequence of
144 Ser205 from RRGs to RHGS, suggesting a potential change of Ser205
145 phosphorylation and a subsequent loss of regulation (Fig. 3a). To test whether
146 GFAT-1 R203H can be phosphorylated at Ser205, we purified wild type and
147 R203H GFAT-1 and performed *in vitro* phosphorylation with PKA, followed by
148 LysC protease digest and untargeted as well as targeted mass spectrometric
149 analyses (Fig. 3b). In the untargeted analysis, we identified a number of
150 phosphorylated sites, among them the PKA phosphorylation sites Ser205 and
151 Ser 235^{32,33} (Supplementary Table 1). For the targeted analysis, heavy isotope
152 labeled phosphorylated peptides (Fig. 3b) of wild type or R203H GFAT-1 were
153 spiked in, allowing more sensitive relative quantification of the Ser205
154 phosphorylation. Untreated insect cell-derived wild type and R203H GFAT-1
155 preparations showed low detectable levels of phosphorylation at Ser205. After
156 *in vitro* PKA treatment, phosphorylation of wild type GFAT-1 Ser205 was highly
157 induced (Fig. 3c, d). In contrast, the increase of Ser205 phosphorylation was
158 drastically reduced in the presence of the R203H substitution (Fig. 3c, d). The
159 second known PKA phosphorylation site of GFAT-1 at Ser235³³ was used as a
160 positive control. For this site, we found a significant increase in the abundance
161 of phosphorylated peptides corresponding to Ser235 in both wild type and
162 R203H GFAT-1, proving full activity of PKA in our setup (Supplementary Fig. 3).
163 Thus, disruption of the PKA consensus sequence by the R203H substitution
164 prevents PKA-mediated phosphorylation at Ser205. Taken together, in addition
165 to a lower sensitivity to UDP-GlcNAc inhibition, the GFAT-1 R203H substitution
166 revealed a second gain-of-function mechanism: a loss of PKA-dependent
167 regulation through strongly reduced phosphorylation at Ser205.

168 **PKA phosphorylation at Ser205 modulates UDP-GlcNAc inhibition of**
169 **GFAT-1**

170 The loss of PKA phosphorylation in the GFAT-1 gain-of-function R203H variant
171 suggests that the Ser205 phosphorylation might be inhibitory. Until now, the
172 effect of phosphorylation at Ser205 has been controversially reported as either
173 activating³² or inhibiting³³. We aimed to mechanistically understand the effect
174 of Ser205 phosphorylation and generated a GFAT-1 S205D mutant to mimic
175 the phosphorylation. Importantly, wild type insect cell-derived GFAT-1 showed
176 almost no phosphorylation at Ser205 (Fig. 3c, d), permitting the direct
177 comparison with the S205D variant in activity assays. We observed reduced
178 k_{cat} values for L-Glu and GlcN6P production resulting in lower catalytic
179 efficiency (k_{cat}/K_m) for both active sites (Fig. 4a, b, Table 2). Overall GlcN6P
180 synthesis was decreased approximately 5-fold in the phospho-mimic mutant
181 (k_{cat}/K_m : $4.4 \text{ mM}^{-1} \text{ sec}^{-1}$) compared to wild type GFAT-1 (k_{cat}/K_m :
182 $21.3 \text{ mM}^{-1} \text{ sec}^{-1}$) (Table 2). Strikingly, the dose-dependent inhibition by UDP-
183 GlcNAc was completely abolished in the S205D mutant (Fig. 4c). Taken
184 together, these data suggest that PKA-dependent phosphorylation at Ser205
185 reduces GFAT-1 activity *in vitro*. The additional loss of UDP-GlcNAc feedback
186 inhibition, however, maintains the activity of Ser205-phosphorylated GFAT-1
187 when UDP-GlcNAc concentrations are high. At low UDP-GlcNAc
188 concentrations up to approximately $30 \mu\text{M}$, the phosphorylation is inhibiting,
189 while at high UDP-GlcNAc concentrations above approximately $30 \mu\text{M}$, the
190 phosphorylation is activating.

191 Understanding the effect of Ser205 PKA phosphorylation on GFAT-1 activity,
192 we next analyzed the relevance of the phosphorylation *in vivo*. For that purpose,
193 we generated HEK293 cell lines, which stably overexpress either wild type,
194 R203H, or S205D GFAT-1 with an internal His₆-tag (Fig. 4d) and measured
195 UDP-GlcNAc and UDP-GalNAc levels in the cell lysates (Fig. 4e,
196 Supplementary Fig. 4). The cells were cultured at high glucose concentrations
197 of 25 mM , to avoid any glucose limitation. Strikingly, GFAT-1 overexpression
198 did not elevate UDP-GlcNAc or UDP-GalNAc concentrations. Likewise, the
199 R203H GFAT-1 cell line did not show increased UDP-GlcNAc or UDP-GalNAc
200 levels. This suggests that UDP-GlcNAc feedback inhibition is effective in fully
201 suppressing the activity of wild type and R203H GFAT-1 even during

202 overexpression (Fig. 4e, Supplementary Fig. 4). In contrast, we found
203 significantly elevated levels of UDP-GlcNAc and UDP-GalNAc when the
204 phospho-mimetic S205D variant of GFAT-1 was overexpressed (Fig. 4e,
205 Supplementary Fig. 4). These data support an interference of the S205D
206 substitution with the UDP-GlcNAc inhibition, which rendered GFAT-1
207 constitutively active. In summary, our data suggest that PKA controls GFAT-1
208 activity by interference with its UDP-GlcNAc feedback inhibition.

209 **PKA phosphorylation at Ser205 modulates GFAT-1 domain stability**

210 Next, we assessed the thermal stability of GFAT-1 in thermal shift assays. Full-
211 length GFAT-1 showed two clearly distinguishable melting points at 53.0 °C
212 and 65.0 °C, which are termed “low” and “high” hereafter (Fig. 5a,
213 Supplementary Fig. 5a). Most likely, these two melting points represent the two
214 domains, which possess different stabilities: the isolated isomerase domain
215 melted at 64.0 °C suggesting that the high melting point corresponded to the
216 isomerase domain, while the low melting point was assigned to the glutaminase
217 domain (Fig. 5a, Supplementary Fig. 5a). To investigate their relative
218 interactions, rising salt concentrations were then used to destabilize the
219 domains. For full-length GFAT-1, high salt (0.5 - 1.0 M) lowered the melting
220 point of the glutaminase domain by 3.0-4.0 °C, and it lowered the melting point
221 of the isomerase domain by 1.7-2.0 °C (Fig. 5b). In contrast, the isolated
222 isomerase domain showed a much stronger reduction of the melting
223 temperature of up to -10 °C (Fig. 5b). Together, these results show that in full-
224 length GFAT-1 the isomerase domain is stabilized by the glutaminase domain,
225 thus indicating interdomain interactions. We conclude that analysis of the
226 melting points in GFAT-1 variants can reveal changes in the interaction
227 between its domains. To independently test whether thermal shift assays can
228 indeed indicate interdomain interactions, we generated the C-tail lock mutant
229 L405R of human GFAT-1 and characterized it structurally, as well as in thermal
230 shift assays and in kinetic measurements. The well-conserved C-tail
231 (C-terminal residues 670-681) is the major mobile element between the
232 isomerase and glutaminase domain of GFAT. It bears residues of both active
233 sites and mediates interactions between glutaminase and isomerase domain¹⁹.
234 In *E. coli*, its flexibility is restricted by a salt-bridge between Arg331 and

235 Glu608⁴³. Miszkiel and Wojciechowski showed in molecular dynamic
236 simulations that the C-tail lock mutation, which introduces a salt-bridge similar
237 as observed in *E. coli*, limits the C-tail's flexibility⁴³. We solved the crystal
238 structure (resolution 2.38 Å, Table 1) and confirmed that the L405R substitution
239 indeed formed a salt-bridge with the C-terminal Glu681 and additionally
240 interacted with Asp428 (Supplementary Fig. 5b-d). The kinetics (L-Gln and
241 Frc6P) and the UDP-GlcNAc inhibition remained unaffected by the C-tail lock
242 mutation (Table 2, Supplementary Fig. 5e-g). Importantly, the L405R mutant
243 clearly showed a single melting point at 56.9 ± 0.1 °C in thermal shift assays
244 (Supplementary Fig. 5h, i), indicating a changed stability of both domains,
245 which can only be explained by the altered interactions between the two
246 domains. Therefore, thermal shift assays are a valuable tool to analyze
247 interdomain interactions of GFAT-1.

248 We next used the thermal shift assay to test the role of the phospho-mimic
249 S205D substitution regarding the interdomain interaction of GFAT-1. At
250 physiological pH, phosphorylation introduces a di-anionic phosphate group that
251 causes a repulsion with other negative charges and enables the formation of
252 electrostatic interactions with positively charged residues. The PKA phospho-
253 site Ser205 is located in a loop of the glutaminase domain pointing towards the
254 glutaminase active site and the interdomain cleft between the glutaminase and
255 isomerase domains (Fig. 5c). Ser205 is thus optimally positioned to induce a
256 potential domain movement upon phosphorylation. Notably, GFAT-1 S205D did
257 not form any crystals in the standard GFAT-1 crystallization condition, indicating
258 a structural change compared to wild type GFAT-1 that prevented crystal
259 packing. Compared to wild type GFAT-1, the S205D mutant showed strongly
260 reduced thermal stability of the glutaminase domain, as indicated by the specific
261 reduction of the lower melting point (Fig. 5d, Supplementary 5j, k). The stability
262 of the isomerase domain (high melting point) was not affected (Fig. 5d,
263 Supplementary 5j, k). To rule out major structural changes in GFAT-1 S205D,
264 we performed circular dichroism (CD) measurements of wild type and S205D
265 GFAT-1. Both proteins showed very similar spectra indicating proper folding
266 (Fig. 5e). Together, these data demonstrate a destabilization of the
267 glutaminase domain in the GFAT-1 S205D variant, suggesting altered
268 interdomain interactions through changed domain orientation. Thus, PKA

269 phosphorylation at Ser205 might affect GFAT-1 activity and block the UDP-
270 GlcNAc inhibition through phosphorylation-induced domain movement.

271 **Discussion**

272 Here we present the GFAT-1 mutant R203H and decipher its two gain-of-
273 function mechanisms: (1) strongly reduced PKA-dependent phosphorylation at
274 Ser205 due to a disrupted consensus sequence and (2) a weaker sensitivity to
275 UDP-GlcNAc feedback inhibition. The current study resolves the contradicting
276 reports regarding the effect of PKA phosphorylation at Ser205 in GFAT-1 and
277 provides a model for phosphorylation-induced domain movement. Strikingly,
278 the phospho-mimetic S205D substitution lowered GFAT-1 activity and
279 abolished UDP-GlcNAc inhibition. Importantly, we demonstrate a modulation of
280 the UDP-GlcNAc feedback inhibition of GFAT-1 by PKA.

281 We describe two independent gain-of-function mechanisms of GFAT-1 R203H.
282 First, when UDP-GlcNAc levels are low, the lack of phosphorylation at Ser205
283 results in higher GFAT-1 activity when compared to wild type GFAT-1 with the
284 latter having a reduced GlcN6P production upon phosphorylation. Second,
285 when UDP-GlcNAc levels are higher, the reduced sensitivity to feedback
286 inhibition in R203H GFAT-1 elevates cellular UDP-GlcNAc levels. The
287 observation of elevated UDP-GlcNAc levels in whole worm lysates in the
288 *gfat-1(dh783)* R203H mutant points to the predominant role of reduced
289 sensitivity to feedback inhibition as the key gain-of-function mechanism.

290 R203H GFAT-1 showed reduced sensitivity to UDP-GlcNAc feedback
291 inhibition, indicating a functional role of Arg203 in UDP-GlcNAc inhibition. In
292 wild type GFAT-1, Arg203 is implicated in a salt-bridge network, which might
293 help to position the glutaminase and isomerase domains (Fig. 5f). Previously,
294 we proposed that UDP-GlcNAc promotes a catalytically unfavorable domain
295 orientation, inhibiting GFAT-1⁴⁰. In general, domain movements are common in
296 the family of glutamine amidotransferases and the function of GFAT depends
297 on the relative orientation of the glutaminase and isomerase domains⁴⁴.
298 Mouilleron *et al.* reported a structure of the *E. coli* GFAT active site mutant C1A
299 (PDB 3OOJ) with a drastically changed orientation of the glutaminase domain
300 relative to the isomerase domain, where one monomer adopts an inactive
301 orientation without forming the ammonia channel⁴⁵. This structure underpins
302 the high flexibility of the two domains relative to each other. In wild type *E. coli*
303 GFAT, the glutaminase domain adopts a specific position relative to the

304 isomerase domain after Frc6P binding¹⁶. The presence of Frc6P activates the
305 glutaminase function of GFAT more than 100-fold in *E. coli* and 70-fold in
306 *C. albicans* and it is very likely that this substrate-induced activation occurs in
307 the human GFAT as well^{18,41}. Also, L-Gln binding induces a rotation of the
308 glutaminase domain relative to the isomerase domain in *E. coli* GFAT¹⁹. These
309 domain movements upon substrate binding are required to enable specific
310 interactions between the isomerase and glutaminase domains that are
311 necessary for catalysis. It is well-established that phosphorylation can introduce
312 conformational changes within loops or domains through altered electrostatic
313 interactions⁴⁶. A well-studied example for a phosphorylation-induced change in
314 domain orientation is rabbit muscle glycogen phosphorylase, whose domains
315 are shifted by approximately 50° relative to each other after
316 phosphorylation^{47,48}. The phosphorylation of GFAT-1 at Ser205 would
317 introduce, at physiological pH, a negatively charged phosphate group,
318 potentially affecting ionic interactions in close proximity. Arg202 of the
319 glutaminase domain and Glu425 of the isomerase domain likely form a salt-
320 bridge between the two domains and this interaction might be affected upon
321 Ser205 phosphorylation (Fig. 5f).

322 Thermal shift assays of wild type and phospho-mimetic GFAT-1 showed a
323 destabilization of the glutaminase domain in the S205D mutant that indicated
324 an altered interdomain interaction. We propose that Ser205 phosphorylation
325 suppresses UDP-GlcNAc inhibition by stabilization of the glutaminase and
326 isomerase domains in a catalytically productive orientation (Fig. 5g). While this
327 conformation does not permit the high catalytic rate of wild type GFAT-1, the
328 fixed conformation of the domains would prevent a UDP-GlcNAc induced
329 domain orientation that might be needed for inhibition.

330 Our data reveal a UDP-GlcNAc concentration-dependent effect on the activity
331 of GFAT-1 that is phosphorylated at Ser205. This finding explains the previous
332 contradicting reports about the effect of the Ser205 phosphorylation for
333 GFAT-1: Zhou *et al.* observed an activation³², while Chang *et al.* reported an
334 inhibition³³. In both studies, cells were treated with forskolin to activate PKA and
335 the GFAT-1 activity was analyzed in cell lysates. These lysates contained
336 unknown UDP-GlcNAc concentrations that might have affected the results.
337 Moreover, Chang *et al.* confirmed the inhibitory effect *in vitro* with GST-tagged

338 GFAT-1 in the absence of UDP-GlcNAc. However, the data obtained from a
339 GST-tagged GFAT-1 should be taken with caution, because tagging GFAT at
340 the N- or C-terminus interferes with the catalytic reactions^{33,49,50}.

341 Very few enzymes are known in which phosphorylation interferes with feedback
342 inhibition⁵¹. This uncoupling of the feedback loop by GFAT-1 phosphorylation
343 is an elegant mechanism to rapidly modulate its enzymatic activity after a
344 stress-induced signal. The cAMP-PKA signaling pathway acts downstream of
345 G protein-coupled receptors (GPCR) to mediate signals of neurotransmitters
346 and hormones, such as glucagon or adrenaline⁵²⁻⁵⁵. The downstream protein
347 targets of the cAMP-PKA signaling pathway regulate glucose homeostasis by
348 inhibition of glycolysis and glycogen synthesis, as well as by triggering glucose
349 release through stimulation of glycogenolysis and gluconeogenesis⁵⁵. Our
350 previous data indicated that GFAT-1 is under a constant UDP-GlcNAc inhibition
351 *in vivo*⁴⁰. Presumably, uncoupling of the feedback inhibition maintains GFAT-1
352 activity and ensures steady UDP-GlcNAc production when PKA activity is high.
353 Given that UDP-GlcNAc is an essential building block and precursor for all
354 glycosylation reactions in mammals, this mechanism is optimally positioned to
355 ensure a constant UDP-GlcNAc supply. In all, our findings illuminate how the
356 different means of GFAT-1 regulation, kinase signaling, and metabolic
357 feedback, are coordinated at the molecular level to fine tune metabolic flux in
358 the HP.

Methods

***C. elegans* strains and culture**

All *C. elegans* strains were maintained at 20°C on nematode growth medium (NGM) agar plates seeded with the *E. coli* strain OP50⁵⁶. To provide an isogenic background, the mutant strain was outcrossed against the wild type Bristol N2 strain. The strains used in this study are: N2 (Bristol), *gfat-1(dh783)*.

Developmental tunicamycin resistance assay

Gravid adult nematodes were bleached to obtain a synchronized population of eggs, which were transferred to NGM plates containing 10 mg/mL tunicamycin (Sigma-Aldrich) seeded with freeze-killed OP50 *E. coli*. Freeze-killed OP50 bacteria were obtained by three cycles of snap-freezing and thawing of pelleted overnight OP50 bacterial cultures. Worms were kept at 20°C and after four days, healthy day 1 adults were scored, whereas sick larvae were not counted. Throughout the experiment, strain identity was unknown to researchers. Data were assembled upon completion of the experiment.

Mutant Hawaiian SNP mapping and sequence analysis

Genomic DNA was prepared using the QIAGEN Genra Puregene Kit according to the manufacturer's protocol. Whole genome sequencing was conducted on the Illumina HiSeq2000 platform. Paired-end 100 bp reads were used; the average coverage was larger than 16-fold. Sequencing outputs were analyzed using the CloudMap Hawaiian and Variant Discovery Mapping on Hawaiian Mapped Samples (and Variant Calling) Workflow_2-7-2014 pipeline on Galaxy^{42,57}. The WS220/ce10 *C. elegans* assembly was used as reference genome.

Small molecule LC/MS/MS Analysis

UDP-HexNAc concentrations were measured as described previously³⁹. In brief, *C. elegans* or HEK293 cells were lysed in water by freeze/thaw cycles, and subjected to chloroform/methanol extraction. Absolute UDP-HexNAc levels were determined using an Acquity UPLC connected to a Xevo TQ Mass Spectrometer (both Waters) and normalized to total protein content.

Site-directed mutagenesis

A pFL vector for the generation of baculoviruses for the expression of human GFAT-1 isoform 2 (hGFAT-1) with internal His₆-tag between Gly299 and Asp300 was cloned previously⁴⁰. The mutations R203H, S205D, and L405R were introduced into pFL-hGFAT1-His299 by site-directed mutagenesis as described previously⁵⁸ (primers: hGFAT1_R203H_for CAAGGCacGGTAGCCCTCTGTTGATTGG, hGFAT1_R203H_rev GAGGGCTACCgtGCCTTGTGCCAACTG, hGFAT1_S205D_for CAAGGCGAGGTgaCCCTCTGTTGATTGG, hGFAT1_S205D_rev GAGGGtcACCTCGCCTTGTGCCAACTG, hGFAT1_L405R_for GTGACTTCCgtGACAGAAACACACCAG, hGFAT1_L405R_rev GTGTTTCTGTCacGGAAGTCACTTGCTAG).

Baculovirus generation and insect cell expression of full-length GFAT-1

Sf21 (DSMZ no. ACC 119) suspension cultures were maintained in SFM4Insect™ HyClone™ medium with glutamine (GE Lifesciences) in shaker flasks at 27 °C and 90 rpm in an orbital shaker. GFAT-1 variants were expressed in *Sf21* cells using the MultiBac baculovirus expression system⁵⁹. In brief, hGFAT-1 variants (from the pFL vector) were integrated into the baculovirus genome via Tn7 transposition and maintained as bacterial artificial chromosome in DH10EMBacY *E. coli* cells. Recombinant baculoviruses were generated by transfection of *Sf21* with bacmid DNA. The obtained baculoviruses were used to induce protein expression in *Sf21* cells.

Bacterial expression of GFAT-1 isomerase domain

The isomerase domain of human GFAT-1 isoform 2 (residues 316-681) was integrated in the plasmid pET28a(+) using NdeI and HindIII restriction sites (primers: hGFAT1-ISO_NdeI_FOR gagCATATGatcatgaagggcaacttcagttcat ttatgc, hGFAT1_HindIII_REV gagAAGCTTcactctacagtcacagattggca agattc). This vector was used to recombinantly express the isomerase domain with N-terminal His₆ tag and a thrombin cleavage site under the control of the T7 promoter in Rosetta (DE3) *E. coli*. LB cultures were incubated at 37°C and 180 rpm until an OD₆₀₀ of 0.4-0.6 was reached. Then, protein expression was induced by addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranosid and

incubated for 3 h at 37°C and 180 rpm. Cultures were harvested and pellets stored at -80°C.

GFAT-1 purification

Sf21 cells (full-length GFAT-1) or *E. coli* (isomerase domain) were lysed by sonication in lysis buffer (50 mM Tris/HCl pH 7.5, 200 mM NaCl, 10 mM Imidazole, 2 mM Tris(2-carboxyethyl)phosphin (TCEP), 0.5 mM Na₂Frc6P, 10% (v/v) glycerol, supplemented with complete EDTA-free protease inhibitor cocktail (Roche) and 10 µg/ml DNaseI (Sigma)). Cell debris and protein aggregates were removed by centrifugation and the supernatant was loaded on a Ni-NTA Superflow affinity resin (Qiagen). The resin was washed with lysis buffer and the protein eluted with lysis buffer containing 200 mM imidazole. For the isomerase domain, the His₆-tag was proteolytically removed using 5 U of thrombin (Sigma-Aldrich) per mg protein overnight at 4°C. The isomerase domain was again purified by IMAC in order to remove undigested His₆-tagged protein. Full-length GFAT-1 and the isomerase domain were further purified according to their size on a HiLoad™ 16/60 Superdex™ 200 prep grade prepacked column (GE Healthcare) using an ÄKTAprime chromatography system at 4°C with a SEC buffer containing 50 mM Tris/HCl, pH 7.5, 2 mM TCEP, 0.5 mM Na₂Frc6P, and 10% (v/v) glycerol.

Crystallization

For crystallization experiments, the SEC buffer was supplemented with 50 mM L-Arg and 50 mM L-Glu to improve protein solubility⁶⁰. GFAT-1 was crystallized at a concentration of 8 mg/ml in sitting-drops by vapor diffusion at 20°C. Crystals grew in the PACT *premier*™ HT-96 (Molecular Dimensions) screen with a reservoir solution containing 0.1 M Bis tris propane pH 8.5, 0.2 M Potassium sodium tartrate and 20% (w/v) PEG3350 and were further optimized. The optimization screen was set up with drop ratios of 1.5 µl protein solution to 1.5 µl precipitant solution and 2 µl protein solution to 1 µl precipitant solution. Best crystals grew in a broad range of 0.1 M Bis tris propane pH 8.5 to 9.0, 0.2 to 0.4 M potassium sodium tartrate, and 20 % (w/v) PEG3350. Data were collected from crystals cryoprotected with reservoir solution supplemented with 15% (v/v) glycerol.

Data collection and refinement

X-ray diffraction measurements were performed at beamline X06DA at the Swiss Light Source, Paul Scherrer Institute, Villigen (Switzerland). The mutant GFAT-1 structures were determined by molecular replacement with phenix.phaser^{61,62} using the model of the wild type human GFAT-1 (PDB 6R4E) as search model. GFAT-1 was further manually built using COOT⁶³ and iterative refinement rounds were performed using phenix.refine⁶². Geometry restraints for ligands were generated with phenix.elbow software⁶². Structures were visualized using PyMOL (Schrödinger).

GDH-coupled activity assay and UDP-GlcNAc inhibition

GFAT's amidohydrolysis activity was measured with a coupled enzymatic assay using bovine glutamate dehydrogenase (GDH, Sigma Aldrich G2626) in 96 well standard microplates (F-bottom, BRAND #781602) as previously described⁵⁰ with small modifications. In brief, the reaction mixtures contained 6 mM Frc6P, 1 mM APAD, 1 mM EDTA, 50 mM KCl, 100 mM potassium-phosphate buffer pH 7.5, 6.5 U GDH per 96 well and for L-Gln kinetics varying concentrations of L-Gln. For UDP-GlcNAc inhibition assays the L-Gln concentration was kept at 10 mM. The plate was pre-warmed at 37°C for 10 min and the activity after enzyme addition was monitored continuously at 363 nm in a microplate reader. The amount of formed APADH was calculated with $\epsilon_{(363 \text{ nm, APADH})} = 9100 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Reaction rates were determined by Excel (Microsoft) and K_m , v_{max} , and IC_{50} were obtained from Michaelis Menten or dose response curves, which were fitted by Prism 7 or 8 software (Graphpad).

GNA-1 expression and purification

The expression plasmid for human GNA-1 with N-terminal His₆-tag was cloned previously⁴⁰. Human GNA-1 with N-terminal His₆-tag was expressed in Rosetta (DE3) *E. coli* cells. LB cultures were incubated at 37°C and 180 rpm until an OD₆₀₀ of 0.4-0.6 was reached. Then, protein expression was induced by addition of 0.5 mM isopropyl- β -D-1-thiogalactopyranosid and incubated for 3 h at 37°C and 180 rpm. Cultures were harvested and pellets stored at -80°C. Human GNA-1 purification protocol was adopted from Hurtado-Guerrero et al.⁶⁴ with small modifications. *E. coli* were lysed in 50 mM HEPES/NaOH pH 7.2,

500 mM NaCl, 10 mM imidazole, 2 mM 2-mercaptoethanol, 5% (v/v) glycerol with complete EDTA-free protease inhibitor cocktail (Roche) and 10 µg/ml DNaseI (Sigma) by sonication. The lysate was clarified by centrifugation and the supernatant loaded on Ni-NTA Superflow affinity resin (Qiagen). The resin was washed with wash buffer (50 mM HEPES/NaOH pH 7.2, 500 mM NaCl, 50 mM imidazole, 5% (v/v) glycerol) and the protein was eluted with wash buffer containing 250 mM imidazole. Eluted protein was then dialyzed against storage buffer (20 mM HEPES/NaOH pH 7.2, 500 mM NaCl, 5% (v/v) glycerol).

GNA-1 and GNA-1-coupled activity assays

The activity of human GNA-1 was measured in 96 well standard microplates (F-bottom, BRAND #781602) as described previously⁶⁵. For kinetic measurements, the assay mixture contained 0.5 mM Ac-CoA, 0.5 mM DTNB, 1 mM EDTA, 50 mM Tris/HCl pH 7.5 and varying concentrations of D-GlcN6P. The plates were pre-warmed at 37°C and reactions were initiated by addition of GNA-1. The absorbance at 412 nm was followed continuously at 37°C in a microplate reader. The amount of produced TNB, which matches CoA production, was calculated with $\epsilon_{(412 \text{ nm, TNB})} = 13800 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Typically, GNA-1 preparations showed a K_m of $0.2 \pm 0.1 \text{ mM}$ and a k_{cat} of $41 \pm 8 \text{ sec}^{-1}$.

GFAT's D-GlcN6P production was measured in a GNA-1-coupled activity assay following the consumption of AcCoA at 230 nm in UV transparent 96 well microplates (F-bottom, Brand #781614) as described by Li et al.⁶⁵. In brief, the assay mixture contained 10 mM L-Gln, 0.1 mM AcCoA, 50 mM Tris/HCl pH 7.5, 2 µg hGNA-1 and varying concentrations of Frc6P. The plates were incubated at 37°C for 4 min and reactions started by adding L-Gln. Activity was monitored continuously at 230 nm and 37°C in a microplate reader. The amount of consumed AcCoA was calculated with $\epsilon_{(230 \text{ nm, AcCoA})} = 6436 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. As UDP-GlcNAc absorbs light at 230 nm, the GNA-1-coupled assay cannot be used to analyze UDP-GlcNAc effects on activity.

Alignments

The protein sequence alignments were created with following UniProt IDs: *Caenorhabditis elegans*: Q95QM8, *Homo sapiens* isoform 2: Q06210-2, *Mus*

musculus isoform 2: P47856-2, *Candida albicans*: P53704, *Escherichia coli*: P17169. The alignment of *H. sapiens* and *C. elegans* GFAT was formatted with the ESPript3 server (esript.ibcp.fr/)⁶⁶.

For the structural superposition, the wild type human GFAT-1 structure in complex with L-Glu and Glc6P (PDB ID 6R4E) was aligned with the structures of mutant GFAT-1 R203H and L405R. UDP-GlcNAc was displayed in the structures after superposition with the structure of wild type human GFAT-1 in complex with L-Glu, Glc6P, and UDP-GlcNAc (PDB ID 6SVP).

***In vitro* PKA phosphorylation for Protein Mass Spectrometry**

Protein Kinase A Catalytic Subunit from bovine heart (PKA, EC 2.7.11.11, Sigma) was reconstituted in bi-distilled water containing 6 mg/ml DTT at a concentration of 50 µg/ml. Purified GFAT-1 variants were phosphorylated in an assay mixture containing 10 mM MgCl₂, 2 mM Na-ATP, and 20 U PKA in 100 µl GFAT-1 SEC buffer (50 mM Tris/HCl, pH 7.5, 2 mM TCEP, 0.5 mM Na₂Frc6P, and 10% (v/v) glycerol) for 30 min at 30°C. The samples were stored frozen. For proteomic analysis, 5 µg GFAT-1 was alkylated by 5 mM chloroacetamide, reduced with 1 mM TCEP, and digested by 0.1 µg Lys-C Endoproteinase (MS Grade, ThermoScientific) in 50 mM Tris/HCl, pH 8.3 overnight in a waterbath at 37°C. The digest was acidified by addition of formic acid (end concentration 0.1%) and the resulting peptides were purified using C-18 STAGE (STop And Go Extraction) tips⁶⁷.

Untargeted protein Mass Spectrometry

One fifth of the STAGE tip purified peptides were separated on a 25 cm, 75 µm internal diameter PicoFrit analytical column (New Objective) packed with 1.9 µm ReproSil-Pur 120 C18-AQ media (Dr. Maisch HPLC GmbH) using an EASY-nLC 1200 (Thermo Fisher Scientific). The column was maintained at 50°C. Buffer A and B were 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile. Peptides were separated on a segmented gradient from 6% to 31% buffer B for 57 min and from 31% to 44% buffer B for 5 min at 200 nl/min. Eluting peptides were analyzed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Peptide precursor m/z was measured at 60000 resolution in the 350 to 1500 m/z range. Precursors with charge state

from 2 to 7 only were selected for HCD fragmentation using 27% normalized collision energy. The m/z values of the peptide fragments were measured at a resolution of 30000 using an AGC target of 2e5 and 80 ms maximum injection time. Upon fragmentation, precursors were put on a dynamic exclusion list for 45 sec.

Heavy synthetic peptides, corresponding to phosphorylated Ser205, read below, were separated on a segmented gradient from 6% to 60% buffer B for 62 min at 200 nl/min. The mass spectrometric analysis was carried out as described above.

Raw data were analyzed with MaxQuant version 1.6.1.0 using the integrated Andromeda search engine^{68,69}. Peptide fragmentation spectra were searched against manually created GFAT-1 fasta file. The database was automatically complemented with sequences of contaminating proteins by MaxQuant. Methionine oxidation, protein N-terminal acetylation and Phospho (STY) were set as variable modifications; cysteine carbamidomethylation was set as fixed modification. The digestion parameters were set to “specific” and “LysC/P,” The minimum number of peptides and razor peptides for protein identification was 1; the minimum number of unique peptides was 0. Protein identification was performed at a peptide spectrum matches and protein false discovery rate of 0.01. For the analysis of the raw data from the synthetic heavy peptides, Lys8 was set as “Standard” and used as a variable modification.

Targeted protein Mass Spectrometry

The phosphorylated (Ser205, pS), heavily labeled (K*) reference peptides Maxi SpikeTides L (wild type: SVHFPGQAVGTRRG-pS-PLLIGVRSEH-K* and mutant R203H: SVHFPGQAVGTRHG-pS-PLLIGVRSEH-K*) were purchased from JPT Peptide Technologies (Berlin, Germany). Heavy peptides were dissolved in 50% acetonitrile, 0.05% formic acid in water and sonicated in a water bath for one minute. The solution was further diluted using 0.1% formic acid in water for a final peptide concentration of 7 nM. The solution was aliquoted and stored at -20°C. For analysis, desalted GFAT-1 peptides were dissolved in 10 µl 0.1% formic acid in water and 2 µl of the peptide solution were combined with 2 µl of the tenfold diluted heavy peptide solution; 2 µl were analyzed by targeted mass spectrometry.

For targeted analysis, peptides were separated as described above. Targeted analysis was performed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). The m/z values for charge states 4 and 5 from the heavy and light peptide were chosen for targeted fragmentation across the entire LC run. The isolation width was 1.6 and collision energy was set to 27%. Fragment m/z values were measured in the Orbitrap in profile mode, at a resolution of 60K, an AGC target of $5e4$, and a maximum injection time of 118 ms.

Raw data was analyzed using Skyline⁷⁰ version 19.1.0.193. A library was built from the untargeted analysis of the heavy peptides. The library ion match tolerance was set to 0.05 m/z , the 10 most intense product ions were picked. Ions with charges 1 or 2 and type y and b were used for quantification. An isotope modification of type "Label" was created using the following modification: $^{13}C(6)^{25}N(2)$ (K). Isotope label type and internal standard type were set to "heavy". For quantification, MS level was set to 2. Results at the peptide and the precursor level were exported and used for downstream data analysis and visualization.

Thermal shift assay

The thermal stability of proteins was analyzed by thermal shift (thermofluor) assays. For this purpose, the proteins were incubated with SYPRO orange dye (Sigma-Aldrich), which binds specifically to hydrophobic amino acids leading to an increased fluorescence at 610 nm when excited with a wavelength of 490 nm. The melting temperature is defined as the midpoint of temperature of the protein-unfolding transition⁷¹. This turning point of the melting curve was extracted from the derivative values of the RFU curve, where a turning point to the right is a minimum. The thermal stability of the isomerase domain of hGFAT-1 and hGFAT-1 variants, were determined in SEC buffer. The influence of increasing salt concentrations (0 to 1 M) in the SEC buffer was assessed for the isomerase domain of hGFAT-1 and full-length hGFAT-1. The reaction mixtures were pipetted in white RT-PCR plates and contained 5 μ l SYPRO orange dye (1:500 dilution in ddH₂O) and 5-10 μ g protein in a total volume of 50 μ l. The plates were closed with optically clear tape and placed in a BioRad CFX-96 Real-Time PCR machine. The melting curves were measured at

1°C/min at the FRET channel and the data analyzed with CFX Manager™ (BioRad).

Circular dichroism spectroscopy

For CD measurements, GFAT-1 was dialyzed in 10 mM potassium phosphate buffer pH 7.5, 0.5 mM TCEP, 0.5 mM Na₂Frc6P, and 10% (v/v) glycerol, and the protein concentration was adjusted to 0.2 mg/ml. The UV spectra in the range of 195–260 nm were recorded with a J-715 CD spectropolarimeter (Jasco, Gross-Umstadt, Germany) at 20°C using a quartz cuvette with 0.1 cm path length. The buffer baseline was recorded separately and subtracted from each sample spectrum. The obtained ellipticity (θ , deg) was converted to mean residue ellipticity $[\theta]$ using: $[\theta] = \theta / (10 * n * c * l)$ in deg*cm²*dmol⁻¹ (n is the number of amino acids, c the protein concentration in mol/l, and l the path length of the cuvette).

Mammalian cell culture and stable cell line generation

HEK293 cells (ATCC) were cultured at 37°C, 5% CO₂ on treated polystyrene culture dishes (Corning) in DMEM media with high glucose (4.5 g/l; 25 mM) with pyruvate (Gibco, 11995-065) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

Cell lines stably overexpressing GFAT-1 variants were generated by transfection of HEK293 cells with pcDNA3.1 plasmids with human GFAT-1 containing an internal His₆-tag between Gly299 and Asp300 (pcDNA3.1-hGFAT-1-His299). Internally tagged GFAT-1 was subcloned from pFL-hGFAT1-His299 using NheI and HindIII restriction sites. For each variant, one well of a 6 well plate was transfected with 2 µg of plasmid DNA with Lipofectamine® 2000 (Life Technologies™) according to the manufacturer's protocol. The selection was performed with 500 µg/ml G418 for several weeks. For Western blot analysis, cells were washed, collected and the proteins from the pellets were extracted by lysis in RIPA-buffer (120 mM NaCl, 50 mM Tris/HCl, 1% (v/v) NP40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS; pH 7.5). The cell debris was removed by centrifugation and the protein concentration of the supernatants were determined using Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) according to manufacturer's protocol. 2 µg

cell lysate was separated by reducing SDS-PAGE and transferred to PVDF membranes. Primary antibodies against human GFAT1 and α -TUBULIN were used. Chemiluminescence of the appropriate secondary rabbit or mouse HRP-conjugated antibodies after incubation with ECL HRP substrate (Immobilon™ Western HRP Substrate, Millipore) was detected using a Chemi Doc™ Quantity One® system (Bio-Rad).

Antibodies

The following antibodies were used in this study: GFAT1 (rb, EPR4854, Abcam ab125069, 1:1000), α -TUBULIN (ms, DM1A, Sigma T6199, 1:50000, rabbit IgG (gt, LifeTechnologies G21234, 1:5000), and mouse IgG (gt, LifeTechnologies G21040, 1:5000).

Data availability

Structural data reported in this study have been deposited in the Protein Data Bank with the accession codes 6ZMJ and 6ZMK. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁷² partner repository with the dataset identifier PXD020451. All other data supporting the presented findings are available from the corresponding authors upon request.

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Acknowledgments

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Author contributions

S.R. and M.S.D. designed the project. S.R. performed the biochemical and crystallization experiments, as well as the cell culture experiments. F.M. performed the experiments related to *C. elegans*. S.M. helped with the mammalian cell culture experiments. I.A. did the protein mass spectrometry measurements and analysis. S.R., M.S.D., and U.B. wrote the manuscript. S.R. prepared the figures.

Competing interests

The authors declare no competing interest.

Figures

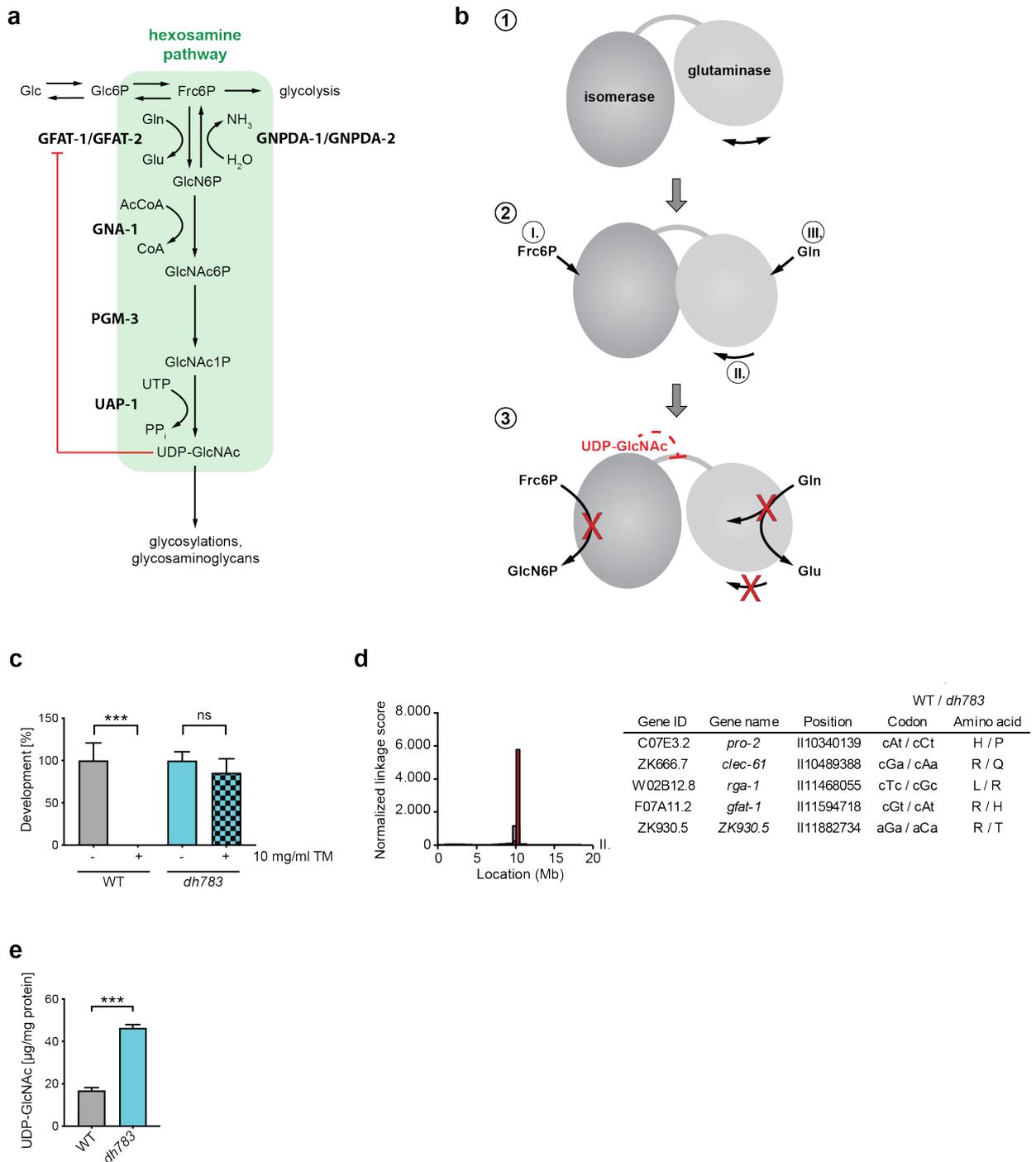


Fig. 1: Characterization of *gfat-1(dh783)* *C. elegans* mutants.

a, Schematic representation of the hexosamine pathway (green box). The enzymes in the pathway are glutamine fructose-6-phosphate amidotransferase (GFAT-1/-2), glucosamine-6-phosphate N-acetyltransferase (GNA-1), phosphoglucomutase (PGM-3), UDP-N-acetylglucosamine pyrophosphorylase (UAP-1), and glucosamine-6-phosphate deaminase (GNPDA-1/-2). UDP-GlcNAc inhibits eukaryotic GFAT (red line).

b, Catalytic scheme of one GFAT monomer. (1) Before catalysis: The glutaminase

domain does not adopt a fixed position. (2) Substrate binding: I. Frc6P binds, II. the glutaminase domain adopts a specific position, III. L-Gln binds. (3) Catalysis and UDP-GlcNAc inhibition. Catalysis: L-Gln is hydrolyzed to L-Glu and the released ammonia is shuttled through an ammonia channel from the glutaminase to the isomerase domain. There, Frc6P is isomerized to Glc6P and the ammonia is transferred to build GlcN6P. UDP-GlcNAc inhibition: UDP-GlcNAc binds to the isomerase domain, interacts with the interdomain linker, and inhibits the glutaminase function and thereby the GlcN6P production. **c**, *C. elegans* (N2 wild type and *gfat-1(dh783)*) developmental resistance assay on NGM plates containing 10 mg/ml tunicamycin. **d**, Frequency plot of normalized parental alleles on chromosome II of *dh783*. The CloudMap Hawaiian Variant Mapping with WGS tool displays regions of linked loci where pure parental allele SNP positions instead of allele positions containing Hawaiian SNPs are over-represented. Gray bars represent 1 Mb and red bars represent 0,5 Mb sized bins. Table: Candidate non-synonymous SNPs between 10 and 12 Mb on chromosome II of *gfat-1(dh783)* animals. **e**, UDP-GlcNAc levels in N2 wild type and *gfat-1(dh783)* animals (mean +SD, n=5, *** p<0.001, unpaired t-test).

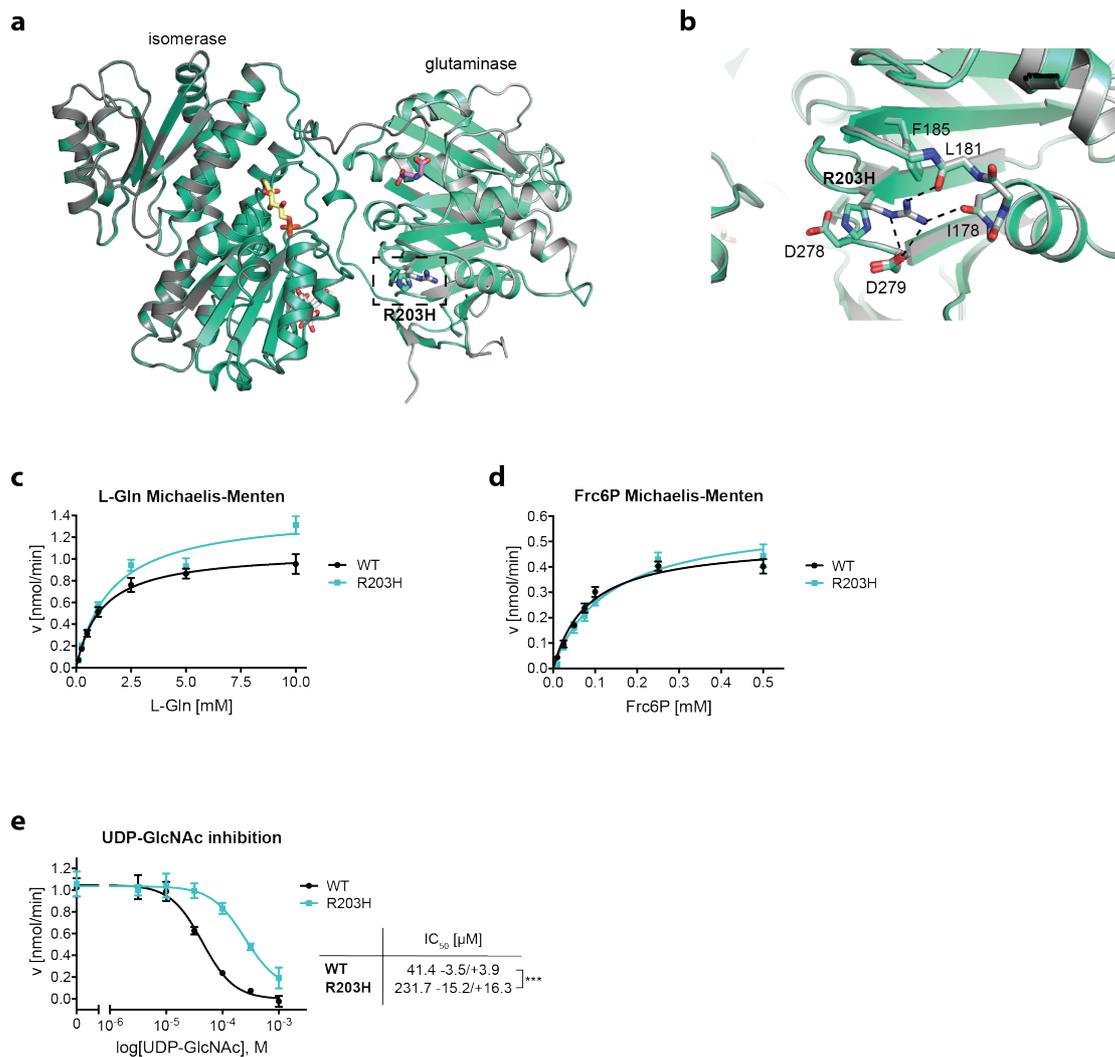


Fig. 2: The GFAT-1 R203H gain-of-function substitution perturbs UDP-GlcNAc feedback inhibition.

a, b, Position of the R203H mutation in the structure of GFAT-1. Proteins are presented as cartoons. Superposition of wild type GFAT-1 (light gray/dark gray) and R203H GFAT-1 (green-cyan, teal). Glc6P (yellow sticks), L-Glu (violet sticks), and UDP-GlcNAc (white sticks) are highlighted, as well as the position of R203H (black box). **a**, Overview. R203H (sticks) is located at the glutaminase domain of GFAT-1 (dashed box). **b**, Close-up view of the position of R203H focusing on residues in close proximity. The R203H mutation and residues in close proximity to the mutation are highlighted with sticks. Arg203 interacts with the neighboring loops (dashed lines). **c**, L-Gln kinetic of wild type (WT, black circle) and R203H (cyan square) GFAT-1 (mean \pm SEM, WT n=5, R203H n=4). **d**, Frc6P kinetic of wild type (black circle) and R203H (cyan square) GFAT-1 (mean \pm SEM, WT n=5, R203H n=4). **e**, Representative UDP-GlcNAc dose response assay of wild type (black circle) and R203H (cyan square) GFAT-1 (mean \pm SD, n=3). Table: IC₅₀ UDP-GlcNAc values (mean \pm SEM, n=4, ***p<0.001, unpaired t-test).

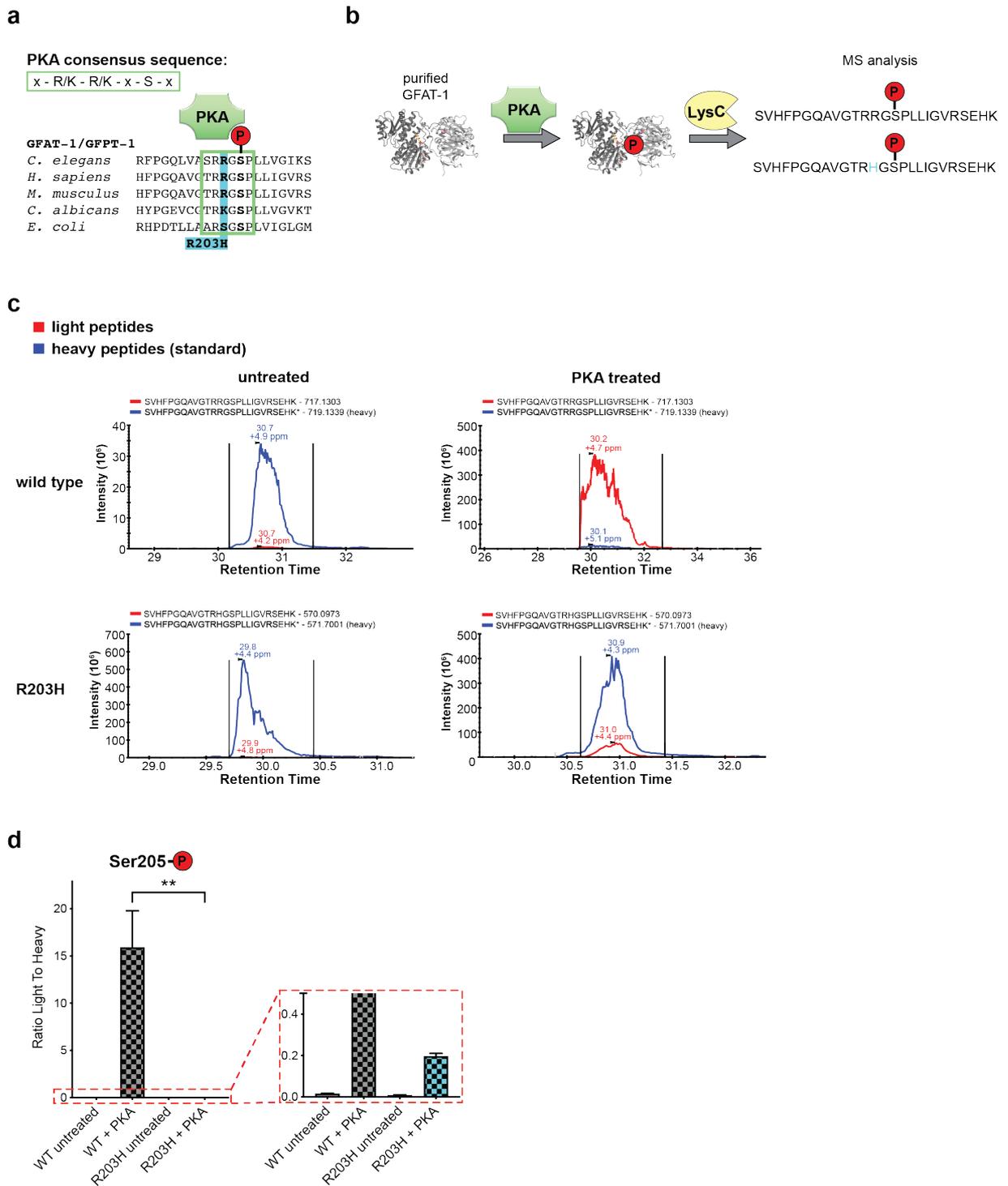


Fig. 3: The GFAT-1 gain-of-function substitution R203H disturbs PKA phosphorylation at Ser205.

a, Position of gain-of-function mutation R203H in a protein sequence alignment of GFAT-1. Mutation R203H (cyan) disrupts the PKA consensus sequence at Ser205. **b**, Workflow for the *in vitro* phosphorylation analysis. **c**, Representative quantification of the light and heavy peptides by mass spectrometry. **d**, Quantification of the Ser205 phosphorylation of wild type GFAT-1 (gray) and R203H (cyan) before and after treatment with PKA (mean +SEM, n=4, ** p<0.01, one-way ANOVA).

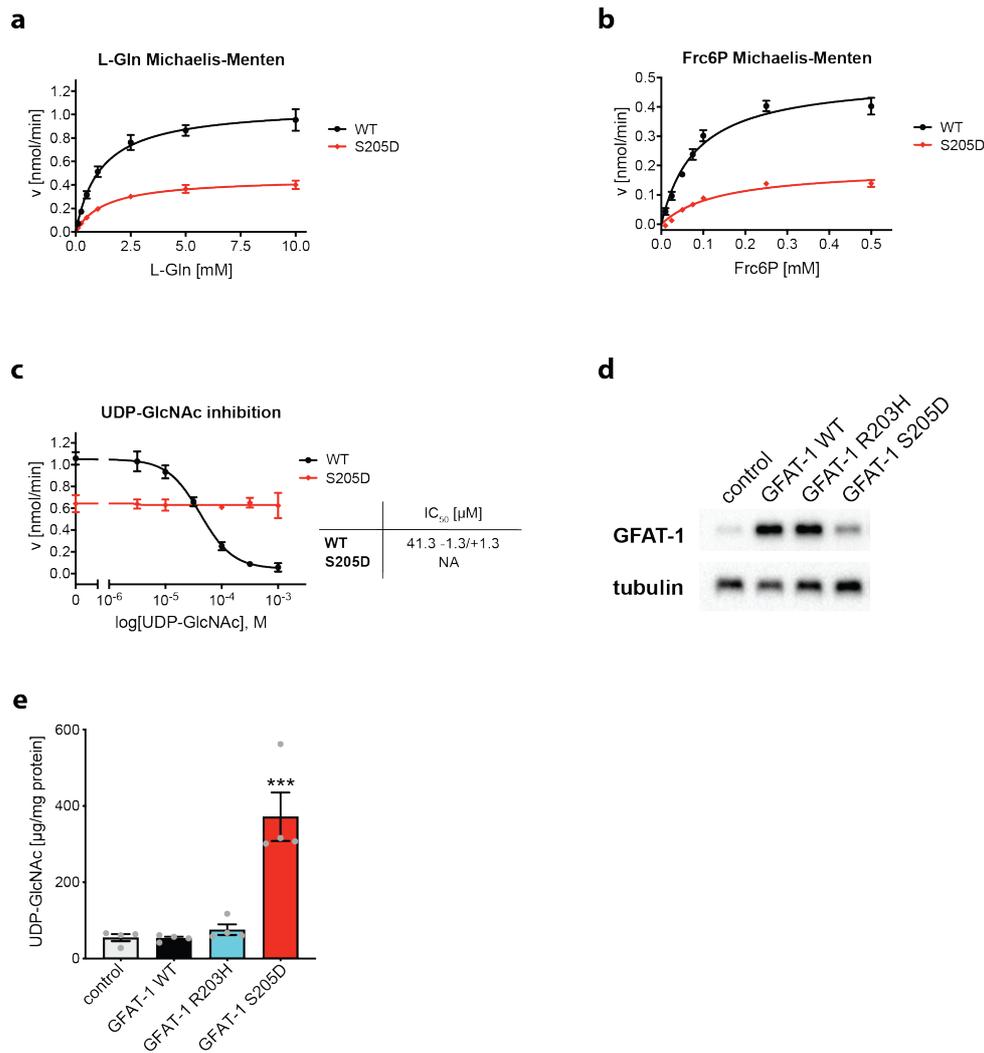


Fig. 4: PKA phosphorylation at Ser205 modulates UDP-GlcNAc inhibition of GFAT-1.

a, L-Gln kinetic of wild type (black circles) and S205D (red diamonds) GFAT-1 (mean \pm SEM, WT n=5, S205D n=6). **b**, Frc6P kinetic of wild type (black circles) and S205D (red diamonds) GFAT-1 (mean \pm SEM, WT n=5, S205D n=4). **c**, Representative UDP-GlcNAc inhibition of wild type (black circles) and S205D (red diamonds) GFAT-1 (mean \pm SD, n=3). Table: IC₅₀ UDP-GlcNAc values (mean \pm SEM, n=3). **d**, Western blot analysis of GFAT-1 protein levels in control HEK293 cells and HEK293s cell stably overexpressing the indicated GFAT-1 variants. **e**, LC/MS measurement of UDP-GlcNAc normalized to protein content presented as means +SEM with n=4, *** p < 0.001 versus WT, one-way ANOVA.

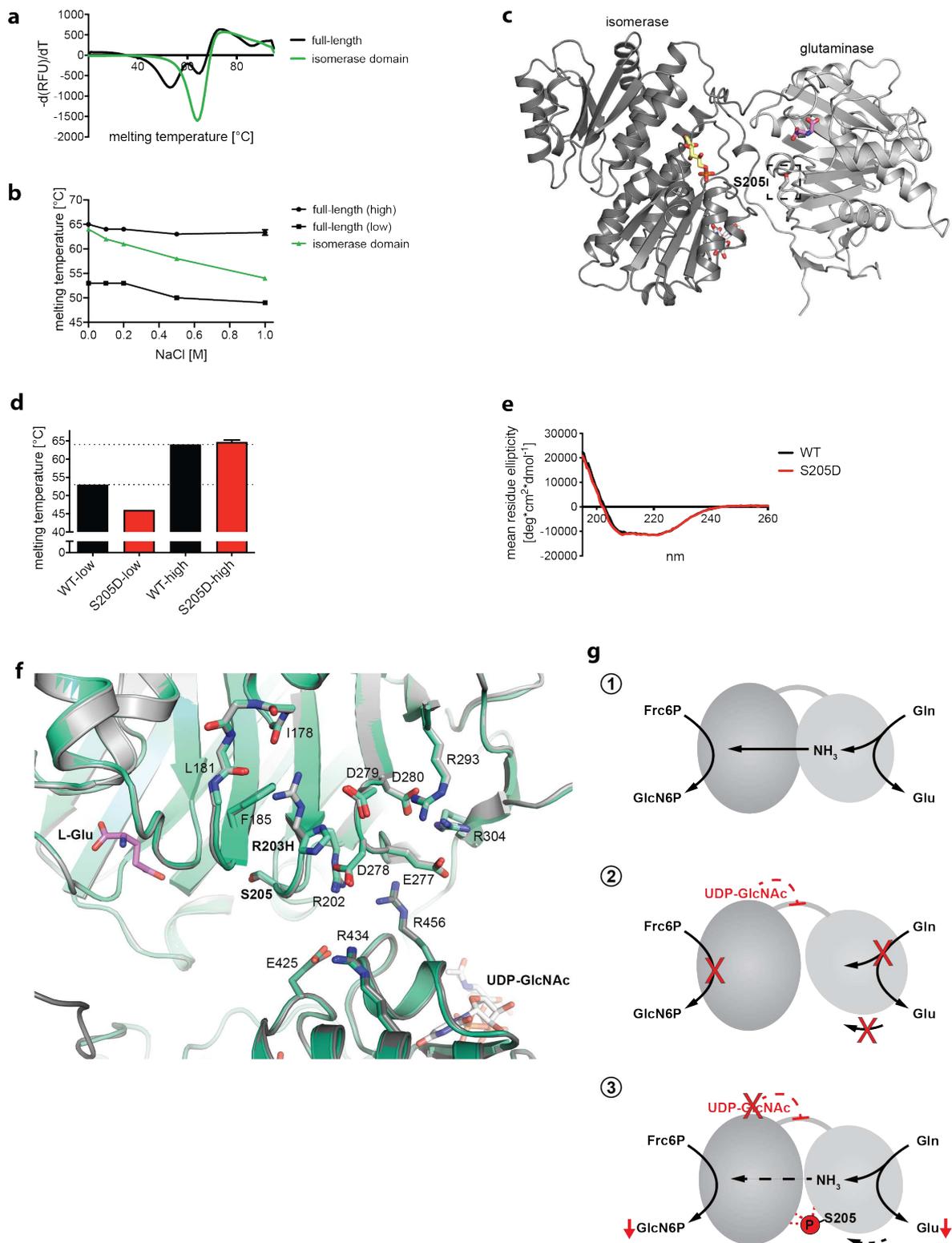


Fig. 5: Altered domain stability of GFAT-1 after PKA phosphorylation at Ser205.

a, Representative derivative melting curves of full-length GFAT-1 (black) and the isolated GFAT-1 isomerase domain (green) in standard SEC buffer without NaCl. **b**, Melting temperatures of full-length GFAT-1 (black) and the isolated GFAT-1 isomerase domain (green) GFAT-1 in SEC buffer with rising NaCl concentrations (mean +SD, n=3). **c**, Overview of the position of the phosphorylation site Ser205 in GFAT-1 in cartoon representation. Glc6P (yellow sticks), L-Glu (violet sticks), and UDP-GlcNAc (white

sticks) are highlighted, as well as the PKA site Ser205 (dashed box). **d**, Melting temperatures of wild type GFAT-1 (black) and GFAT-1 S205D (red) (mean +SD, n=3). **e**, CD spectra of wild type GFAT-1 (black) and GFAT-1 S205D (red). **f**, Superposition of the structures of wild type (gray) and R203H (green cyan) GFAT-1 in cartoon representation. L-Glu (violet sticks) and UDP-GlcNAc (white sticks) are highlighted. Ser205, the R203H substitution and residues that might form salt bridges in close proximity to the mutation are highlighted with sticks. **g**, Schematic models of one GFAT monomer. (1) Catalysis: L-Gln is hydrolyzed to L-Glu and the released ammonia is shuttled through an ammonia channel from the glutaminase to the isomerase domain. (2) UDP-GlcNAc inhibition: UDP-GlcNAc binds to the isomerase domain, interacts with the interdomain linker, and inhibits the glutaminase function and thereby the GlcN6P production. (3) Ser205 phosphorylation: Upon phosphorylation, the catalytic activity is reduced and the UDP-GlcNAc inhibition is abolished.

Table 1. Data collection and refinement statistics.

| | GFAT-1 R203H | GFAT-1 L405R |
|---------------------------------------|-----------------------------------|-----------------------------------|
| Wavelength (Å) | 1.00 | 1.00 |
| Resolution range (Å) | 48.65 - 2.77 (2.87 - 2.77) | 48.74 - 2.38 (2.47 - 2.38) |
| Space group | P 4 ₁ 2 ₁ 2 | P 4 ₁ 2 ₁ 2 |
| a, b, c (Å) | 152.8 152.8 164.8 | 152.9 152.9 166.2 |
| α, β, γ (°) | 90 90 90 | 90 90 90 |
| Total reflections | 660165 (61036) | 1059136 (102791) |
| Unique reflections | 49792 (4773) | 78975 (7737) |
| Multiplicity | 13.3 (12.8) | 13.4 (13.3) |
| Completeness (%) | 99.8 (97.9) | 99.9 (99.2) |
| Mean I/sigma(I) | 14.05 (1.16) | 20.15 (1.23) |
| Wilson B-factor | 62.73 | 55.09 |
| R_{merge} (%) | 22.0 (230.5) | 11.1 (202.3) |
| R_{meas} (%) | 22.9 (240.0) | 11.5 (210.4) |
| R_{pim} (%) | 6.2 (66.5) | 3.1 (57.2) |
| CC_{1/2} (%) | 99.7 (45.0) | 99.9 (50.1) |
| CC* (%) | 99.9 (78.8) | 100 (81.7) |
| Reflections used in refinement | 49787 (4773) | 78970 (7737) |
| Reflections used for R-free | 1993 (187) | 1983 (187) |
| R_{work} (%) | 20.1 (32.6) | 19.3 (30.0) |
| R_{free} (%) | 23.9 (36.0) | 21.1 (30.0) |
| CC_{work} (%) | 94.1 (68.3) | 96.2 (74.3) |
| CC_{free} (%) | 92.2 (49.4) | 96.4 (76.3) |
| Number of non-hydrogen atoms | 10329 | 10529 |
| macromolecules | 10277 | 10384 |
| ligands | 32 | 32 |
| solvent | 20 | 113 |
| Protein residues | 1299 | 1313 |
| RMS (bonds) (Å) | 0.002 | 0.002 |
| RMS (angles) (°) | 0.45 | 0.45 |
| Ramachandran favored (%) | 96 | 96 |
| Ramachandran allowed (%) | 4 | 3.5 |
| Ramachandran outliers (%) | 0.078 | 0.23 |
| Rotamer outliers (%) | 0.088 | 0.26 |
| Clashscore | 0.78 | 0.67 |
| Average B-factor | 95.73 | 84.61 |
| macromolecules | 95.97 | 85.11 |
| ligands | 52.14 | 49.82 |
| solvent | 42.46 | 48.53 |
| Number of TLS groups | 4 | 4 |
| PDB code | 6ZMJ | 6ZMK |

Statistics for the highest-resolution shell are shown in parentheses.

Table 2. Kinetic parameters.

| | L-Glu production | | | D-GlcN6P production | | |
|------------------|---------------------|-----------------------------------|--|---------------------|-----------------------------------|--|
| | K_m L-Gln [mM] | k_{cat} [sec ⁻¹] | k_{cat}/K_m [mM ⁻¹ sec ⁻¹] | K_m Frc6P [mM] | k_{cat} [sec ⁻¹] | k_{cat}/K_m [mM ⁻¹ sec ⁻¹] |
| Wild type | 1.1 ± 0.2 | 3.6 ± 0.2 | 3.3 | 0.08 ± 0.01 | 1.7 ± 0.1 | 21.3 |
| R203H | 1.6 ± 0.27 | 4.8 ± 0.26 | 3.0 | 0.13 ± 0.02 | 2.0 ± 0.15 | 15.4 |
| S205D | 1.3 ± 0.23 | 1.5 ± 0.08 | 1.2 | 0.18 ± 0.04 | 0.8 ± 0.07 | 4.4 |
| L405R | 1.0 ± 0.10 | 3.0 ± 0.08 | 3.0 | 0.07 ± 0.02 | 1.9 ± 0.15 | 27.1 |

Supplementary figures

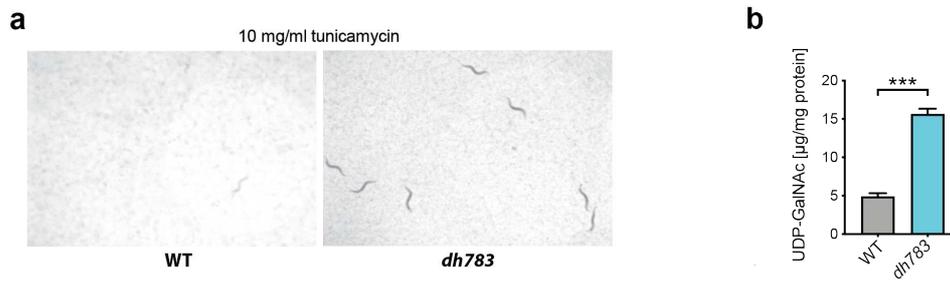


Fig. 1 Supp.: Characterization of *gfat-1(dh783)* *C. elegans* mutants.

a, Representative images of wild type (N2) and *gfat-1(dh783)* animals 4 days post-hatch on NGM plates containing 10 mg/ml tunicamycin. **b**, UDP-GalNAc levels in N2 wild type and *gfat-1(dh783)* animals (mean +SD, n=5, *** p<0.001, unpaired t-test).

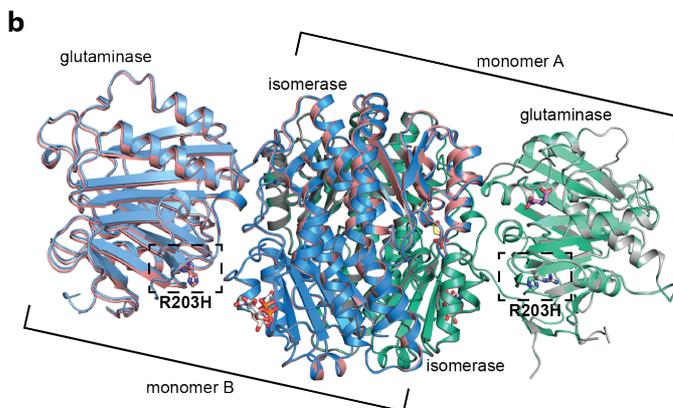


Fig. 2 Supp: The GFAT-1 R203H gain-of-function substitution perturbs UDP-GlcNAc feedback inhibition.

a, Protein sequence alignment of GFAT-1. Red boxes indicate identical residues, red letters indicate similar residues. **b**, Position of the R203H mutation in the dimeric structure of GFAT-1. Proteins are presented as cartoons. Superposition of wild type GFAT-1 (light gray/dark gray) and R203H GFAT-1 (green-cyan, teal). Glc6P (yellow sticks), L-Glu (violet sticks), and UDP-GlcNAc (white sticks) are highlighted, as well as the position of R203H (black box).

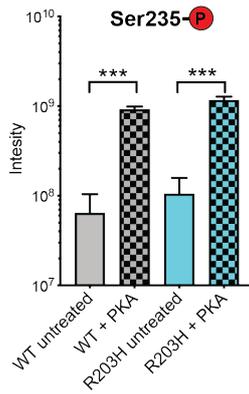


Fig. 3 Supp.: *In vitro* PKA treatment of GFAT-1 results in phosphorylation at Ser235.

Intensity of phosphorylated peptides at Ser235 of wild type GFAT-1 (gray) and R203H (cyan) before and after treatment with PKA normalized to protein abundance (mean +SEM, n=4, *** p<0.001, one-way ANOVA).

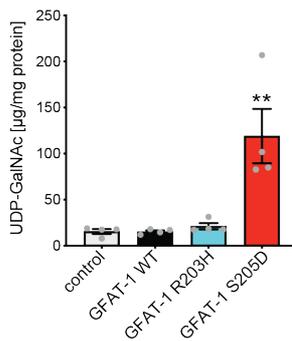


Fig. 4 Supp: PKA phosphorylation at Ser205 modulates UDP-GlcNAc inhibition of GFAT-1.

LC/MS measurement of UDP-GalNAc normalized to protein content presented as means +SEM with n=4, ** p<0.01 versus WT, one-way ANOVA.

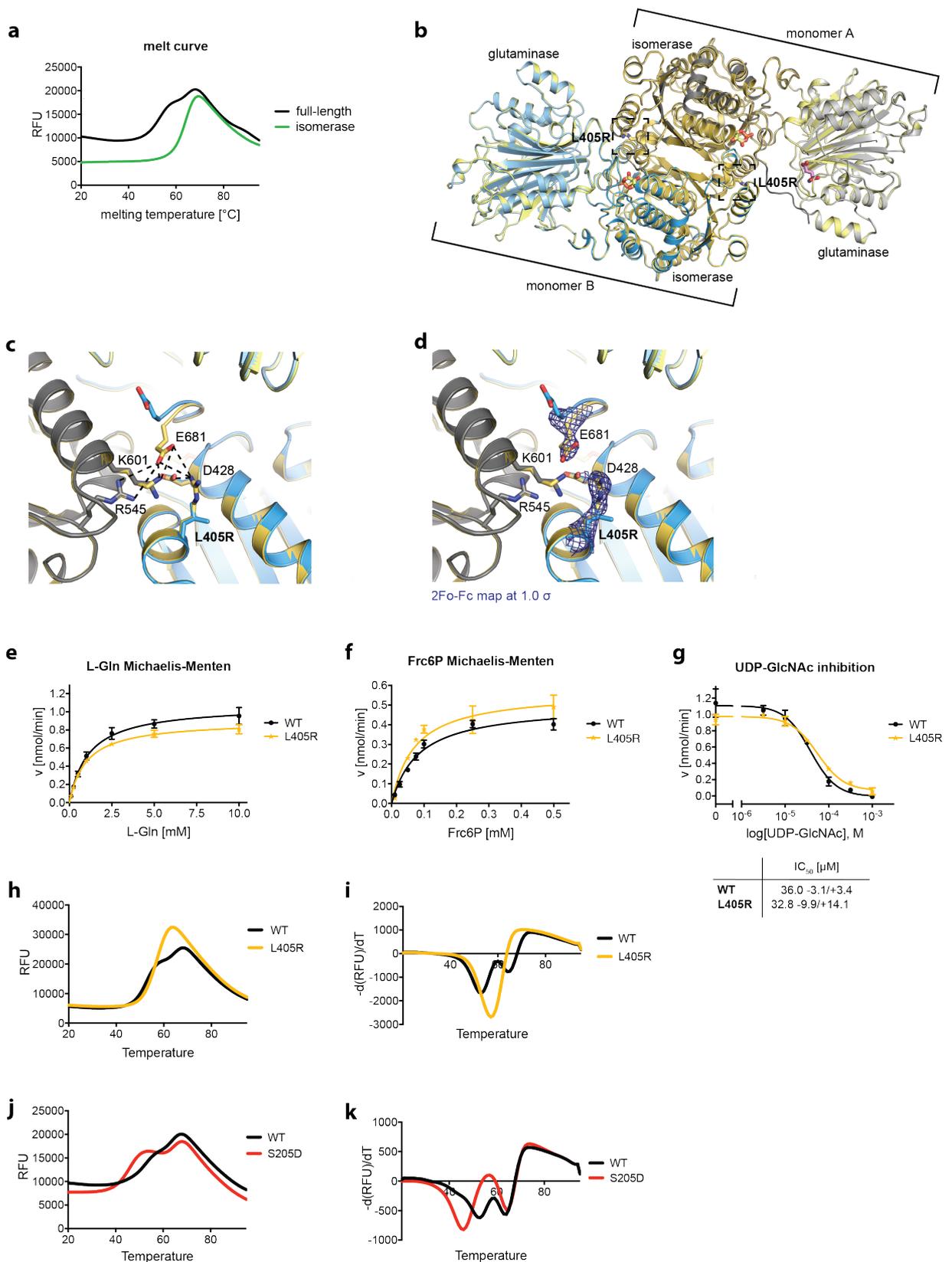


Fig. 5 Supp: Altered domain stability of GFAT-1 after PKA phosphorylation at Ser205.

a, Representative melting curves of full-length (black) and isolated isomerase domain (green) in standard SEC buffer without NaCl. **b-d**, Position of the L405R mutation in the

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Figures

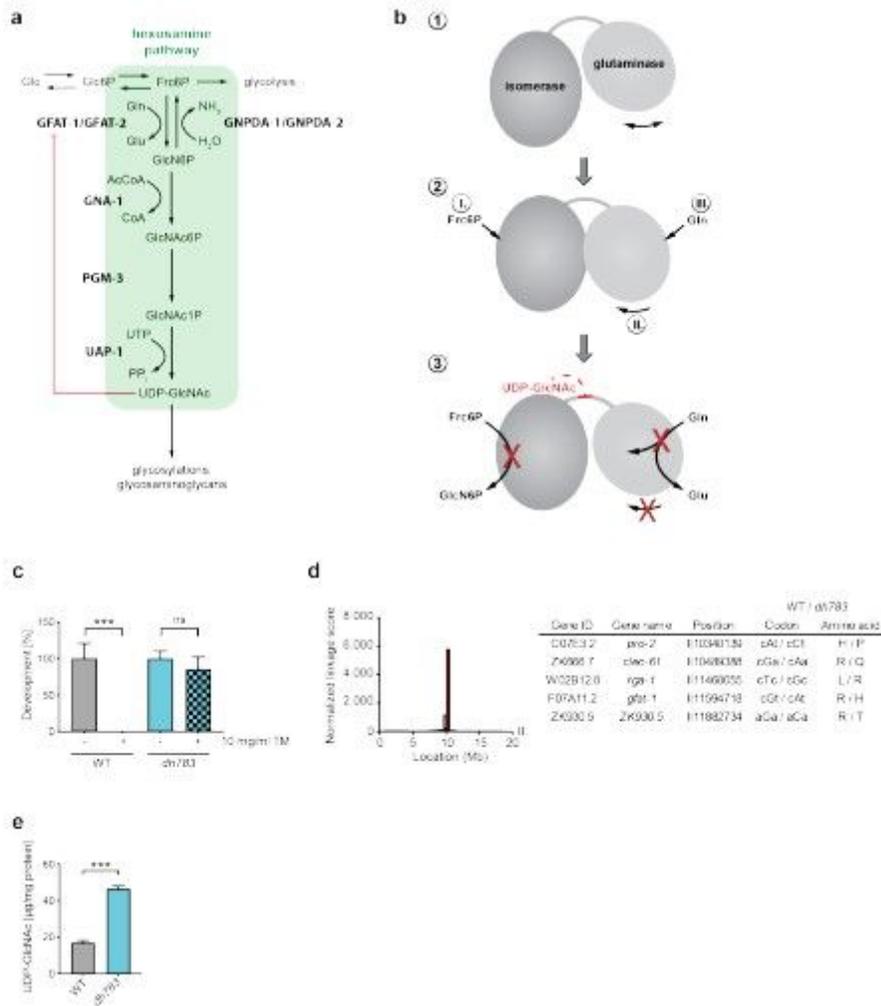


Figure 1

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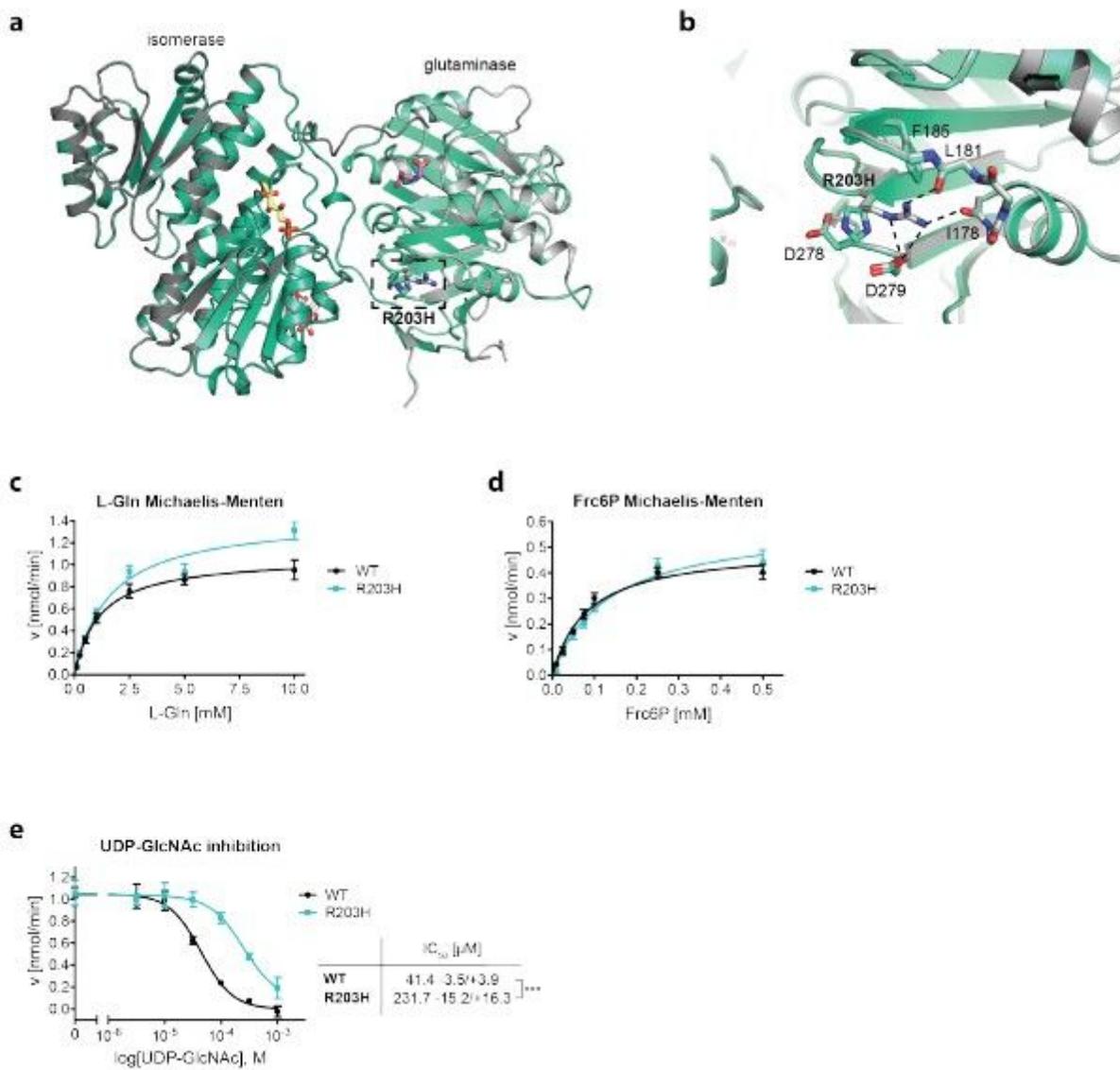


Figure 2

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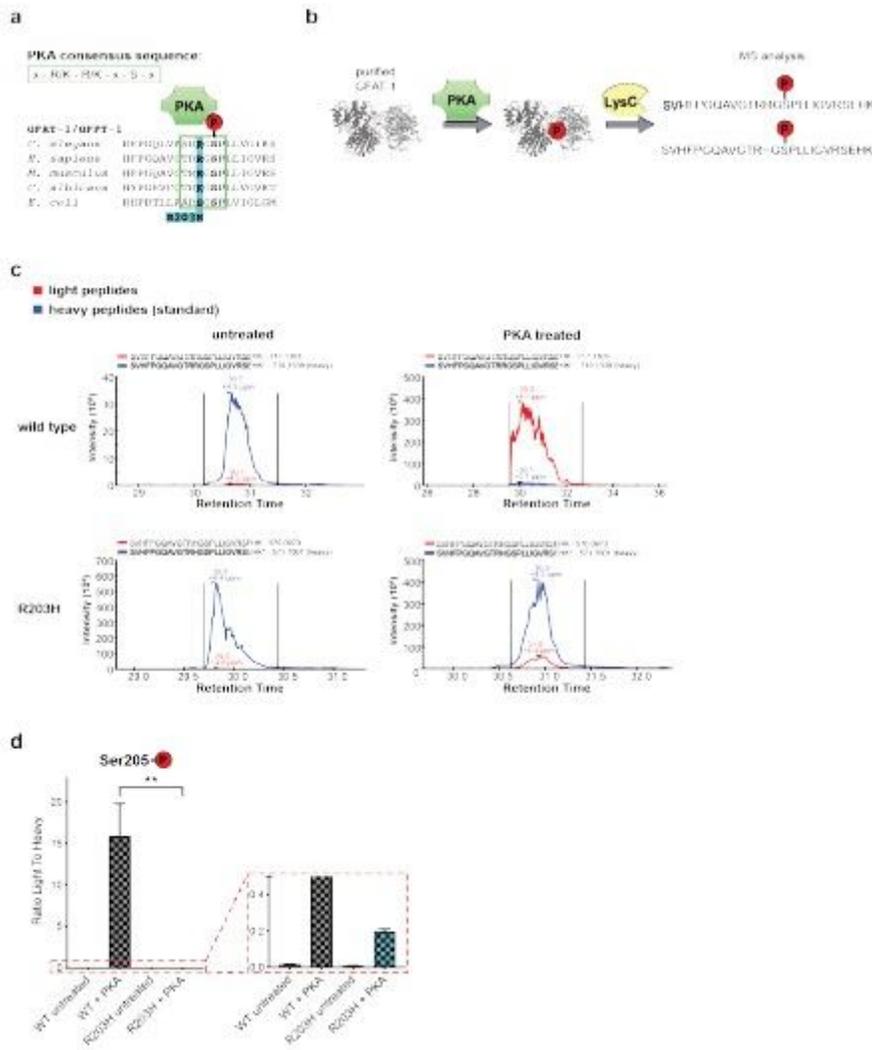


Figure 3

The GFAT-1 gain-of-function substitution R203H disturbs PKA phosphorylation at Ser205. a, Position of gain-of-function mutation R203H in a protein sequence alignment of GFAT-1. Mutation R203H (cyan) disrupts the PKA consensus sequence at Ser205. b, Workflow for the in vitro phosphorylation analysis. c, Representative quantification of the light and heavy peptides by mass spectrometry. d, Quantification of the Ser205 phosphorylation of wild type GFAT-1 (gray) and R203H (cyan) before and after treatment with PKA (mean \pm SEM, n=4, ** p<0.01, one-way ANOVA).

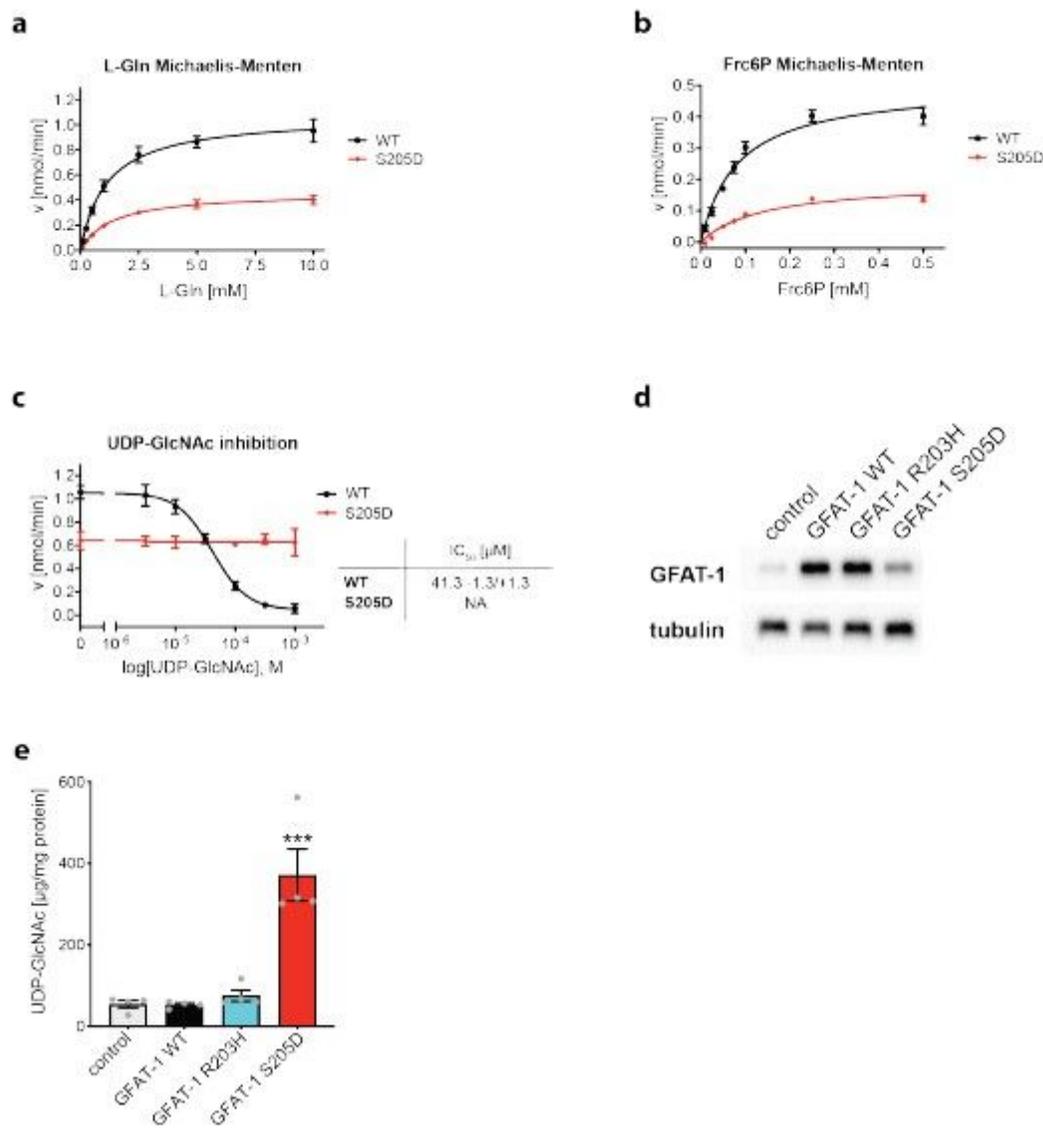


Figure 4

PKA phosphorylation at Ser205 modulates UDP-GlcNAc inhibition of GFAT-1. a, L-Gln kinetic of wild type (black circles) and S205D (red diamonds) GFAT-1 (mean ±SEM, WT n=5, S205D n=6). b, Frc6P kinetic of wild type (black circles) and S205D (red diamonds) GFAT-1 (mean ±SEM, WT n=5, S205D n=4). c, Representative UDP-GlcNAc inhibition of wild type (black circles) and S205D (red diamonds) GFAT-1 (mean ±SD, n=3). Table: IC₅₀ UDP-GlcNAc values (mean ±SEM, n=3). d, Western blot analysis of GFAT-1 protein levels in control HEK293 cells and HEK293s cell stably overexpressing the indicated GFAT-1 variants. e, LC/MS measurement of UDP-GlcNAc normalized to protein content presented as means +SEM with n=4, *** p < 0.001 versus WT, one-way ANOVA.

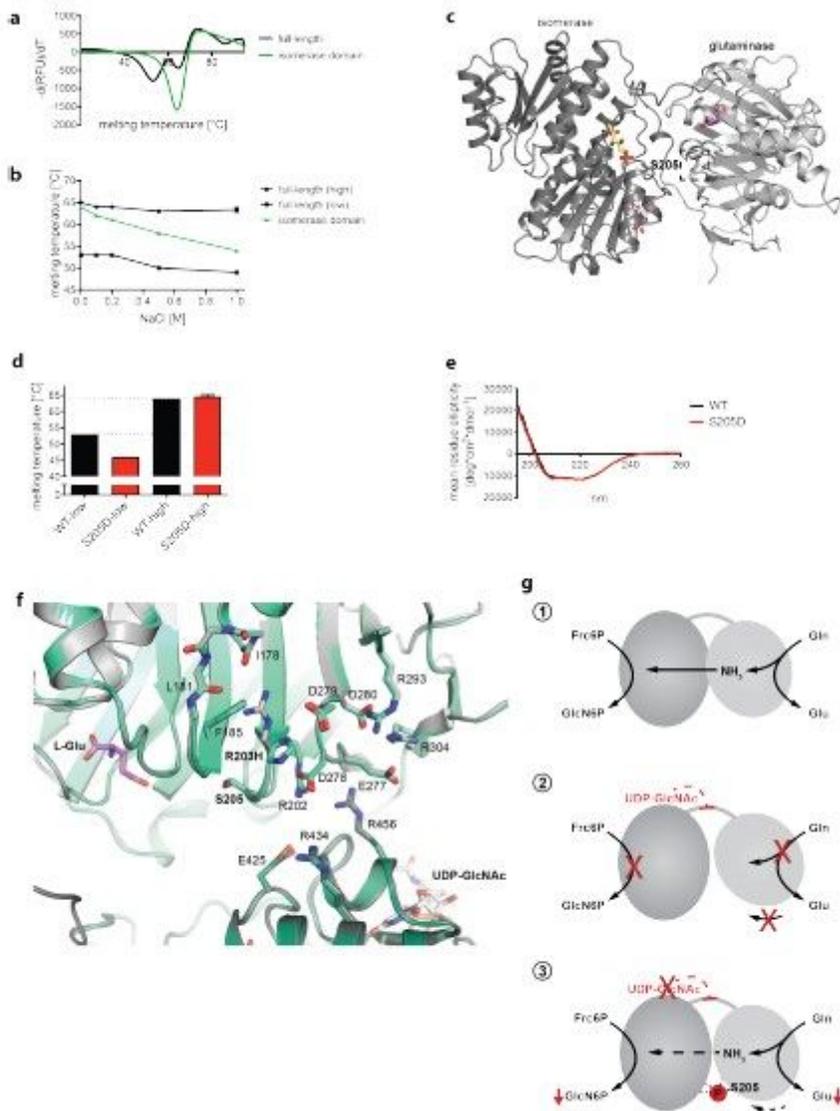


Figure 5

Altered domain stability of GFAT-1 after PKA phosphorylation at Ser205. a, Representative derivative melting curves of full-length GFAT-1 (black) and the isolated GFAT-1 isomerase domain (green) in standard SEC buffer without NaCl. b, Melting temperatures of full-length GFAT-1 (black) and the isolated GFAT-1 isomerase domain (green) GFAT-1 in SEC buffer with rising NaCl concentrations (mean +SD, n=3). c, Overview of the position of the phosphorylation site Ser205 in GFAT-1 in cartoon representation. Glc6P (yellow sticks), L-Glu (violet sticks), and UDP-GlcNAc (white 35 sticks) are highlighted, as well as the PKA site Ser205 (dashed box). d, Melting temperatures of wild type GFAT-1 (black) and GFAT-1 S205D (red) (mean +SD, n=3). e, CD spectra of wild type GFAT-1 (black) and GFAT-1 S205D (red). f, Superposition of the structures of wild type (gray) and R203H (green cyan) GFAT-1 in cartoon representation. L-Glu (violet sticks) and UDP-GlcNAc (white sticks) are highlighted. Ser205, the R203H substitution and residues that might form salt bridges in close proximity to the mutation are highlighted with sticks. g, Schematic models of one GFAT monomer. (1) Catalysis: L-Gln is hydrolyzed to L-Glu and the released ammonia is shuttled through an ammonia channel from the glutaminase to the isomerase domain. (2) UDP-GlcNAc

inhibition: UDP-GlcNAc binds to the isomerase domain, interacts with the interdomain linker, and inhibits the glutaminase function and thereby the GlcN6P production. (3) Ser205 phosphorylation: Upon phosphorylation, the catalytic activity is reduced and the UDP-GlcNAc inhibition is abolished.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [validationreportstructureGFAT1L405R.pdf](#)
- [validationreportstructureGFAT1R203H.pdf](#)
- [Supplementarytable12.xlsx](#)
- [FigureS1.png](#)
- [FigureS2.jpg](#)
- [FigureS3.jpg](#)
- [FigureS4.jpg](#)
- [FigureS5.jpg](#)