Loss of GFAT-1 feedback regulation activates the hexosamine pathway that modulates protein homeostasis

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Glutamine fructose-6-phosphate amidotransferase (GFAT) is the key enzyme in the hexosamine pathway (HP) that produces uridine 5′-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc), linking energy metabolism with posttranslational protein glycosylation. In Caenorhabditis elegans, we previously identified gfat-1 gain-of-function mutations that elevate UDP-GlcNAc levels, improve protein homeostasis, and extend lifespan. GFAT is highly conserved, but the gain-of-function mechanism and its relevance in mammalian cells remained unclear. Here, we present the full-length crystal structure of human GFAT-1 in complex with various ligands and with important mutations. UDP-GlcNAc directly interacts with GFAT-1, inhibiting catalytic activity. The longevity-associated G451E variant shows drastically reduced sensitivity to UDP-GlcNAc inhibition in enzyme activity assays. Our structural and functional data point to a critical role of the interdomain linker in UDP-GlcNAc inhibition. In mammalian cells, the G451E variant potently activates the HP. Therefore, GFAT-1 gain-of-function through loss of feedback inhibition constitutes a potential target for the treatment of age-related proteinopathies.
A progressive decline of physiological functions limits healthspan and survival in most organisms. The aging process is modulated by specific signaling pathways and their manipulation can result in lifespan extension, suggesting that aging is a coordinated process. Relevant pathways include insulin/IGF-1 signaling, mTOR, and the AMPK cascade. While the plasticity of the aging process suggests that pathways could be targeted to prolong life and postpone age-related diseases in humans, few drug candidates have emerged. For example rapamycin, which targets mTOR, and metformin that acts through yet incompletely understood mechanisms, have entered clinical trials with the goal to slow aging and prevent age-related phenotypes. Thus, there is a clear need for the identification of druggable targets and respective drugs in gerontology.

The ubiquitous hexosamine pathway (HP) is essential for aminosugar biosynthesis (Fig. 1a). Increased HP activity extends lifespan and ameliorates pathology in multiple proteotoxic disease models in the nematode Caenorhabditis elegans. The HP converts the glycolysis intermediate fructose-6-phosphate (Frc6P) to uridine 5′-diphosphate-N-acetylglucosamine (UDP-GlcNAc), using between 1% and 3% of total cellular glucose. UDP-GlcNAc is a precursor for multiple important roles in the extracellular matrix and N-glycosylation reactions in mammals. Mucin-type O-glycosylation plays an important role in the extracellular matrix and N-glycosylation contributes to cellular protein homeostasis by governing the protein folding process in the endoplasmic reticulum (ER). Additionally, GlcNAc can be transferred as a single moiety to serine or threonine residues of target proteins. This so-called O-GlcNAcylation competes with phosphorylation and serine or threonine residues of target proteins. Interestingly, O-GlcNAcylation potentially plays a critical role in several neurodegenerative disorders. In Alzheimer’s disease, hyperphosphorylation of tau protein triggers aggregation, which is a hallmark of pathological processes governing cellular physiology. Importantly, GFAT engages with both of its substrates, L-Gln and Frc6P, and its bacterial homolog, glucosamine-6-phosphate synthase (GlmS), in the presence of the feedback inhibitor UDP-GlcNAc and revealed the UDP-GlcNAc binding site within the isomerase domain. This binding site was confirmed in human GFAT. Although UDP-GlcNAc binds to GFAT’s isomerase domain, it inhibits the glutaminase function and thus GlcN6P production, suggesting interdomain communication. Interfering with GFAT regulation might open an avenue to pharmacological modulation of the HP.

Here, we present the full-length human GFAT structure and delineate how single amino acid substitutions modulate GFAT activity. Structural and functional analyses of point mutants show that their gain-of-function results from loss of UDP-GlcNAc inhibition. Going beyond in vitro assays, we demonstrate the relevance of the GFAT gain-of-function substitution in regulating the HP in mammalian cells.

Results

Structure of full-length human GFAT-1. To understand HP regulation at the molecular level, we determined the crystal structure of active full-length human GFAT-1. As N- or C-terminal tags interfere with GFAT-1 activity, we inserted an internal His6-tag between Gly299 and Asp300 (Supplementary Fig. 1a), which does not interfere with GFAT-1 kinetic properties. We established a protocol for large-scale production of active, internally His6-tagged GFAT-1 using the MultiBac baculovirus expression system with subsequent purification via immobilized metal affinity chromatography and size-exclusion chromatography. Tetragonal GFAT-1 crystals formed within a few days and diffracted to a resolution limit of 2.4 Å. Data collection and refinement statistics are given in Tables 1 and 2. Two GFAT-1 monomers were present in the asymmetric unit, which were termed monomer A and B according to the chain identifier in the PDB files. The complete structure was modeled into the electron density map except for two flexible loops of the glutaminase domain (residues 228–239 and 295–299) that include the internal His6-tag. The two GFAT-1 monomers in the asymmetric unit form an asymmetric dimer through direct interactions of the isomerase domains while the glutaminase domains point outward to opposite sides (Fig. 1b).

Structural comparison of human GFAT-1 with its homologs. The human isomerase domain consists of two sugar isomerase (SIS) sub-domains: both are composed of five-stranded parallel β-sheets flanked by two or three α-helices on both sides (Fig. 1c). Overall, the isomerase domain is very similar to the respective structures from E. coli, C. albicans, and the previously published isolated human isomerase domain (Supplementary Fig. 1b). The structure of the glutaminase domain of human GFAT-1 shows a typical N-terminal nucleophile (Ntn) hydrolase fold with two β-sheets composed of seven and five antiparallel β-strands sandwiched between two layers of three α-helices (αβα-core, Fig. 1c). Two short antiparallel β-strands (residues 221–223 (β9) and 303–305 (β14), Fig. 1c, Supplementary Fig. 1a), the latter originating from the linker between the two domains, cover one side of the αβα-core. The overall αβα-core of the human GFAT-1 glutaminase domain is similar to E. coli GlmS, while β-strands and loops connecting the α-helices and β-sheets are more extended in the human enzyme (Supplementary Fig. 1a, c). At least two phosphorylation sites, S235 and S243, are located within...
these extended loops and S243 was found phosphorylated in both mass spectrometry analysis and the crystal structure (Supplementary Fig. 1a, d).

GFAT-1 active sites are conserved from bacteria to humans. GFAT-1 was crystallized in the presence of its substrate Frc6P and the product L-Glu. Corresponding electron density was found in both active sites. At the given resolution, however, we cannot unambiguously determine whether the substrate Frc6P or Glc6P, which is formed in the absence of L-Gln, was present in the isomerase active site. As the presence of the linear sugar suggests isomerization activity and the equilibrium constant for Frc6P-Glc6P-isomerization favors Glc6P formation38, we decided to model the product Glc6P in our crystal structures (Supplementary Fig. 1e). Residues of both isomerase domains of the GFAT-1 dimer contribute to each Glc6P-binding site. Thr376, Ser474, and Lys558 of one monomer, and His577* of the other monomer coordinate the hydroxyl groups of Glc6P through hydrogen bonding. Further hydrogen bonds are formed between the sugar phosphate group and Ser377, Ser421, Gln422, and Thr426.

**Fig. 1 Structure of human GFAT-1, the key enzyme of the hexosamine pathway.** 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 Overall structure of the human GFAT-1 dimer in cartoon representation. The N-terminal glutaminase domains are colored in light blue and light gray, and the C-terminal isomerase domains in marine and dark gray. Glc6P (yellow sticks) and L-Glu (violet sticks) are highlighted, as well as important loops discussed in this manuscript: R-loop (green), Q-loop (red), and C-loop (orange). c Secondary structure elements of human GFAT-1. β-Sheets are colored in red and α-helices in blue. Glc6P (yellow sticks) and L-Glu (violet sticks) are highlighted. The isomerase domain (left) consists of two sugar isomerase (SIS) sub-domains. The glutaminase domain (right) is composed of a Ntn-hydrolase fold (αββα-core) with two short antiparallel β-sheets covering one side (β9 and β14). d, e Active sites of human GFAT-1. The protein is in cartoon representation; residues involved in substrate binding or catalysis are highlighted as sticks, and dashed lines indicate key interactions. d Frc6P-binding site formed by both isomerase domains. e L-Gln-binding site in one glutaminase domain.
Thus, both binding pockets and the catalytic residues are evolutionary conserved from *E. coli* to human GFAT-1, suggesting a similar reaction mechanism as described in bacteria.  

**GFAT-1 forms an asymmetric dimer.** The two crystallographically independent GFAT-1 monomers form a dimer. The dimer is asymmetric because the two glutaminase domains are oriented differently relative to their respective isomerase moieties, which form a symmetric assembly (Figs. 1b and 2a). The position of the glutaminase domain of monomer A is stabilized by crystal contacts. In contrast, the glutaminase domain of monomer B makes no crystal contacts and is more flexible resulting in higher B-factors (Supplementary Fig. 2a) and partially low electron density (Supplementary Fig. 2b). Due to the conformational shift of the glutaminase domains relative to their isomerase domains, the cleft between the two domains is more open in monomer A than in monomer B. This open conformation in monomer A allows conformational changes at the active sites with loop movements, which do not occur in the closed conformation in

(Fig. 1d, Supplementary Fig. 1f). The catalytically relevant residues Lys558, Glu561, His577, and Lys676 are conserved from *E. coli* to humans (Supplementary Fig. 1a)39.

Mass spectrometry analysis of the glutaminase domain revealed that the initial methionine is removed, resulting in a free α-amino group of the catalytic Cys2, as published previously. The product L-Glu is bound to the glutaminase active site in one monomer (monomer A), but is absent in the second monomer (monomer B). In the L-Glu-free state, Cys2 forms hydrogen bonds to Thr94, Asn123, and to the δ-carboxyl-group of L-Glu. L-Glu binding pocket itself is further formed by Arg95, Trp96, Thr98, His99, His108, and Gly124 (Fig. 1e, Supplementary Fig. 1f). The catalytically relevant residues involved in glutamine hydrolysis (Cys2, Asn123, Gly124, and Thr679) are fully conserved (Supplementary Fig. 1a)17,27.  

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### Table 1 Data collection and refinement statistics of wild type GFAT-1.

|                  | GFAT-1 WT w/o Glu +Glc6P | GFAT-1 WT w/o Glu +GlcN6P | GFAT-1 WT w/o Glu +Glc6P +UDP|GFAT-1 WT w/o Glu +GlcN6P +UDP |
|------------------|--------------------------|--------------------------|-----------------------------|-----------------------------|
| Wavelength (Å)   | 0.98                     | 0.98                     | 1.00                        | 1.00                        |
| Resolution range (Å) | 40.13–2.35              | 48.33–2.33               | 48.81–2.50                  | 46.36–2.50                  |
| Space group      | P 4₁ 2₁ 2                | P 4₁ 2₁ 2                | P 4₁ 2₁ 2                   | P 4₁ 2₁ 2                   |
| a, b, c (Å)      | 153.9 153.9 166.3        | 152.8 152.8 165.4        | 152.4 152.4 169.3           | 152.6 152.6 166.5           |
| Total reflections | 1,068,061 (96,281)       | 871,831 (70,057)         | 891,471 (74,962)            | 868,152 (78,008)            |
| Unique reflections | 82,721 (7933)           | 84,017 (8181)            | 81,961 (7811)               | 99,839 (9437)               |
| Multiplicity     | 12.9 (12.1)              | 22.3 (20.8)              | 12.9 (11.1)                 | 9.9 (9.7)                   |
| Completeness (%) | 99.6 (98.8)              | 99.8 (98.8)              | 99.9 (98.9)                 | 99.9 (98.9)                 |
| Mean I/σ(I)      | 18.10 (15.7)             | 18.80 (10.8)             | 20.61 (11.1)                | 19.93 (13.9)                |
| Wilson B-factor  | 61.6 (60.1)              | 60.1                     | 62.8                        | 58.8                        |
| Rmerge (%)       | 8.4 (133.7)              | 11.8 (230.2)             | 10.1 (189.9)                | 9.6 (146.2)                 |
| Rfree (%)        | 8.8 (139.5)              | 12.0 (235.9)             | 10.5 (199.1)                | 10.1 (154.3)                |
| CCfree (%)       | 2.4 (39.6)               | 2.5 (51.1)               | 2.9 (59.3)                  | 3.2 (48.7)                  |
| CCфree (%)       | 99.9 (68.6)              | 99.9 (55.8)              | 99.9 (45.2)                 | 99.9 (55.6)                 |
| Reflections used in refinement | 82,715 (7933) | 83,998 (8181) | 69,156 (6673) | 69,139 (6736) |
| Reflections used for R-free | 1991 (193) | 1927 (185) | 1936 (189) | 1935 (189) |
| Rwork (%)        | 17.5 (25.8)              | 19.0 (31.7)              | 19.4 (31.4)                 | 19.0 (30.7)                 |
| Rfree (%)        | 20.9 (31.5)              | 22.5 (36.9)              | 24.1 (32.9)                 | 24.1 (32.6)                 |
| CCwork (%)       | 96.9 (81.8)              | 96.7 (72.8)              | 95.9 (68.6)                 | 96.4 (71.5)                 |
| CCфree (%)       | 93.2 (71.1)              | 94.2 (62.4)              | 96.6 (70.9)                 | 95.5 (59.4)                 |
| Number of non-hydrogen atoms | 10,552                  | 10,574                   | 10,339                      | 10,481                      |
| Macromolecules   | 10,440                   | 10,440                   | 10,236                      | 10,296                      |
| Ligands          | 32                       | 32                       | 32                          | 112                         |
| Solvent          | 80                       | 102                      | 71                          | 71                          |
| Protein residues | 1323                     | 1321                     | 1297                        | 1304                        |
| RMS (bonds) (Å)  | 0.002                    | 0.002                    | 0.003                       | 0.002                       |
| RMS (angles) (°) | 0.45                     | 0.46                     | 0.49                        | 0.45                        |
| Ramachandran favored (%) | 97                     | 96                       | 96                          | 96                           |
| Ramachandran allowed (%) | 3.2                   | 3.5                      | 4.1                         | 3.6                           |
| Ramachandran outliers (%) | 0.076                 | 0.15                     | 0.23                        | 0.15                          |
| Rotamer outliers (%) | 0.17                  | 0.17                     | 0.27                        | 0.26                          |
| Clashscore       | 0.57                     | 0.72                     | 0.73                        | 0.67                          |
| Average B-factor | 90.33                    | 89.70                    | 103.10                      | 90.04                        |
| Macromolecules   | 90.68                    | 90.13                    | 103.53                      | 90.58                        |
| Ligands          | 60.92                    | 57.45                    | 70.99                       | 66.57                        |
| Solvent          | 57.06                    | 55.88                    | 54.91                       | 49.71                        |
| Number of TLS groups | 4                      | 4                        | 4                           | 4                            |
| PDB code         | 6R4E                     | 6SVO                     | 6R4F                        | 6R4G                         |

*Statistics for the highest-resolution shell are shown in parentheses.*
monomer B. l-Glu was found bound to the glutaminase domain of the open monomer A, while in monomer B no l-Glu was detected (Fig. 1b). In monomer B, Glc6P binds to the isomerase domain and the nine C-terminal residues, the so-called C-loop (Fig. 2b, Supplementary Fig. 2c). The C-loop is stabilized by interactions with the C-loop backbone (Fig. 2b, Supplementary Fig. 2d, e). Although both active sites are occupied by ligands in monomer A, no ammonia channel connecting the glutaminase and the isomerase site is formed. These data suggest that the C-loop covers the glutaminase active site upon l-Glu binding, but as it is the product, it cannot induce the coupling of both active sites by ammonia channel formation. We wondered how the presence of l-Glu and GlcN6P would affect the structure and co-crystallized GFAT-1 in the presence of both product molecules. The GlcN6P-bound structure formed the same asymmetric dimer as the Glc6P-bound structure and a linear sugar was present in the active site (Supplementary Fig. 2f, g, h). Compared to human GFAT-1, the GlcN6P-bound E. coli

Table 2 Data collection and refinement statistics of wild type and point mutant GFAT-1.

|                  | GFAT-1 WT | GFAT-1 G461E | GFAT-1 G461E + UDPGlcNAc | GFAT-1 G451E | GFAT-1 G451E + UDPGlcNAc |
|------------------|-----------|--------------|--------------------------|--------------|--------------------------|
| Wavelength (Å)   | 1.00      | 0.97         | 0.97                     | 1.00         | 1.00                     |
| Resolution range (Å) | 49.15–2.48 | 48.68–2.59   | 48.21–2.72               | 48.98–2.24   | 48.73–2.42               |
| Space group      | P 4 1 2 2 | P 4 1 2 2 | P 4 1 2 2 | P 4 1 2 2 | P 4 1 2 2 |
| a, b, c (Å)      | 152.0 152.0 165.8 | 152.8 152.8 166.0 | 152.5 152.5 164.9 | 154.1 154.1 162.9 | 153.2 153.2 162.5 |
| Total reflections | 601,542 | 613,756 | 464,957 | 690,080 | 992,398 |
| Unique reflections | 68,982 | 61,581 | 52,752 | 93,588 | 93,581 |
| Multiplicity     | 8.7 (8.6) | 10.0 (10.1) | 8.8 (9.0) | 7.4 (7.2) | 13.4 (12.7) |
| Completeness (%) | 99.8 (98.3) | 99.6 (97.2) | 97.6 (98.9) | 99.7 (97.5) | 99.9 (99.9) |
| Mean I/σ (I)     | 14.75 (1.15) | 17.49 (1.41) | 16.86 (1.44) | 16.87 (1.54) | 18.36 (1.54) |
| Wilson B-factor  | 57.5      | 65.0        | 71.5        | 45.7         | 49.7         |
| Rmerge (%)       | 11.0 (157.4) | 9.7 (144.5) | 10.0 (142.9) | 8.0 (109.8) | 12.9 (163.2) |
| Rfree (%)        | 11.7 (167.4) | 10.2 (152.2) | 10.6 (151.7) | 8.6 (118.3) | 13.4 (170.0) |
| Rwork (%)        | 3.9 (56.1)  | 3.2 (47.0)  | 3.5 (50.2)  | 3.2 (43.5)  | 3.6 (47.4)  |
| Rfree (%)        | 99.9 (49.6) | 99.9 (54.1) | 99.9 (54.9) | 99.9 (62.7) | 99.9 (59.9) |
| CC1/2 (%)        | 100 (81.5) | 100 (83.8) | 100 (84.2) | 100 (87.8) | 100 (86.5) |
| Reflections used in refinement | 68,971 (6701) | 61,566 (5916) | 52,734 (5145) | 93,581 (9029) | 74,006 (7226) |
| Reflections used for R-free | 1935 (177) | 1967 (181) | 1940 (209) | 1976 (192) | 1991 (195) |
| Rwork (%)        | 18.9 (29.3) | 19.2 (29.2) | 20.5 (30.6) | 18.8 (27.1) | 19.4 (27.9) |
| Rfree (%)        | 22.2 (33.5) | 21.9 (31.1) | 23.4 (31.6) | 20.4 (31.8) | 22.3 (30.9) |
| CC1/2 (%)        | 96.1 (71.8) | 96.2 (73.5) | 95.2 (70.1) | 96.5 (78.3) | 95.9 (77.8) |
| CCfree (%)       | 94.5 (47.9) | 93.9 (66.5) | 93.6 (68.9) | 96.0 (70.9) | 94.9 (79.8) |
| Number of non-hydrogen atoms | 10,565 | 10,463 | 10,270 | 10,617 | 10,570 |
| Macromolecules   | 10,390 | 10,367 | 10,228 | 10,454 | 10,332 |
| Ligands          | 112    | 32    | 32    | 32    | 112    |
| Solvent          | 63     | 64    | 10    | 131   | 126    |
| Protein residues | 1316   | 1310   | 1292   | 1320   | 1304   |
| RMS (bonds) Å    | 0.003  | 0.002  | 0.002  | 0.003  | 0.002  |
| RMS (angles) °   | 0.48   | 0.45   | 0.44   | 0.53   | 0.44   |
| Ramachandran favored (%) | 96 | 96 | 96 | 98 | 97 |
| Ramachandran allowed (%) | 3.8 | 3.7 | 4.1 | 2 | 2.4 |
| Ramachandran outliers (%) | 0.077 | 0.15 | 0 | 0.15 | 0.23 |
| Rotamer outliers (%) | 0.087 | 0.17 | 0.089 | 0.17 | 0.088 |
| Clausescor        | 0.48   | 0.72   | 0.83   | 0.67   | 0.77   |
| Average B-factor  | 87.35  | 109.55  | 115.46  | 70.93  | 78.97  |
| Macromolecules   | 87.83  | 109.98  | 115.69  | 71.37  | 79.63  |
| Ligands          | 64.10  | 78.68   | 62.07   | 44.41  | 54.72  |
| Solvent          | 49.35  | 56.18   | 53.11   | 42.56  | 46.88  |
| Number of TLS groups | 4   | 4    | 4    | 4    | 4    |
| PDB code         | 6SVM   | 6R4I    | 6SVQ    | 6R4H   | 6R4J   |

Statistics for the highest-resolution shell are shown in parentheses.
structure shows the pyranose ring GlcN6P in the active site and no electron density for the glutaminase domain is observed, although the full-length protein was crystallized. While in the E. coli structure the product might have already left the active site, destabilizing the glutaminase domain, our structure represents the last step of catalysis, just before departure of the GlcN6P product.

**Structural alterations in the absence of glutamate.** Given that the occupancy of the glutaminase site by L-Glu is linked to the conformation of the Q- and R-loops, we wanted to know the conformation of these loops in the absence of L-Glu. Since crystallization without glutamate yielded poorly diffracting crystals, we crystallized GFAT-1 in the presence of glutamate and subsequently removed it in several dilution steps.

The removal of glutamate, as indicated by the electron density (Supplementary Fig. 3a), leads to major changes in the glutaminase domain of the previously L-Glu-bound monomer A (Fig. 3a): first, lower electron density and higher B-factors of the Q-loop indicate higher flexibility and a destabilization of this loop at the glutaminase site (Fig. 3b, Supplementary Fig. 3b). This is consistent with the observation that residues of the Q-loop stabilize glutamate binding. Second, the R-loop changes orientation with large side chain movements of Arg33 and Tyr35 (Fig. 3a, c). In the presence of glutamate, Arg33 points to the cleft between both domains and forms a salt bridge with Asp262 of the glutaminase domain. In the absence of glutamate, however, Arg33 is rotated towards the isomerase domain and forms a salt bridge with Asp667 of the isomerase domain. The Arg33 movement frees space between Tyr32 and the catalytically active Cys2, which is filled by Tyr35 (Fig. 3c). Third, in the absence of glutamate, the C-loop could not be modeled due to the lack of electron density indicating its high flexibility (Fig. 3a, b). However, the removal of glutamate did not affect the conformation of monomer B. Together, the glutamate-free structure of monomer A suggests a communication of the glutaminase site through Arg33 with the isomerase domain.

**Interaction of UDP-GlcNAc and UDP-GalNAc with human GFAT-1.** The main distinctive feature between prokaryotic and eukaryotic GFAT is the feedback inhibition by the HP product UDP-GlcNAc, which inhibits the glutaminase function. To mechanistically understand GFAT-1 feedback inhibition, we next analyzed UDP-GlcNAc interaction with GFAT-1. Co-crystallization of GFAT-1 with UDP-GlcNAc did not yield well-diffacting crystals. However, we successfully soaked UDP-GlcNAc into the human GFAT-1 crystals and analyzed the crystal structure. UDP-GlcNAc was bound to both GFAT-1 monomers...
in the asymmetric unit. As shown previously, UDP-GlcNAc binds with a cation to the GFAT-1 isomerase domain (Fig. 4a, Supplementary Fig. 4a, b)\(^3\). UDP-GlcNAc binding is stabilized by hydrogen bonds to Gly355, Thr447, Thr458, Gly461, as well as ionic interactions between the pyrophosphates and Arg343 and His463 (Fig. 4c, Supplementary Fig. 4b). Additionally, we identified hydrogen bonds between UDP-GlcNAc and Gln310 of the interdomain linker in monomer A (Fig. 4c, Supplementary Fig. 4b). These were not observed in monomer B due to an increased distance caused by the shift of the glutaminase domain relative to the isomerase domain (Fig. 2a) and poor electron density of Gln310. Binding of UDP-GlcNAc induced local side chain reorientations of His463 and Asn465 in both monomers (Fig. 4d). While the UDP moiety of UDP-GlcNAc interacts with the isomerase domain through multiple amino acid residues, there are only two interactions of the \(N\)-acetylglucosamine moiety with the protein (Fig. 4c, Supplementary Fig. 4b). To test the role of the sugar in GFAT-1 inhibition, we used the UDP-GlcNAc epimer uridine 5′-diphospho-\(N\)-acetyl-\(D\)-gallactosamine (UDP-GalNAc), which differs only in the orientation of the hydroxyl group at C4 of the sugar ring that is pointing towards the interdomain linker. UDP-GalNAc was detected in the UDP-GlcNAc binding site after crystal soaking and induced the same side chain movements (Fig. 4e, Supplementary Fig. 4c–e). To investigate its role in GFAT-1 inhibition, we next analyzed GFAT-1 inhibition in activity assays.

**GFAT-1 activity and feedback inhibition.** To analyze the activity of GFAT-1 in vitro, we used activity assays for each domain: \(L\)-Glu production by the glutaminase domain was measured by a coupled activity assay with glutamate dehydrogenase (GDH). \(D\)-GlcN6P synthesis of the isomerase domain was monitored using a coupled activity assay including glucosamine-6-phosphate \(N\)-acetyltransferase (GNA-1), the second enzyme of the HP (Supplementary Fig. 4f). In kinetic measurements, we observed both \(L\)-Gln hydrolysis and \(D\)-GlcN6P synthesis (Table 3, Fig. 4f, Supplementary Fig. 4g). These numbers are in accordance with previous studies\(^4\). The GDH-coupled glutaminase domain activity assay was used to characterize UDP-GlcNAc and UDP-GalNAc effects. As a negative control we generated a GFAT-1 variant that prevents UDP-GlcNAc binding by mutating Gly461, which is located at the bottom of the UDP-GlcNAc-binding pocket (Fig. 4c). Indeed, UDP-GlcNAc did not bind to the G461E mutant of GFAT-1 after crystal soaking (Supplementary Fig. 4h). Kinetic measurements showed that GFAT-1 G461E has similar kinetic properties like wild type GFAT-1 for \(L\)-Glu and \(D\)-GlcN6P synthesis (Table 3, Supplementary Fig. 4g, i). In vitro, wild type human GFAT-1 was inhibited by UDP-GlcNAc in a dose-dependent manner with an IC\(_{50}\) of 43.3 (±2.3/±2.5) \(\mu\)M (Fig. 4g). GFAT-1 G461E did not respond to UDP-GlcNAc treatment, as expected from the crystal structure (Supplementary Fig. 4j). Of note, in wild type GFAT1, UDP-GalNAc showed an inhibitory effect only at much higher doses compared to UDP-GlcNAc.
Taken together, GFAT-1 was responsive to UDP-GlcNAc and feedback inhibition of UDP-GlcNAc involves a critical interaction between the sugar’s C4 hydroxyl group and the interdomain linker.

Inhibition of regulatory feedback by a single point mutation. Having solved the structure of GFAT-1, we aimed to understand the mechanism of GFAT-1 activation through a single amino acid substitution (G451E) that elevated HP flux and extended lifespan in C. elegans. Positioned in an evolutionary conserved region of the isomerase domain, Gly451 is in close proximity to the UDP-GlcNAc-binding site, suggesting that the mutation might interfere with UDP-GlcNAc inhibition (Supplementary Fig. 1a, 5a). Crystal structure analysis of GFAT-1 G451E revealed no major structural changes compared to wild type GFAT-1 (Fig. 5a, b).
Fig. 4 GFAT-1 inhibition by UDP-GlcNAc and UDP-GalNAc. a–d Structural analysis of UDP-GlcNAc binding to wild type-GFAT-1. Proteins are presented as cartoons. UDP-GlcNAc-bound GFAT-1 is depicted in green and UDP-GlcNAc-free GFAT-1 is colored in purple. Glc6P (yellow sticks), L-Glu (violet sticks), UDP-GlcNAc (white sticks), and Mg$^{2+}$ (cyan sphere) are highlighted. a Stereo image of GFAT-1 monomer A. The 2Fo–Fc maps (blue) of the ligands have a contour level of 1.0 RMSD. b Superposition of UDP-GlcNAc-free and UDP-GlcNAc-bound GFAT-1. c UDP-GlcNAc binding site at the isomerase domain. Binding is mediated by residues of the isomerase domain, as well as interactions with Gln310 from the interdomain linker region. Residues involved in UDP-GlcNAc binding are highlighted as sticks, and dashed lines indicate the most important interactions. d Locally occurring side chain movements upon UDP-GlcNAc binding. e Superposition of UDP-GlcNAc-binding site at the GFAT-1 isomerase domain in the presence of UDP-GlcNAc (green structure) or UDP-GalNAc (blue structure). UDP-GlcNAc (white sticks), UDP-GalNAc (black sticks), and Mg$^{2+}$ (cyan sphere) are highlighted. f L-Gln kinetic of wild type (WT, black circle) GFAT-1 (mean ± SEM, n = 5). g Representative UDP-GlcNAc (black circle) and UDP-GalNAc (blue triangle) inhibition of wild type GFAT-1 (mean ± SD, n = 5). Table: IC$_{50}$ UDP-GlcNAc values (mean ± SEM, n = 3). Source data are provided as a Source Data file.

### Table 3 Kinetic parameters.

|                  | L-Glu production | D-GlcN6P production |
|------------------|------------------|---------------------|
|                  | $k_m$ [mM]      | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [mM$^{-1}$ s$^{-1}$] | $k_m$ Frc6P [mM] | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [mM$^{-1}$ s$^{-1}$] |
| Wild type        | 1.1 ± 0.2        | 3.6 ± 0.2           | 3.3                                  | 0.08 ± 0.01 | 1.7 ± 0.1 | 21.3                                  |
| G461E            | 0.5 ± 0.1        | 3.1 ± 0.1           | 6.2                                  | 0.09 ± 0.01 | 2.1 ± 0.1 | 23.3                                  |
| G451E            | 1.3 ± 0.2        | 2.2 ± 0.1           | 1.7                                  | 0.04 ± 0.01 | 0.8 ± 0.04 | 20.0                                  |
| Q307A            | 0.6 ± 0.04       | 2.6 ± 0.04          | 4.3                                  | 0.15 ± 0.01 | 2.1 ± 0.1 | 14.0                                  |
| Q307A/G451E      | 1.0 ± 0.05       | 2.1 ± 0.03          | 2.1                                  | 0.05 ± 0.01 | 1.1 ± 0.04 | 22.0                                  |

Wild type, G461E, and G451E: mean ± SEM, n = 5; Q307A and Q307A/G451E: mean ± SEM, n = 3

Notably, Glu451 forms a new hydrogen bond to Gln307 of the interdomain linker in the mutant structure (Fig. 5b). Analysis of the kinetic properties of the G451E mutant revealed a comparable $K_m$ for L-Gln-hydrolysis but had a reduced $k_{cat}$ compared to the wild type enzyme (Table 3, Fig. 5c). Furthermore, the $k_{cat}/K_m$ ratio for n-GlcN6P synthesis is comparable to the wild type, although both $K_m$ and $k_{cat}$ are smaller (Table 3, Supplementary Fig. 5e, f). Unexpectedly, Q307A showed a higher sensitivity to UDP-GlcNAc compared to G451E (Table 3, Supplementary Fig. 5d) at the high concentrations employed in this experiment. UDP-GlcNAc also induced the local side chain movements described above for the wild type within the binding pocket (Fig. 5). To further analyze the role of the additional hydrogen bond between G451E and Gln307 of the interdomain linker, we characterized the mutant Q307A and the double-mutant Q307A/G451E. Q307A showed similar kinetic properties as wild type, while the kinetic parameters of Q307A/G451E were similar to G451E (Table 3, Supplementary Fig. 5e, f). Unexpectedly, Q307A showed a higher sensitivity to UDP-GlcNAc compared to wild type (Fig. 5d). In contrast to the G451E variant, the double mutant Q307A/G451E was inhibited at a lower UDP-GlcNAc dose (Fig. 5d). We conclude that GFAT-1 gain-of-function in the G451E variant results from a loss of regulation by UDP-GlcNAc-mediated feedback inhibition. Moreover, the increased UDP-GlcNAc sensitivity of the interdomain linker mutant Q307A again points to a critical role of the interdomain linker in UDP-GlcNAc inhibition.

### Evolutionary conservation of GFAT-1

As UDP-GlcNAc inhibition is a feature of eukaryotic GFAT-1, we analyzed the evolutionary conservation of the interdomain linker from bacteria to higher organisms. To this end, we generated a sequence alignment comparing eukaryotic and prokaryotic GFATs and used the ConSurf server to highlight conserved regions within the structure. Quite unsurprisingly, both isomerase and glutaminase active sites are fully conserved between pro- and eukaryotes (Fig. 6a). In contrast, the interdomain linker showed a high heterogeneity within the prokaryotes, while the eukaryotic interdomain linker was well conserved (Fig. 6b). This further supports a key role of the interdomain linker in UDP-GlcNAc-mediated GFAT-1 inhibition.

Loss of feedback inhibition activates GFAT-1 in vivo. Understanding GFAT-1 gain-of-function by a specific point mutation, we next assessed the relevance of this mutation in mammalian cells. For this, we introduced the GFAT-1 G451E substitution in N2a mouse neuroblastoma cells by editing the endogenous locus using CRISPR/Cas9. Two independent cell lines carrying the homozygous GFAT-1 mutation were generated (Fig. 7a, Supplementary Fig. 6a). Given that HP activation confers strong tunicamycin resistance in C. elegans, we assessed cellular survival upon tunicamycin treatment in the engineered N2a cells. Compared to wild type control cells, both GFAT-1 G451E lines were resistant to tunicamycin (Fig. 7b, Supplementary Fig. 6b). Further, we measured absolute levels of UDP-GlcNAc and UDP-GalNAc (together UDP-HexNAc) from cellular extracts by liquid chromatography coupled mass spectrometry. Both GFAT-1 G451E lines showed markedly increased steady-state levels of UDP-HexNAc, compared to the wild type control line (Fig. 7c, Supplementary Fig. 6c). To rule out that elevated UDP-GlcNAc levels were a consequence of increased GFAT-1 expression in the mutant cells, we quantified mRNA and protein. We found that GFAT-1 expression was decreased upon the G451E mutation (Fig. 7d–f, Supplementary Fig. 6d–f), indicating that the gain-of-function resulted from constant GFAT-1 activity due to the lack of feedback inhibition in vivo.

### Discussion

Here we present the full-length crystal structure of human GFAT-1 and present mechanistic insights into its regulation that affects protein homeostasis though the HP. We performed soaking experiments with the physiological inhibitor UDP-GlcNAc and its epimer UDP-GalNAc. Both bind to the GFAT-1 isomerase...
domain, leading to local structural changes. However, we did not observe any conformational changes at the glutaminase site, which would explain the inhibition of amidohydrolysing activity. Interestingly, while UDP-GlcNAc was a potent GFAT-1 inhibitor, its closely related epimer UDP-GalNAc emerged as a weak GFAT-1 inhibitor. The epimers differ only in the orientation of the sugar’s C4 hydroxyl group, suggesting that GFAT-1 differentiates between the epimers with high fidelity. 

**Fig. 5 GFAT-1 gain-of-function mutation perturbs UDP-GlcNAc inhibition.** a, b Effect of G451E mutation on GFAT-1 structure. Proteins are presented as cartoons. Superposition of wild type GFAT-1 (light gray/dark gray) and G451E GFAT-1 (yellow/orange). Glc6P (yellow sticks) and L-Glu (violet sticks) are highlighted. a G451E (sticks) is located at the isomerase domain of GFAT-1 pointing towards the interdomain linker (black box). b Superposition of the wild type and the G451E GFAT-1 structure focusing on residues in close proximity to the mutation. c L-Gln kinetic of wild type (WT, black circle) and G451E (red triangle) GFAT-1 (mean ± SEM, n = 5). d Representative UDP-GlcNAc inhibition of wild type (black circle), G451E (red triangle), Q307A (blue triangle), and Q307A/G451E (green square) GFAT-1 (mean ± SD, n = 3). Table: IC50 UDP-GlcNAc values (mean ± SEM, n = 3). e, f Superposition of UDP-GlcNAc-bound G451E GFAT-1 (yellow/orange) and wild type GFAT-1 in the absence of UDP-GlcNAc (gray). Proteins are presented as cartoons. Glc6P (yellow sticks), L-Glu (violet sticks), UDP-GlcNAc (white sticks), Mg2+ (cyan sphere), and G451E (sticks) are highlighted. e Overall structure with Fo–Fc omit map (green) of UDP-GlcNAc binding to G451E GFAT-1 at a contour level of 3.0 RMSD. f Close-up of the UDP-GlcNAc-binding pocket with local side chain movements in G451E GFAT-1. Source data are provided as a Source Data file.
The open conformation observed in monomer A, L-Glu was bound to the isomerase and glutaminase domain relative to each other. In the potential importance for the regulation of eukaryotic GFAT.

Similar residues. Source data are provided as a Source Data file.

gain-of-function mutation, G451E, which causes HP activation and longevity in C. elegans, revealed that gain-of-function results from a drastically reduced sensitivity to UDP-GlcNAc inhibition. Finally, we showed that the gain-of-function mechanism leads to elevated HP flux in mammalian cells.

While the isomerase domain of the full-length structure is very similar to previously crystallized bacterial and eukaryotic isomerase domains, our structure of the eukaryotic glutaminase domain revealed major differences in extended β-sheets and longer loops connecting α-helices and β-sheets. These differences might allow for the design of antibacterial drugs targeting the glutaminase domain. Two phosphorylation sites, S235 and S243, are positioned within these extended loops, indicating their potential importance for the regulation of eukaryotic GFAT.

We observed an asymmetric GFAT-1 dimer, which allowed the characterization of an open and closed conformation of the isomerase and glutaminase domain relative to each other. In the open conformation observed in monomer A, L-Glu was bound to GFAT-1, which stabilizes an inactive orientation of the catalytic Cys2 pointing away from the active site, as well as a Q-loop movement closing the glutaminase site and inducing a rotation of Trp96. Previously, the Q-loop movement was reported for the E. coli enzyme, where after binding of the competitive inhibitor DON, an analog of the glutamine substrate, the rotation of Trp96 equivalent Trp74 opens the ammonia channel. In contrast, no ammonia channel was formed after L-Glu binding in our structure. These data suggest that L-Glu binding induces some changes similar to L-Gln binding, but as it is the product and not substrate of the reaction, it switches Cys2 to a catalytic incompetent rotamer and does not provoke the formation of the ammonia channel. Although L-Glu was present in high excess, it was not detected in monomer B, where the Q-loop does not seal the glutaminase active site. Here, the L-Glu product has probably diffused out of the active site owing to its greater accessibility to the solvent space.

Moreover, we describe a glutamate-free GFAT-1 structure where the R-loop at the glutaminase site of monomer A showed major structural changes, flipping into the cleft between glutaminase and isomerase site. The conserved R-loop arginines were previously suggested to be key elements in maintaining a functional glutaminase site and to be involved in interdomain communication in bacterial GFAT and other Ntn-hydrolases (PURF, ASNB)17,27,44,45. In E. coli residue Arg26, which is the equivalent of Arg33 in the human enzyme, is thought to keep Cys2 in an active position pointing towards the active site after glutamine binding and was suggested to mediate communication between the two active sites. Our study reveals structural evidence for a direct interaction of Arg33 from the glutaminase site with Asp667 of the isomerase domain, which is in close proximity to the catalytically important C-loop (residues 670–681). Presumably, the salt bridge between Arg33 and Asp667 keeps the two domains in an open conformation. After disruption of the salt bridge by reorientation of the R-loop upon glutamine binding, the cleft could close. This allows the C-loop to interact with both active sites, which is necessary for catalysis. Thereby, the R-loop might signal the presence of substrate in the glutaminase active site to the isomerase domain. Taken together, these results clearly point to an important role of Arg33 in interdomain communication during the GFAT-1 catalytic cycle.

To understand GFAT-1 regulation, UDP-GlcNAc and UDP-GalNAc were soaked into GFAT-1 crystals. We detected binding to both monomers and observed local structural changes at the binding site of the isomerase domains. However, binding had no consequences at the glutaminase site. The two active sites of GFAT-1 are coupled and GFAT-1 function depends on the communication and relative orientation of the two domains. For the bacterial homolog GlnS, it is reported that the glutaminase domain adopts a fixed position relative to the isomerase domain upon Frc6P binding, and that the glutaminase function is activated in the presence of Frc6P by 100-fold27,41. The Frc6P-dependent activation of the glutaminase activity was also reported for the C. albicans homolog44. It is therefore very likely that this also happens in the human enzyme. We propose that UDP-GlcNAc could disturb the tight coupling of the active sites by interference with the orientation of the two domains relative to each other. There are several lines of evidence that support a key role of the interdomain linker in this process. First, activity assays comparing UDP-GlcNAc and UDP-GalNAc inhibition revealed that the orientation of the sugar’s C4 hydroxyl group, which is positioned to interact with the interdomain linker, is sufficient to modulate the inhibition (Fig. 7g). Second, in the G451E gain-of-function mutant, which showed a reduced sensitivity to UDP-GlcNAc-dependent feedback inhibition, Glu451 interacts with Gln307 from the interdomain linker and the Q307A mutant showed an increased sensitivity to UDP-GlcNAc. Third, while both active sites are highly conserved from prokaryotes to eukaryotes, the interdomain linker is only conserved among eukaryotes, whose GFAT-1 is susceptible to UDP-GlcNAc inhibition. Fourth, previous publications indicated a specific role of the N-acetyl moiety of UDP-GlcNAc in inhibition, which points towards the interdomain linker: Assirri et al.33 reported that UDP and UDP-Glc do not inhibit GFAT-1, but are able to bind with a similar KD as for UDP-GlcNAc. Moreover, Walter et al.46 generated metabolic chemical reporters with large azide- or alkyne-
residues at the N-acetyl position, which also failed to inhibit GFAT-146. Together, these publications emphasize the role of the N-acetyl group in inhibition whose only possible interaction partner are residues from the interdomain linker, especially Gln307 (Fig. 7g). Taken together, our current study suggests a previously unknown role of the interdomain linker in UDP-GlcNAc inhibition.

While the orientation of the two domains with their respective active sites is relevant for catalysis, the correct orientation of the UDP-GlcNAc-bound isomerase and the interdomain linker seem to be important for UDP-GlcNAc inhibition. In our mutants, we observed altered sensitivities to UDP-GlcNAc, which we interpret as follows: in the gain-of-function mutant G451E, which showed a reduced sensitivity to UDP-GlcNAc inhibition, the hydrogen bond between Glu451 and Gln307 stabilizes the linker and both residues might function as spacers between the interdomain linker and the UDP-GlcNAc-bound isomerase domain, preventing a close interaction. Furthermore, the G451E mutation might interfere with UDP-GlcNAc binding as inhibition can only be observed at very high concentrations. The enhanced sensitivity to

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**Fig. 7** The G451E substitution activates GFAT-1 in mammalian cells. a Sanger sequencing results of wild type (WT) and G451E genomic Gfpt1 engineered in mouse neuroblastoma cells (N2a). b Cell viability (XTT assay) of WT and GFAT-1 G451E-engineered N2a cells after a 48 h treatment with 0.5 µg/ml tunicamycin (TM) (mean + SEM, n = 3, **p < 0.01, unpaired t-test). c UDP-HexNAc level in WT and GFAT-1 G451E N2a cells (mean + SEM, n ≥ 25, ***p < 0.001, unpaired t-test). d Relative Gfpt1 mRNA-level (qPCR) of GFAT-1 G451E N2a cells normalized to WT controls (mean + SEM, n = 3). e, f Western blot analysis of endogenous GFAT-1 protein levels from WT and GFAT-1 G451E N2a cells (e), including quantification relative to tubulin and the WT control cells (f, mean + SD, n = 4, *p < 0.05, unpaired t-test). g Close-up of the interdomain linker and UDP-GlcNAc-binding site in GFAT-1 monomer A. The protein is presented as a cartoon. The isomerase domain is colored in dark gray and the glutaminase domain is colored in light gray. UDP-GlcNAc (white sticks) and UDP-GalNAc (black sticks) are superimposed. Residues of the interdomain linker (sticks) and the position of G451 (red star) are highlighted. The NAc and C4 hydroxyl groups of UDP-GlcNAc/UDP-GalNAc are pointing towards the interdomain linker (black boxes). Source data are provided as a Source Data file.
UDP-GlcNac of mutant Q307A might be due an increased flexibility of the interdomain linker and the smaller side chain would allow a closer interaction of the interdomain linker with the UDP-GlcNac-bound isomerase domain. Interestingly, the double-mutant Q307A/G451E showed a ten times weaker response to UDP-GlcNac than the wild type, thus indicating that the G451E substitution might be sufficient to disturb inhibition even in the presence of mutation Q307A. In the double mutant, the Glu451 side chain might disturb UDP-GlcNac binding and prevent a close interaction between the UDP-GlcNac-bound isomerase domain with the interdomain linker.

In mammalian cells, loss of regulation by UDP-GlcNac feed-back inhibition upon G451E mutation constitutively activated GFAT-1, which increased HP flux. Similar to the situation in the nematode C. elegans, this increased HP flux goes along with elevated tolerance to tunicamycin-induced ER stress. Notably, GFAT-1 protein and mRNA levels were reduced in the mutant compared to wild type control cells, demonstrating that the observed increase in HP flux stems from higher GFAT-1 activity, not abundance. Since UDP-GlcNac is nearly completely inhibited by GFAT-1 G451E activity in vitro is not observed increase in HP.

**Methods**

**Plasmids and site-directed mutagenesis.** An internal His6-tag was introduced into human GFAT-1 isoform 2 in between Gly299 and Asp300 in a FLAG-HA-hGFAT-1-pcDNA3.1 plasmid (pcDNA3.1+; Thermo Fisher Scientific; v79020) by site-directed mutagenesis using the following primers: hGFAT1-299-His6_for (5'-ctgcgtcctgccgttcatgccga-3') and hGFAT1-299-His6_rev (5'-gtcccggtgtggttatctgcgagcagtcg-3').

The hGFAT-1 gene with internal His6-tag was subsequently subcloned into the pFL vector using xhoI and HindIII entry sites. The mutations Q307A, G451E, and G461E were introduced into pFL-HA-GFAT1-His299 by site-directed mutagenesis as described above. Mutants were then confirmed by sequencing. In the double mutant, the G451E substitution might be sufficient to disturb interaction of the interdomain linker with the UDP-GlcNac-bound isomerase domain as a search model. After a first round of autobuilding using the ARP/wARP Web Service, GFAT-1 was further manually built using COOT and iterative refinement rounds were performed using phenix.refine. One of the glutaminase domains (chain B) was not well defined in the structure. After placing initial strads, the domain was completed by superposition with the glutaminase domain of chain A. Structures of GFAT-1 variants and UDP-GlcNac/UDP-GaINac soaked crystals were solved by molecular replacement using the full-length GFAT-1 structure as a search model. Geometry constraints for ligands were generated with phenix.elbow software or the Grade Web Server. Structures were visualized using PyMOL (Schrödinger) and 2D ligand-protein interaction diagrams were generated using LigPLOT.
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 match and protein false discovery rate of 0.01.
The “second peptide” option was on. Extracted ion chromatograms were generated
using Qual Browser version 2.2. Data visualization was done using ggplot2.

GDH-coupled activity assay and UDP-GlcNAc inhibition. GDPAT’s amidohy-
drolase activity was measured with a coupled enzymatic assay using bovine glu-
tamate dehydrogenase (GDH, Sigma Aldrich, G2626) in 96-well standard
microplates (F-bottom, BRAND #781602) as described previously30 with small
modifications. In brief, the reaction mixtures contained 6 mM Fru6P, 1 mM APAD,
1 mM EDTA, 50 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
εmax,APADH = 9100 mol·cm−1·mol−1.
Reaction rates were determined by Excel (Microsoft) and Km, Vmax, and Kfco
were obtained from Michaelis Menten or dose response curves, which were
fitted by Prism 7 or 8 software (Graphpad).

GNA1 cloning. In order to receive a second Ndel restriction site, a silent muta-
tion, H77H, was introduced into human GNA1 in the FLAG-HA-hGNA1-
cDNA3.1 plasmid by site-directed mutagenesis48 (primers: hGNA1-H77H_for
5′-cttttgagcaCatgaagaaatctgggg, hGNA1-H77H_rev 5′-cttcatGtgctcaaaagatttcataaattgttc).
Subsequently, GNA1 was cloned into the pET28a expression vector (Merck
Millipore) using Ndel restriction sites, a silent muta-
tion was engineered in N2a cells using the CRISPR/Cas9 technology
of formed APADH was calculated with
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Author contributions

S.R., U.B. and M.S.D. designed the project. S.R. performed all biochemical and crystallization experiments. M.H. performed all experiments related to CRISPR/Cas9 and mammalian cell culture. C.P. supported the biochemical and crystallization experiments. K.A. helped with the tissue culture experiments. S.R., M.H., M.S.D. and U.B. wrote the manuscript. S.R. prepared the figures.

Competing interests

The authors declare no competing interests.

Additional information

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