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The *Drosophila* Neurally Altered Carbohydrate Mutant Has a Defective Golgi GDP-fucose Transporter*

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Background: The defect underlying reduced HRP epitope expression in *Drosophila nac*[1] mutants has not been identified.
Results: *nac*[1] flies have a defective GDP-fucose transporter.
Conclusion: The defective *nac*[1] transporter cannot support normal *N*-glycan core fucosylation, leading to reduced HRP epitope expression.
Significance: *nac*[1] flies are a valid model for the human congenital disorder of glycosylation, CDG-IIc, which is caused by a similar molecular defect.

Studying genetic disorders in model organisms can provide insights into heritable human diseases. The *Drosophila* neurally altered carbohydrate (*nac*) mutant is deficient for neural expression of the HRP epitope, which consists of *N*-glycans with core α1,3-linked fucose residues. Here, we show that a conserved serine residue in the Golgi GDP-fucose transporter (GFR) is substituted by leucine in *nac*[1] flies, which abolishes GDP-fucose transport *in vivo* and *in vitro*. This loss of function is due to a biochemical defect, not to destabilization or mistargeting of the mutant GFR protein. Mass spectrometry and HPLC analysis showed that *nac*[1] mutants lack not only core α1,3-linked, but also core α1,6-linked fucose residues on their *N*-glycans. Thus, the *nac*[1] *Gfr* mutation produces a previously unrecognized general defect in *N*-glycan core fucosylation. Transgenic expression of a wild-type *Gfr* gene restored the HRP epitope in neural tissues, directly demonstrating that the *Gfr* mutation is solely responsible for the neural HRP epitope deficiency in the *nac*[1] mutant. These results validate the *Drosophila nac*[1] mutant as a model for the human congenital disorder of glycosylation, CDG-IIc (also known as LAD-II), which is also the result of a GFR deficiency.

Congenital disorders of glycosylation (CDGs)[2] are a phenotypically diverse group of heritable diseases caused by mutations in genes functioning in glycosylation (recently reviewed in Refs. 1–3). The first congenital disorders that were recognized to have altered glycosylation patterns were identified in humans (4–7). These disorders were later found to involve deficiencies in *N*-glycan biosynthesis and processing (8–11) that were caused by mutations in genes encoding those functions (12–15).

The first CDG reported in a non-human organism was *nac* (neurally altered carbohydrate), which was identified in *Drosophila*. *nac* mutants have reduced levels of a neural carbohydrate epitope that is produced by α1,3-linkage of a fucose residue to the *N*-glycan core (16–18). Due to its identification as a dominant epitope in the plant glycoprotein horseradish peroxidase, this core α1,3-fucosylated *N*-glycan is also known as the horseradish peroxidase (HRP) epitope (19, 20).

In *Drosophila,* the HRP epitope is expressed mainly in the central nervous system (CNS) (21–23), where it is produced by a fucosyltransferase designated FucTA (24–26). FucTA is a Golgi-resident enzyme that transfers fucose from the donor substrate GDP-fucose to the proximal *N*-acetylglucosamine residue of *N*-glycans (Fig. 1*A*). GDP-fucose is produced in the cytoplasm (27) and transported into the Golgi apparatus by GFR, a specific GDP-fucose transporter, in exchange for GMP (Fig. 1*A*) (28, 29). The *Drosophila Gfr* gene is homologous to the human *GFR* gene, which is defective in a congenital disorder of glycosylation known as CDG-IIc and also known as Type II leukocyte adhesion deficiency (LAD-II) (30, 31) or SLC35C1-CDG (32).

The original *nac* mutant, which was later redesignated *nac*[1] to distinguish it from other alleles, has a temperature-insensitive loss of the neural HRP epitope associated with other cold-sensitive phenotypes expressed at 18 °C but not 25 °C (33, 34). Katz *et al.* (33) cytogenetically mapped the *nac*[1] mutation to the region between 84F4 and 84F11-12, which includes about 32 genes. Subsequent work showed that the *Gfr* gene, which encodes a Golgi GDP-fucose transporter, is located in this region (28). This finding hinted that a *Gfr* mutation might be responsible for *nac*[1] because a defect in the ability to transport GDP-fucose into the Golgi apparatus could account for the reduced neural HRP epitope in the *nac*[1] fly. This speculation was strengthened by data showing that homozygous *Gfr* knock-out flies have temperature-sensitive

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[2] The abbreviations used are: CDG, congenital disorder of glycosylation; ER, endoplasmic reticulum; ESI, electrospray ionization; NST, nucleotide sugar transporter; RP-HPLC, reverse phase HPLC; eGFP, enhanced GFP; UAS, upstream activating sequence.

Notch-like wing phenotypes (35), which are similar to the temperature-sensitive scalloped wing phenotype observed in the *nac*[1] mutant (34). However, neither the gene(s) mutated in *nac*[1] flies nor the precise nature of the mutation have been elucidated. Thus, we examined the *Gfr* gene in the *Drosophila nac*[1] mutant and found that *nac*[1] flies have a mutant *Gfr* gene, which encodes a defective Golgi GDP-fucose transporter that is solely responsible for its neural HRP epitope deficiency.

EXPERIMENTAL PROCEDURES

Genomic DNA Analysis—nac[1] homozygous flies were obtained from the Bloomington *Drosophila* Stock Center (Indiana University) and maintained at 28 °C. Genomic DNA was extracted from a single adult fly, as described previously (36). Briefly, the fly was homogenized in a lysis buffer containing RNase A, and the homogenate was incubated at 55 °C for 1 h. The lysate was briefly centrifuged to remove debris, and the DNA was precipitated. The DNA was dissolved in TE buffer and extracted once with phenol/chloroform, and 1 μl of the resulting DNA preparation was used as the template for a PCR with primers that flanked the *Gfr* gene transcript (AAGGGATGGGGCCAAGAAGC and AATCCACCCCCGCACTCAAC). All PCRs were performed using Phusion™ DNA polymerase (New England Biolabs). Agarose gel electrophoresis showed that the PCR yielded a single major amplification product of the expected size, which was gel-purified using the Qiaquick gel purification kit (Qiagen) and directly sequenced with the primers used for the PCR.

Expression Plasmids and Baculoviruses—All plasmid constructs derived directly from PCRs were sequence verified and amplimers for TOPO cloning were gel-purified and then treated with TaqDNA polymerase (New England Biolabs). Total RNA was isolated from the *Drosophila* WT Canton-S strain using TRIzol® reagent (Invitrogen), and cDNA was synthesized using SuperScript® III RT (Invitrogen) and oligo(dT). The cDNA was used as the template to amplify the WT *Gfr* ORF (primers TCAGGCCTTCTGGGTGGCGGTGCT and CACCATGTACAAGAATCTGGAGGAGCAC), which was cloned into pcDNA™3.1/V5-His-TOPO® (Invitrogen), and a sequence-verified clone was designated pcDNA-DmGFR-WT. This plasmid was used as the template for PCR mutagenesis with the additional primers GTGCACCTTGATATTGACGGTATTCG and GTCAATATCAAGGTGCACCAGTAGAGC to produce the *nac*[1] *Gfr* ORF by overlap PCR. The amplimer was cloned into pcDNA™3.1/V5-His-TOPO®, and a clone encoding the *nac*[1] mutant *Gfr* was designated pcDNA-DmGFR-nac.

Baculovirus transfer plasmids were produced by cloning the BamHI-NotI fragment encoding the WT or *nac*[1] mutant *Gfr* gene from pcDNA-DmGFR-WT and pcDNA-DmGFR-nac, respectively, into the BglII-NotI sites of pAcp(+)IE1TV3 (37), resulting in production of the pAcp(+)DmGFR-WT and pAcp(+)DmGFR-nac transfer plasmids, respectively.

Plasmids encoding C-terminally eGFP-tagged GFR proteins were constructed by PCR overlap extension. The WT or *nac*[1] mutant *Gfr* ORFs minus their stop codons were PCR-amplified using the respective pcDNA plasmids as the template, respectively, with the primers CACCATGTACAAGAATCTGGAGGAGCAC and GCTCACCATGGCCTTCTGGGTGGCGGT. The eGFP ORF was PCR-amplified using peGFP-N1 (Clontech) as the template with the primers CAGAAGGCCATGGTGAGCAAGGGCGAG and CTACTTGTACAGCTCGTCCATGC. The reaction products were gel-purified and used as the template in PCR overlap extension reactions. The reaction products were cloned into pcDNA™3.1/V5-His-TOPO®, and clones encoding the C-terminally GFP-tagged WT and *nac*[1] *Gfr* were designated pcDNA-DmGFR-WT-GFP and pcDNA-DmGFR-nac-GFP, respectively. The fused ORFs were excised from these plasmids using BamHI and NotI and cloned into the BglII-NotI sites of pAcp(+)IE1TV3 (37), yielding pAcp(+)IE1TV3-DmGFR-WT-GFP and pAcp(+)IE1TV3-DmGFR-nac-GFP, respectively.

Transfer plasmids were used to produce recombinant baculoviruses by a standard allelic transplacement method (38, 39) with BestBac viral DNA (Expression Systems) as the target for homologous recombination. All recombinant baculoviruses were plaque-purified once, amplified in Sf9 cells, and titered by plaque assay on Sf9 cells, as described previously (39). *Autographa californica* nucleopolyhedrovirus strain E2 served as a negative control for some of the experiments included in this study.

Transient Expression in CDG-IIc (LAD-II) Cells, Lectin Blotting—Primary fibroblast cells from a CDG-IIc (LAD-II) patient were maintained essentially as described (40) in α-minimum essential medium supplemented with 15% FBS in 5% CO_2 at 37 °C. For transfections, cells were seeded in a 75-cm^2 culture flask; grown to confluence; and transfected with pcDNA, pcDNA-DmGFR-WT, or pcDNA-DmGFR-nac using Lipofectamine™ 2000 (Invitrogen). Briefly, cells were transfected using 30 μg of DNA and 75 μl of transfection reagent for 3 h using serum-free α-minimum essential medium according to the manufacturer's protocol. Cells were subsequently incubated for 24 h in α-minimum essential medium with 15% FBS, after which cells were collected by trypsinization, washed twice in PBS, and lysed in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 1.0% Nonidet P-40, 150 mM NaCl, 0.5 mM PMSF, 1 mM EDTA, one Complete Mini™ protease inhibitor mixture tablet (Roche Applied Science)/10 ml of buffer). After vigorous vortexing, the lysate was centrifuged for 10 min at 13,000 × *g*, after which the supernatant was collected and assayed for soluble protein concentration using a commercial BCA assay (Pierce).

CHO cell lysates were prepared as described above from CHO cells cultured as described before (41). Aliquots containing 50 μg of total protein were separated by SDS-PAGE (42) and transferred to an Immobilon-P PVDF membrane (Millipore). The membrane was blocked, probed, and developed essentially as described before (43), except that biotin-conjugated *Aleuria aurantia* lectin (Vector Laboratories) was used as the probe.

Subcellular Localization—Sf9 and *Drosophila* S2 cells were transfected with expression plasmids encoding RFP-tagged *S. frugiperda* MGAT1 (44) and GFP-tagged WT or *nac*[1] mutant GFR proteins (pAcP(+)DmGFR-WT-GFP or pAcP(+)DmGFR-nac-GFP), plated on concanavalin A-coated dishes, and photographed essentially as described before (44). An Olympus FSX100 microscope was used at ×80 magnification, and the

manufacturer's FSX-BSW version 03.01 software was used for image capture at 1360 × 1024 pixels. Images were processed with Photoshop CS3 to reduce background and to provide similar signal intensities for the red and green channels.

GDP-fucose Transport Assays—Fifty ml of Sf9 cells were seeded at a density of 5×10^5 cells/ml in complete TNM-FH medium, grown overnight to 1×10^6 cells/ml, and infected with the appropriate viral stock at a multiplicity of infection of about 2 plaque-forming units/cell. After 22 h, infected cells were pelleted at 500 × *g* for 5 min, resuspended in 25 ml of ice-cold PBS (pH 7.4), and repelleted. The pellet was resuspended in 6.5 ml of lysis buffer (250 mM sucrose, 5 mM imidazole, 0.5 mM mercaptoethanol, pH 7.0, one Complete Mini™ protease inhibitor mixture tablet (Roche Applied Science)/10 ml of buffer). The cells were subsequently homogenized on ice in a Dounce homogenizer with pestle A, after which nuclei and remaining intact cells were pelleted at 4 °C at 1000 × *g* for 10 min. The crude microsomal preparation was then layered onto a sucrose cushion (1.3 M sucrose, 5 mM imidazole, 0.1 mM EDTA, pH 7.0), covered with sucrose overlay (125 mM sucrose, 5 mM imidazole, 0.1 mM EDTA, pH 7.0), and then centrifuged in a Beckman SW28 rotor at 100,000 × g_{av} for 40 min at 4 °C. Subsequently, the microsomal band was harvested, diluted with sucrose overlay, and recentrifuged in an SW28 rotor at 110,000 × g_{av} for 20 min at 4 °C. The microsomal pellet was resuspended in 600 μl of STM buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM DDT, pH 7.5), divided into aliquots, and stored at −80 °C for up to 1 week. To determine total protein concentrations, aliquots were thawed on ice, briefly vortexed, and solubilized by the addition of an equal volume of water containing 1.0% (v/v) Nonidet P-40. These mixtures were then centrifuged at 13,000 × *g* for 10 min, and the concentrations of solubilized protein in the supernatants were determined using a commercial BCA assay (Pierce).

For transport assays, aliquots of the microsomal preparations were thawed on ice and thoroughly vortexed, and transport assay mixtures were prepared by adding 10 μl of the microsomal preparation to 80 μl of STM buffer, cooling the mixture in an ethanol-ice bath (approximately −5 °C), and then adding 10 μl of STM buffer containing 30 nCi of [^{3}H]GDP-fucose, (Fucose-2-^{3}H(N), PerkinElmer Life Sciences; 15–35 Ci/mmol). The mixture was briefly vortexed and quickly returned to the ethanol-ice bath. The mixture was then transferred to a water bath at 18, 25, or 32 °C for precisely 1 min, returned to the ethanol-ice bath, and quenched by the addition of 900 μl of ice-cold STM buffer. The mixtures were then filtered through water-wetted 0.45-μm mixed cellulose ester filters (Type HA; Millipore) using a 1225 Sampling Manifold (Millipore). The disks were washed three times with 5 ml of ice-cold STM buffer, air-dried, placed in 7 ml of Ultima Gold F scintillation mixture (Packard Instrument Co.), and counted for 10 min in a model LS-6500 liquid scintillation spectrometer (Beckman Coulter).

Background counts were determined by counting an unused filter as described above. All samples were assayed at least three times in triplicate ($n = 9$). Raw counts were corrected for background and normalized to 30 μg of soluble protein content. Significant differences were determined by one-way analysis of variance using Microsoft® Excel.

Mass Spectrometry and HPLC—For *nac*^{1} and WT Canton S flies, pepsin glycopeptides were enriched, and *N*-glycans were released with peptide-*N*-glycosidase A prior to pyridylamination and RP-HPLC, MALDI-TOF MS or ESI-MS analysis (45). As a first step, linear MALDI-TOF mass spectra of unlabeled *N*-glycans were obtained prior to pyridylamination using a Thermo Bioanalysis Dynamo mass spectrometer in linear mode with 2,5-dihydroxybenzoic acid as matrix. For ESI-MS of the pyridylaminated glycans with a Micromass Q-TOF Ultima Global mass spectrometer, the $[M + H]^+$ ions were calculated by applying the MassLynx MaxEnt3 software to the raw multiply charged ion data. For reverse phase HPLC analysis of pyridylaminated *N*-glycans, an ODS Hypersil column (250 × 4 mm) with a gradient of 0.3% methanol/min was used with an oligohexose series (3–11 glucose units) as a calibration standard; elution times in terms of glucose units can be compared with previous data on WT fly *N*-glycans (24). Individual RP-HPLC fractions were also analyzed by MALDI-TOF MS and MS/MS using a Bruker Autoflex™ speed instrument in reflectron mode and 6-aza-2-thiothymine as matrix.

Drosophila Transgenesis and Rescue—All *Drosophila* strains (OreR, *w*^{1118}, *elav*-GAL4 inserted on the X chromosome, *nac*^{1}, and balancer stocks) were obtained from the Bloomington *Drosophila* Stock Center. The full WT Canton S *Gfr* ORF was isolated by PCR using the primers GGAATTCCGAAATGTACAAGAATCTG and GGGGTACCTCAGGCCTTCTGGGTGG. The amplimer was purified and cut with EcoRI and KpnI and ligated into the same sites of the pUAST transgenesis plasmid (46). Transgenic stocks carrying UAS-*Gfr* elements on all three chromosomes were generated by injection of pUAST-*Gfr* into precellularized embryos using standard methods (46).

Drosophila Embryo Anti-HRP Staining—Embryos were dechorionated, fixed, devitellinized, stained with antibodies, and staged according to standard methods (47, 48). Antibody dilutions were 1:5000 for rabbit anti-HRP (Jackson Immunoresearch) and 1:2000 for peroxidase-conjugated secondary antibodies (Jackson Immunoresearch). All embryos were processed identically and in parallel (same antibody dilutions, same development time, same day) to facilitate objective comparison of HRP epitope levels in all genotypes.

RESULTS

Gfr Gene Sequence in the nac^1 *Mutant*—The sequence of a PCR amplimer from a genomic region that includes the *Gfr* transcript revealed that the *Gfr* gene in the *nac*^{1} mutant has a single mutation consisting of a cytosine to thymidine transition at position 86 (C86T) of the ORF. This mutation was independently identified in the Jarvis and Wilson laboratories in *nac*^{1} stocks obtained at different times and from different sources. The *nac*^{1} C86T transition results in the substitution of a leucine for a serine residue at position 29 (S29L) in the predicted GFR amino acid sequence. The WT serine residue is fully conserved among all known and putative GDP-fucose transporters throughout the animal kingdom (Fig. 1*B*) and is located in the first predicted transmembrane region (Fig. 1*C*). Because production of the HRP epitope requires GDP-fucose in the Golgi apparatus, where it serves as the donor substrate, these observations were consistent with the idea that *nac*^{1} flies might have

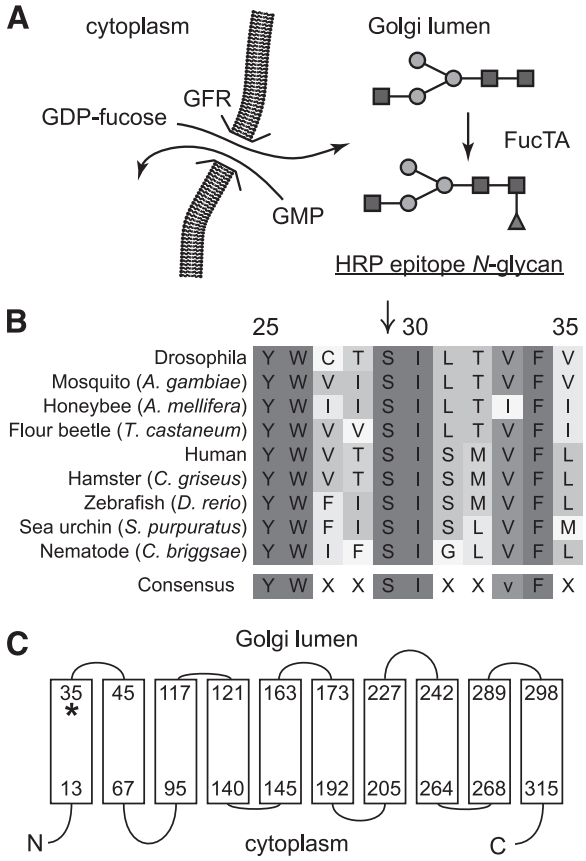

FIGURE 1. **Production of the HRP epitope and the *Drosophila nac*^{1} GFR mutation.** *A*, GDP-fucose is produced in the cytoplasm and transported into the Golgi lumen by the GFR transporter in exchange for GMP. FucTA uses GDP-fucose in the Golgi lumen to produce the HRP epitope consisting of core α1,3-fucosylated *N*-glycans. *Squares*, *N*-acetylglucosamine; *circles*, mannose; *triangles*, fucose. *B*, amino acid sequence comparison of known and predicted GDP-fucose transporters. The *arrow* indicates the conserved serine residue that is mutated to a leucine in *Drosophila nac*^{1} GFR. Amino acid residue numbering is according to the *Drosophila* gene product. GenBank™ accession numbers are as follows: *Drosophila*, NP_649782.1; mosquito, XP_312562.2; honeybee, XP_623632.1; flour beetle, XP_967192.1; human, NP_001138737.1; Chinese hamster, BAE16173.1; zebrafish: NP_001008590.1; sea urchin, XP_798515.1; nematode, XP_002637574.1. *C*, Ser-29 is located in the middle of the first predicted transmembrane domain of *Drosophila* GFR.

a defective Golgi GDP-fucose transporter. Thus, we examined the impact of the *nac*^{1} S29L mutation on the GDP-fucose transport function of the mutant *Gfr* gene product.

GDP-fucose Transport by the nac^1 *Mutant GFR Product*—Our transport assays measured the amount of GDP-fucose transported into microsomes, analogous to previously described nucleotide sugar transport assays employing Golgi-enriched microsomes from cells expressing heterologous NSTs (35, 49). Baculovirus expression vectors were used to express the WT and *nac*^{1} mutant GFRs in Sf9 insect cells, and then Golgi-enriched microsomes were isolated from those cells and used for GDP-fucose transport assays. Microsomes from cells infected with the empty baculovirus vector were used as background controls, and each assay was performed at different temperatures to determine if there were any temperature-dependent differences that could explain *nac*^{1} cold-sensitive phenotypes (34). As compared with the background controls, microsomes containing WT GFR imported more and those containing the *nac*^{1} mutant GFR imported less GDP-fucose at all temperatures examined (Fig. 2*A*). Increasing the assay temperature from 18 to 25 °C did not produce a statistically significant increase in GDP-fucose import in either the background controls or WT GFR samples. However, this temperature shift significantly increased GDP-fucose import in the *nac*^{1} GFR samples. These experiments were extended by performing additional assays at 32 °C to determine if the *nac*^{1} mutant transporter gained even more function at this higher temperature. Indeed, GDP-fucose import with background control, WT GFR, and *nac*^{1} GFR microsomes was increased further at 32 °C (Fig. 2*A*). As with the previous temperature shift, the increase in GDP-fucose import activity was highest in the *nac*^{1} GFR samples, confirming that the *nac*^{1} mutant GFR is more cold-sensitive than WT GFR, which potentially contributes to the cold-sensitive *nac*^{1} phenotypes (34).

We also assessed the function of *nac*^{1} GFR *in vivo* by using cells from a CDG-IIc (LAD-II) patient (40). These cells cannot produce fucosylated *N*-glycans because they have a defective GFR, but their fucosylation-negative phenotype can be rescued by transfection with a WT human *GFR* gene (28, 30, 31). Thus, we transfected CDG-IIc (LAD-II) cells with plasmids encoding the WT or *nac*^{1} *Drosophila Gfr* genes, prepared total cell lysates and CHO cell lysates as a positive control, and probed them with fucose-specific *A. aurantia* lectin (50). *A. aurantia* lectin bound strongly to multiple proteins in the CHO cell lysate but not to any proteins in the empty vector-transfected CDG-IIc (LAD-II) cell lysate, as expected (Fig. 2*B*). *A. aurantia* lectin also bound to multiple proteins in the WT *Gfr*-transfected CDG-IIc (LAD-II) cell lysate but not to any proteins in the *nac*^{1} *Gfr*-transfected CDG-IIc (LAD-II) cell lysate (Fig. 2*B*). The higher level of *A. aurantia* lectin binding observed with the CHO cell lysate as compared with the WT *Gfr*-transfected CDG-IIc (LAD-II) cell lysate probably reflects cell toxicity associated with the transfection because we observed significant cell death at later time points. Alternatively, it might reflect the inefficiencies inherent in the transfection process or the differences between the CHO and LAD-II cell types. Regardless, these results showed that the *nac*^{1} *Gfr* gene failed to rescue the fucosylation-negative phenotype in CDG-IIc (LAD-II) cells, indicating that the *nac*^{1} mutant gene product is defective *in vivo*.

The nac^1 *Mutant GFR Localizes to the Golgi Apparatus*—GDP-fucose transporters typically localize to the Golgi apparatus (28, 40). Hence, the transport defect observed with the *nac*^{1} mutant gene product could have resulted from a direct impact of the mutation on its biochemical function or an indirect impact on its intracellular trafficking. To distinguish between these possibilities, we expressed GFP-tagged forms of the WT and *nac*^{1} GFRs in *Drosophila* S2 cells as well as in Sf9 cells, which had been used for the *in vitro* GDP-fucose transport assays. We used RFP-tagged insect *N*-acetylglucosaminyltransferase I (MGAT1) as a Golgi marker because this enzyme acts immediately upstream of HRP epitope synthesis by producing the FucTA acceptor substrate (44, 51–53). The red and green fluorescence patterns observed in these experiments each had punctate, cytoplasmic distributions typical of the multiple Golgi apparatuses found in lepidopteran insect cells (Fig. 2*C*)

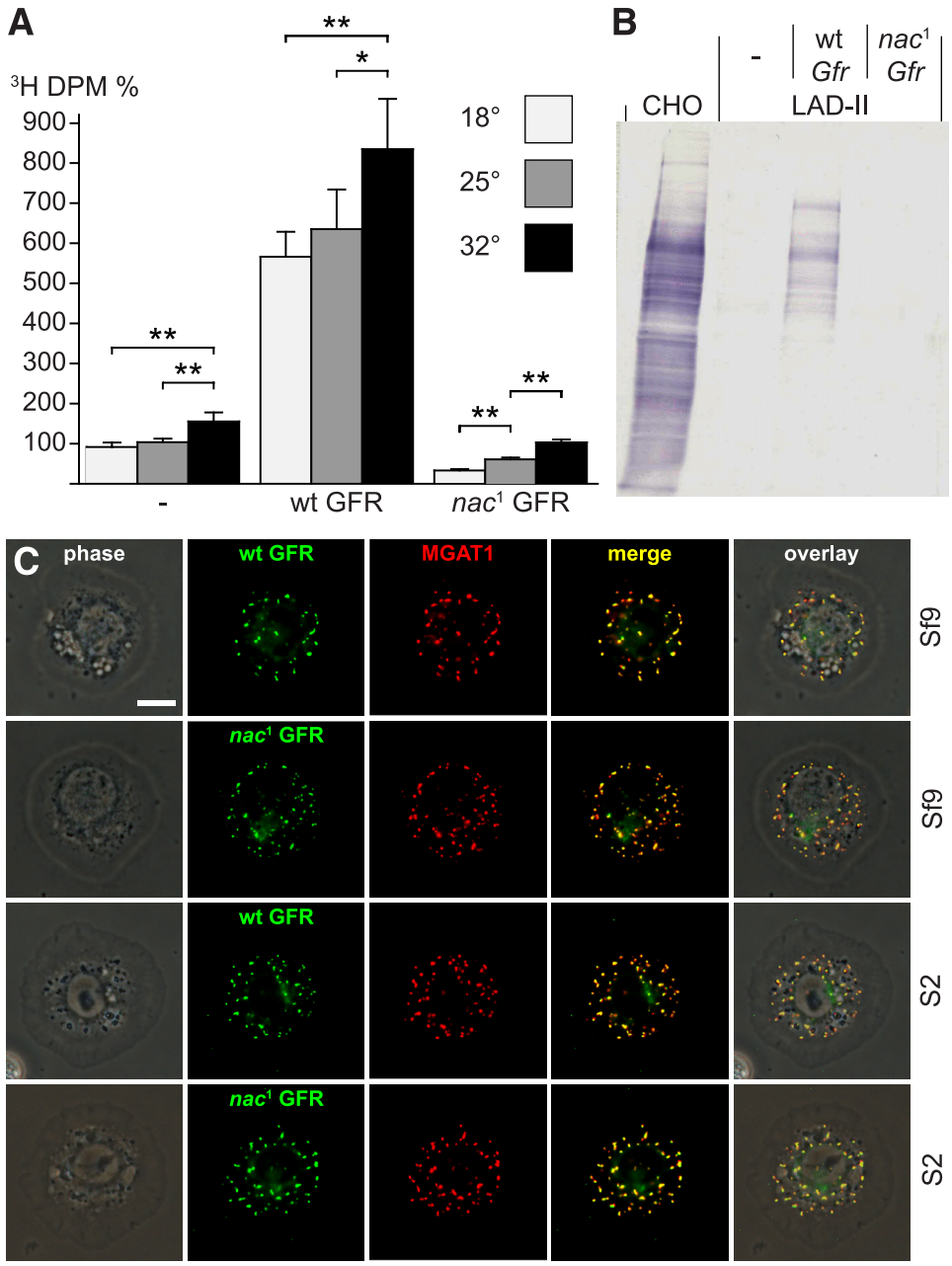

FIGURE 2. **WT, but not *nac*^{1} GFR can transport GDP-fucose *in vitro* and *in vivo*, and both are Golgi-localized.** *A*, [^{3}H]GDP-fucose import activity of Golgi-enriched microsomes from Sf9 cells infected with baculovirus vectors encoding WT GFR, *nac*^{1} GFR, or no exogenous transporter (−) at 18, 25, or 32 °C. Background import at 18 °C (1.5 fmol of GDP-fucose/μg of total protein/min) is set at 100% (−). *Error bars*, 95% confidence interval. *p* values for different samples at the same temperatures were all <0.01. *, $p < 0.05$; **, $p < 0.01$. *B*, *A. aurantia* lectin (*AAL*) blot of CHO cells or CDG-IIc (LAD-II) cells transfected with expression plasmids encoding WT GFR, *nac*^{1} GFR, or nothing (−). *C*, subcellular distribution of WT and *nac*^{1} GFR in Sf9 and *Drosophila* S2 cells. *Columns*, phase-contrast, GFP-tagged GFR, RFP-tagged MGAT1 (insect Golgi marker), GFP and RFP merge, and overlay. *Scale bar*, 10 μm.

(54, 55). Furthermore, there was a close overlap between the GFR and MGAT1 fluorescence patterns in all cases, indicating that these two proteins reside in the same subcellular compartment. The similarity in the fluorescence patterns observed with the WT and *nac*^{1} mutant GFRs and their close overlap with the Golgi marker indicated that the *nac*^{1} mutation does not impact the intracellular trafficking of GFR, which was consistent with the presence of only a single amino acid substitution in the mutant protein. These data also indicated that this mutation does not dramatically reduce GFR stability, although it is possible that the mutant protein was stabilized by being fused to GFP.

Core α1,3- and α1,6-Fucosylation Are Both Reduced in nac^1 *Flies*—Golgi-localized GDP-fucose is required as the donor substrate for both core α1,3- and α1,6-fucosylation. Thus, one might expect a functional knock-out of the *Gfr* gene to reduce both types of core fucosylation in *nac*^{1} flies. To test this expectation, we determined the relative levels of core fucosylated *N*-glycans in WT and *nac*^{1} flies using ESI-MS. The results showed that 21 and 10% of the *N*-glycans from WT (Fig. 3*A*) and *nac*^{1} mutant (Fig. 3*B*) adult flies, respectively, were monofucosylated glycans with the structure $Hex_3HexNAc_2Fuc$. Similarly, the prevalence of monofucosylated *N*-glycans with the structure $Hex_2HexNAc_2Fuc$ was 5% in WT but only 1.4% in

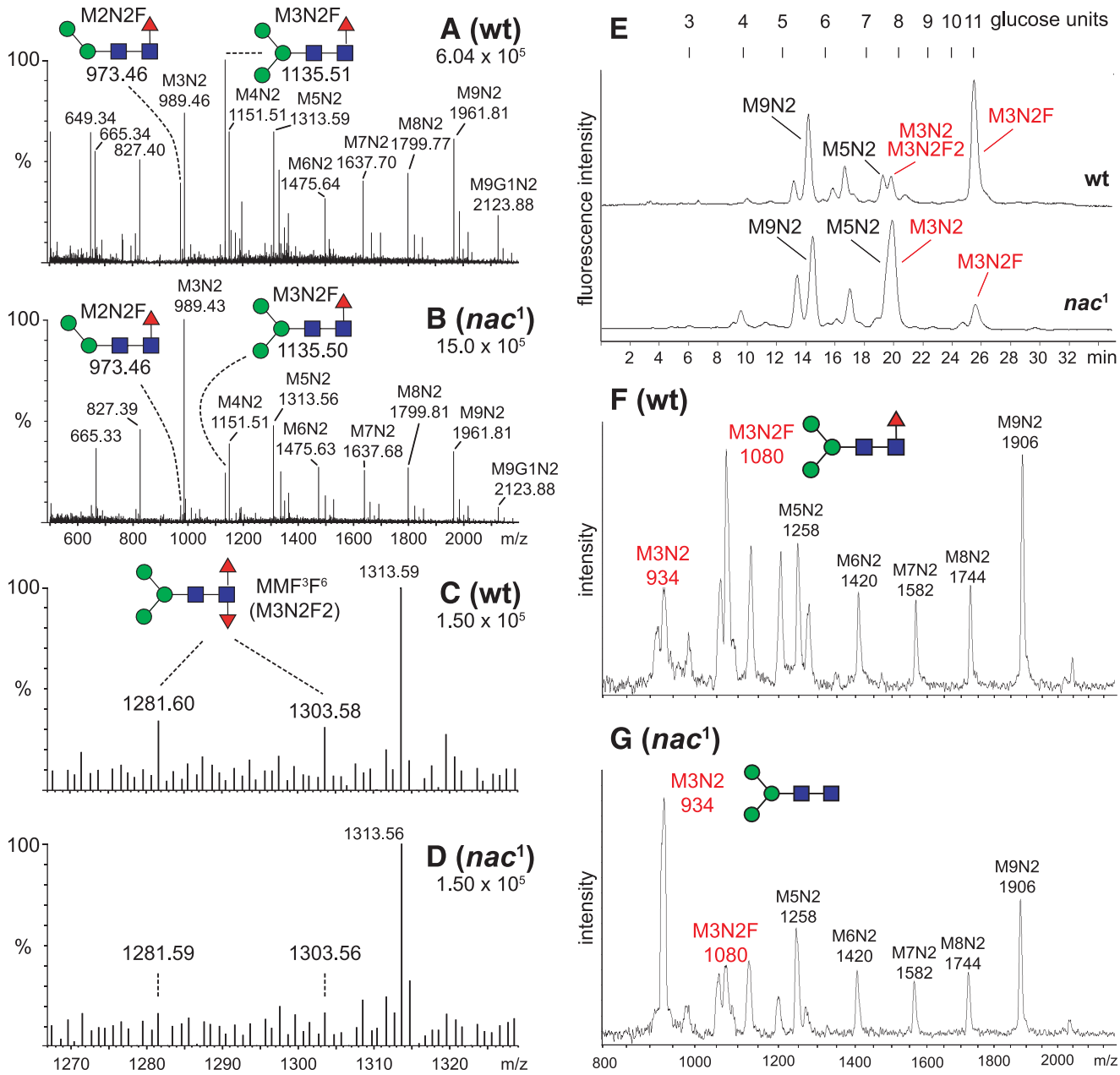

FIGURE 3. **Core di-, α1,3-, and α1,6-fucosylated *N*-glycan levels are strongly reduced in *nac*^{1} flies.** Peptide-*N*-glycosidase A-released *N*-glycans from WT Canton S (*A*, *C*, *E*, and *F*) and *nac*^{1} adults (*B*, *D*, *E*, and *G*) were subjected to analysis by ESI-MS (*A–D*), RP-HPLC (*E*), and MALDI-TOF MS (*F* and *G*). *F*, fucose; *G*, glucose; *M*, mannose; *N*, *N*-acetylhexosamine. *Red triangles*, fucose; *green circles*, mannose; *blue squares*, *N*-acetylglucosamine. The late elution time of M3N2F (*E*) indicates that it is a core α1,6-fucosylated glycan, and its reduced relative intensity in *nac*^{1} flies is shown by all three methods, MALDI-TOF MS analysis (not shown) of individual RP-HPLC fractions indicated trace levels of difucosylated glycans co-eluting with M3N2 in *nac*^{1} flies. The *exploded views* of the ESI-MS spectra (*C* and *D*; *m/z* 1265–1335) are set to the same ion count (*y* axis; 1.5×10^5) and show the almost complete absence in *nac*^{1} flies of the difucosylated HRP epitope MMF^3F^6 glycan in its $[M + H]^+$ and $[M + Na]^+$ forms.

nac^1 mutant adults (Fig. 3*B*). Low levels of difucosylated *N*-glycans bearing both the HRP epitope and core α1,6-linked fucose residues also were detected in WT but not in *nac*^{1} mutant flies (Fig. 3, *C* and *D*). We further assessed the levels of monofucosylated *N*-glycans in *nac*^{1} mutant and WT flies by reverse phase HPLC (Fig. 3*E*) and MALDI-TOF (Fig. 3, *F* and *G*); analysis of individual RP-HPLC fractions by MALDI-TOF MS revealed only trace amounts of the difucosylated glycans $Hex_{2-3}HexNAc_2Fuc_2$ in *nac*^{1} (co-eluting with $Hex_3HexNAc_2$) as compared with WT flies (data not shown). The results obtained using both of these analytical methods confirmed that *nac*^{1} flies have lower levels of monofucosylated *N*-glycans. Thus, three independent methods indicated that the *nac*^{1} mutation reduced both α1,3- and α1,6-linked core fucosylation, as would be expected from the loss of GFR function. In addition, all three methods also revealed a relative increase in the levels of the non-fucosylated *N*-glycan $Hex_3HexNAc_2$ corresponding to the decreased levels of fucosylated *N*-glycans, further confirming the lack of fucosylation.

WT Gfr Expression Rescues the Neural HRP Epitope in nac^1 *Embryos*—Immunohistochemistry with an anti-HRP antibody confirmed that HRP epitope expression in the ventral nerve

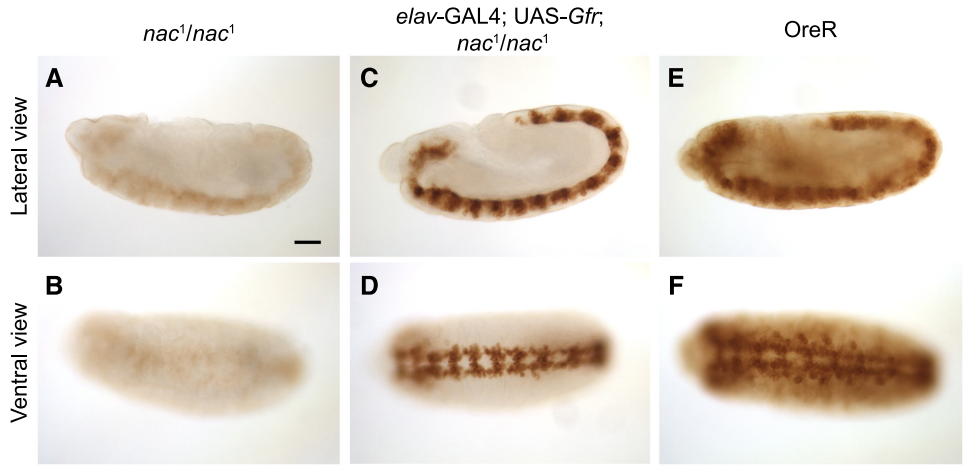

FIGURE 4. **Reduced HRP epitope expression in *nac*^{1} homozygous embryos is rescued by transgenic expression of WT *Gfr*.** *A*, *C*, and *E*, lateral view. *B*, *D*, and *F*, ventral view. All embryos are late stage 12 to early stage 13. In *nac*^{1}/*nac*^{1} embryos (*A* and *B*), HRP epitope expression is reduced in comparison with WT embryos (*E* and *F*). A WT *Gfr* transgene driven by the neuron-specific *elav* promoter rescues neural HRP epitope expression (*C* and *D*). *Scale bar*, 70 μm.

cord was much lower in *nac*^{1} than in WT embryos (Fig. 4, *A*, *B*, *E*, and *F*), as shown previously (21, 33). In order to determine if the *Gfr* C86T mutation was solely responsible for this change, we generated transgenic *Drosophila* stocks designed to express the WT *Gfr* coding sequence in *nac*^{1} embryos using the GAL4/UAS system (46). A second chromosome UAS-*Gfr* transgenic strain and an X chromosome *elav*-GAL4 driver strain were both crossed into the third chromosome *nac*^{1} background, resulting in stocks that were homozygous for *nac*^{1} and either the UAS-*Gfr* or *elav*-GAL4 element. These stocks were crossed to generate embryo collections, which were then stained with the anti-HRP antibody to assess whether transgenic *Gfr* expression could rescue the *nac*^{1} core α1,3-fucosylation defect. As expected for the neural specificity of the *elav*-GAL4 element (56), expression of the HRP-epitope was rescued in differentiating neurons of *elav*-GAL4; UAS-*Gfr*; *nac*^{1}/*nac*^{1} progeny (Fig. 4, *C* and *D*). Thus, reduced HRP epitope expression in *nac*^{1} flies is due solely to a defect in their *Gfr* gene.

Staining with anti-HRP antibody could be detected in late stage 10 rescued embryos, which is substantially earlier than in wild-type embryos, where staining first appeared in early stage 12. This is consistent with the time course of *elav* expression (56), suggesting that *Gfr* expression at least partially limits core α1,3-fucosylation in *Drosophila.* Surprisingly, *elav*-driven expression of *Gfr* resulted in embryonic lethality at mid-embryogenesis. This is probably a result of our use of the very strong *elav*-GAL4 driver, which typically provides highly efficient expression of UAS-transgenes in the embryonic nervous system.

In the course of generating UAS-*Gfr* transgenic stocks, we identified a line that exhibited partially rescued HRP epitope expression in the ventral nerve cord and peripheral nervous system of *nac*^{1} mutant embryos without crossing to a GAL4 driver line (data not shown). This leaky expression line (UAS-*Gfr*^{vk2}) was homozygous viable and fertile in both *nac*^{1} and WT backgrounds, suggesting a significantly lower *Gfr* expression level than was obtained by crossing UAS-*Gfr* lines to the *elav*-GAL4 driver line. Interestingly, the UAS-*Gfr*^{vk2} leaky expression line rescued temperature-sensitive lethality associated with the *nac*^{1} mutant; only 6% of *nac*^{1}/*nac*^{1} adults survived after a shift to 18 °C, whereas 82% of UAS-*Gfr*^{vk2}/UAS-*Gfr*^{vk2}; *nac*^{1}/*nac*^{1} adults survived and reproduced at 18 °C. Thus, whereas overexpression of *Gfr* proved to be embryonic lethal, moderate expression was well tolerated and rescued both HRP epitope expression and developmental arrest defects associated with the *nac*^{1} mutation.

Ser-29 Is Conserved in Other GDP-sugar Transporters—Finally, we compared the amino acid sequences of the *Drosophila* GFR and other known Golgi nucleotide sugar transporters (NSTs) to more generally assess the potential functional relevance of Ser-29 (Fig. 5). We found that plant, fungal, protozoan, and animal GDP-sugar transporters have clear homology to an amino acid sequence in the N-terminal region of the *Drosophila* GFR. Several fungal Golgi GDP-mannose transporters (57–62); a *Leishmania* Golgi GDP-mannose, -fucose, and -arabinose transporter (63, 64); and the *Arabidopsis* and *Volvox* Golgi GDP-mannose transporters (65–67) are clearly similar to *Drosophila* GFR in this region, and each has a serine residue in positions corresponding to *Drosophila* GFR Ser-29. Other multisubstrate transporters that also could transport a GDP-sugar, including human HFRC1 (68), *Drosophila* FRC (69), human UGTrel7 (70), and nematode SQV-7 (71), are similar to *Drosophila* GFR as well, and each has a serine residue at a position corresponding to *Drosophila* GFR Ser-29. In contrast to these GDP-sugar transporters, other Golgi NSTs, including the human UDP-galactose (72), CMP-sialic acid (73), UDP-*N*-acetylglucosamine (74), and UDP-xylose transporters (75), lack significant homology to GFR. Of these, the human UDP-galactose and UDP-*N*-acetylglucosamine transporters have a serine residue at a position corresponding to *Drosophila* GFR Ser-29. However, these serine residues are not conserved in the homologous transporters from most other species, indicating that, unlike *Drosophila* GFR Ser-29, they are probably not essential for functionality. Based on these data, we suggest that a serine residue at a position corresponding to *Drosophila* GFR Ser-29 in Golgi NSTs that also have similarity to an amino acid sequence in the first transmembrane domain of *Drosophila* GFR might predict GDP-sugar transport capacity.

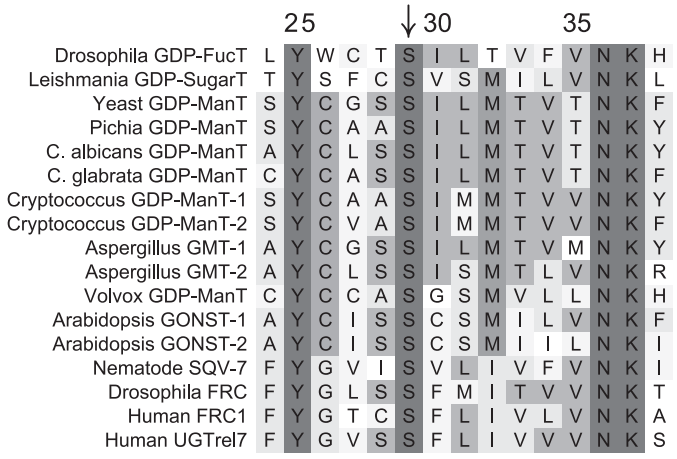

FIGURE 5. ***Drosophila* GFR Ser-29 is conserved in other GDP-sugar transporters.** Shown is an alignment of *Drosophila* GFR with other characterized GDP-sugar transporters from *Leishmania donovani* (GDP-SugarT) (64), *Saccharomyces cerevisiae* (yeast GDP-ManT) (59), *Pichia pastoris* (*Pichia* GDP-ManT) (57), *Candida albicans* (*C. albicans* GDP-ManT) (62), *Candida glabrata* (*C. glabrata* GDP-ManT) (61), *Cryptococcus neoformans* (*Cryptococcus* GDP-ManT-1 and -2) (58), *Aspergillus nidulans* (*Aspergillus* GMT-1 and -2) (60), *Volvox carteri* (*Volvox* GDP-ManT) (67), and *Arabidopsis thaliana* (*Arabidopsis* GONST-1 and -2) (65, 66) and similar NSTs of *Caenorhabditis elegans* (nematode SQV-7) (71), *Drosophila melanogaster* (*Drosophila* FRC) (69), and humans (human FRC1 and hUGTrel7) (68, 70).

DISCUSSION

CDGs are a diverse group of heritable diseases caused by mutations in genes involved in glycosylation. The study of CDGs has been facilitated by the availability of animal models because much of the glycosylation machinery is evolutionarily conserved (76, 77). Since the description of the *nac*^{1} mutant in 1988 by Katz *et al.* (33), *Drosophila* has become established as a model organism for the study of human genetic disorders, including CDGs (78, 79). However, the genetic defect underlying the *nac*^{1} mutation had not yet been elucidated.

In this study, we identified a single nucleotide transition (C86T) that produces a leucine for serine substitution at position 29 of the Golgi GDP-fucose transporter encoded by the *nac*^{1} *Gfr* gene. The mutagen originally used to isolate the *nac*^{1} mutant was ethyl methane sulfonate (33). Mechanistically, ethyl methane sulfonate is expected to produce G/C to A/T transitions (80, 81), and this expectation has been confirmed in mutagenesis studies (82–84). Thus, the C86T transition in the *nac*^{1} *Gfr* gene is fully consistent with the use of ethyl methane sulfonate mutagenesis in producing the *nac*^{1} strain.

There are three currently known missense mutations in the human GDP-fucose transporter that cause CDG-IIc (LAD-II) (T308R, R147C, and Y337C) (30, 85). In each case, these mutations alter a residue analogous to *Drosophila* GFR Ser-29, which is fully conserved among animal GDP-fucose transporters and is located in a predicted transmembrane helix. Like these human GFR missense mutations, the *nac*^{1} S29L mutation also abolishes GDP-fucose transport function *in vitro* and *in vivo*. Serine residues corresponding to *Drosophila* GFR S29 are conserved in GDP-sugar transporters from a wide variety of species but not in other types of NSTs. Thus, we speculate that a serine residue at a position corresponding to *Drosophila* GFR Ser-29 might predict GDP-sugar transporting capacity in Golgi NSTs that are also otherwise similar to *Drosophila* GFR. Interestingly, the first transmembrane domains of human HFRC1 and UGTrel7, like that of *Drosophila* FRC, are similar to GFR, and each has a conserved serine corresponding to GFR Ser-29, unlike any other human or *Drosophila* Golgi NSTs. Thus, it is possible that the low level of fucosylation in *nac*^{1} flies is due to GDP-fucose transport by the *Drosophila* FRC gene product. Similarly, it is possible that the alternative GDP-fucose transport activity observed in CDG-IIc (LAD-II) cells supplemented with fucose is due to this same function of the human HFRC1 or UGTrel7 gene products.

For our *in vitro* GDP transport assays, we used microsomes from Sf9 cells (86), which have endogenous Golgi GDP-fucose transport activity because these cells typically produce α1,6 core fucosylated *N*-glycans (87). Despite the presence of this endogenous activity, we were able to demonstrate a >6-fold increase in transport activity in microsomes from cells infected with a baculovirus encoding WT GFR, as compared with background controls. Surprisingly, microsomes from cells infected with a baculovirus encoding the *nac*^{1} GFR samples had reduced GDP-fucose import activity compared with the controls, indicating a possible dominant negative effect. Considering that GFR dimerization might be necessary to produce a functional transporter (29), co-expression of the *nac*^{1} GFR could have produced a subpopulation of heterodimers consisting of endogenous transporter molecules and recombinant *nac*^{1} GFR molecules, which were less functional than the endogenous transporter homodimers. A similar dominant negative phenotype in which co-expression of a mutant transporter negatively affects transport has been observed with the yeast Golgi GDP-mannose transporter (88), which also functions as a homodimer. Alternatively, it is possible that overexpression of the *nac*^{1} GFR altered the subcellular distribution of the endogenous transporter, thereby reducing the number of transporter molecules in those microsomal preparations.

Non-functional, mutant NSTs that fail to exit the ER typically have frameshift mutations that eliminate one or more transmembrane domains and the C-terminal domain. Two such mutations have been identified in the human GDP-fucose transporter (40, 89). On the other hand, point mutations that change single amino acids typically do not alter the Golgi localization of NSTs, including the GDP-fucose transporter (31, 40, 90). Like the inactivating missense mutations in human GFR, the *nac*^{1} S29L mutation did not affect subcellular distribution because both WT and *nac*^{1} GFR were Golgi-localized.

ESI-MS, RP-HPLC, and MALDI-TOF analyses demonstrated that *nac*^{1} flies have reduced levels of core α1,3-fucosylated and only trace levels of core α1,6-/α1,3-difucosylated *N*-glycans, which is consistent with the original observation that *nac*^{1} flies have significantly reduced levels of the HRP epitope. We also discovered that these flies had reduced levels of core α1,6-fucosylated *N*-glycans, which is consistent with the requirement of a Golgi GDP-fucose transporter for both core α1,6- and α1,3-fucosylation. The residual levels of monofucosylated *N*-glycans indicate that *nac*^{1} flies are still able to transport some GDP-fucose into the Golgi. This suggests the presence of an alternative, functionally redundant GDP-fucose transport mechanism, a notion that is supported by the results of another study, in which faint anti-HRP and *A. aurantia* lec-

tin staining could still be detected in flies with a large deletion in the *Gfr* gene (91). This redundant transport mechanism is not provided by the ER-localized GDP-fucose transporter encoded by the *Efr* gene because flies lacking *Gfr* alone or both *Gfr* and *Efr* have comparable amounts of residual core fucosylated *N*-glycans (91). Similarly, humans also have an alternative but less efficient Golgi GDP-fucose import mechanism because dietary fucose supplementation can restore *N*-glycan core fucosylation in CDG-IIc (LAD-II) patients that are homozygous for a completely non-functional Golgi GDP-fucose transporter (40, 89). The precise nature of the redundant GDP-fucose transport mechanism remains to be determined in both humans and flies; however, its low affinity and non-saturable character suggests that it is not provided by another specific GDP-fucose transporter (92, 93).

Interestingly, *nac*^{1} embryos rescued with *elav*-driven WT *Gfr* expressed the HRP epitope at earlier developmental stages than WT embryos, suggesting that *N*-glycan fucosylation is at least partially limited by transport of GDP-fucose into the Golgi apparatus. The notion that *Gfr* expression limits fucosylation *in vivo* is corroborated by the demonstration that increased *N*-glycan fucosylation in cancer cells correlates with increased *GFR* expression, and that fucosylation can be increased directly by overexpressing *GFR* (94). A surprising observation was that *elav*-driven WT *Gfr* overexpression triggered embryonic lethality. This is probably a pleiotropic phenotype arising from the increased availability of GDP-fucose in the Golgi apparatus for a variety of *N*-linked and *O*-linked fucosylation reactions. Intriguingly, the *nac*^{1} phenotypes and the embryonic lethality observed in *elav*-GAL4; UAS-*Gfr* rescued *nac*^{1} flies suggest that it is biologically necessary to maintain protein fucosylation within a certain range. Furthermore, the relation between *Gfr* expression levels, embryonic lethality, and HRP epitope production suggests that *Gfr* is part of the regulatory system that maintains Golgi GDP-fucose levels within a physiologically acceptable range.

Finally, the observation that transgenic WT *Gfr* expression can restore HRP epitope production in *nac*^{1} flies indicates that the *Gfr* C86T transition is the only genetic defect responsible for the neurally altered carbohydrate phenotype. Hence, the defective Golgi GDP-fucose transporter and the resulting fucosylation deficit in *nac*^{1} flies are analogous to human CDG-IIc (LAD-II). Coupled with our observation that the *nac*^{1} mutant GFR is more cold-sensitive than its WT counterpart, we suggest that the *nac*^{1} fly is a useful model of human CDG-IIc (LAD-II) that could be effectively exploited in a variety of creative ways, such as by using its cold-sensitive phenotypes to titrate *N*-glycoprotein core fucosylation.

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