The DMAP interaction domain of UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase is a substrate recognition module

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UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) is an $\alpha_2\beta_2\gamma_2$ heterohexamer that mediates the initial step in the formation of the mannose 6-phosphate recognition signal on lysosomal acid hydrolases. We previously reported that the specificity of the reaction is determined by the ability of the α/β subunits to recognize a conformation-dependent protein determinant present on the acid hydrolases. We now present evidence that the DNA methyltransferase-associated protein (DMAP) interaction domain of the α subunit functions in this recognition process. First, GST-DMAP pulled down several acid hydrolases, but not nonlysosomal glycoproteins. Second, recombinant GlcNAc-1-phosphotransferase containing a missense mutation in the DMAP interaction domain (Lys732Asn) identified in a patient with mucolipidosis II exhibited full activity toward the simple sugar α-methyl D-mannoside but impaired phosphorylation of acid hydrolases. Finally, unlike the WT enzyme, expression of the K732N mutant in a zebrafish model of mucolipidosis II failed to correct the phenotypic abnormalities. These results indicate that the DMAP interaction domain of the α subunit functions in the selective recognition of acid hydrolase substrates and provides an explanation for the impaired phosphorylation of acid hydrolases in a patient with mucolipidosis II.

The lysosomes of higher eukaryotes contain more than 60 soluble acid hydrolases capable of degrading most biological materials. For the lysosome to effectively perform its function, proper targeting of the hydrolases to this organelle is essential. This process uses the mannose 6-phosphate (Man-6-P) targeting pathway, in which the newly synthesized acid hydrolases acquire Man-6-P residues on their *N*-linked glycans that serve as high-affinity ligands for binding to Man-6-P receptors in the *trans*-Golgi network (1). The hydrolase-receptor complexes are then packaged into transport carriers that deliver the cargo to endosomes and lysosomes. The first step in the synthesis of the Man-6-P recognition signal is mediated by UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase), which transfers GlcNAc-1-P from UDP-GlcNAc to mannose residues on the *N*-linked glycans of the acid hydrolases. The GlcNAc is subsequently excised by a second enzyme to generate the Man-6-P monoester.

The specificity of the phosphorylation reaction for acid hydrolases is determined by the ability of GlcNAc-1-phosphotransferase to recognize a conformation-dependent protein determinant present on the acid hydrolases, but absent on nonlysosomal glycoproteins that contain identical *N*-linked glycans (2).GlcNAc-1-phosphotransferase is an $\alpha_2\beta_2\gamma_2$ heterohexamer encoded by two genes (3–6). The *GNPTAB* gene encodes the α/β precursor, which undergoes a proteolytic cleavage in the Golgi to generate the α and β subunits (7), whereas the γ subunit is encoded by the *GNPTG* gene (4). We have presented evidence that the α/β subunits recognize the protein determinant of acid hydrolases as well as mediate the catalytic function of the enzyme (8). However, the domain or domains of the α/β subunits that performs the recognition function has not been identified. We now report that the DMAP interaction domain of the α subunit is involved in the selective binding of GlcNAc-1-phosphotransferase to acid hydrolases.

Results

DMAP Interaction Domain Binds Acid Hydrolases. The α/β subunits of GlcNAc-1-phosphotransferase contain three identifiable domains: the stealth domain, which consists of 4 regions spread throughout the *GNPTAB* gene; two Notch domains; and a DMAP interaction domain (Fig. 1*A*). The stealth domain is similar to genes found in bacteria that are believed to mediate the synthesis of cell wall polysaccharides and has been proposed to represent the catalytic function of GlcNAc-1-phosphotransferase (9). Notch modules are present in the Notch-receptor family, where they regulate the ligand-induced proteolytic cleavage of the Notch-receptor by an unclear mechanism (10). The DMAP interaction domain of DNA methyltransferase 1 (DNMT1) has been proposed to serve as a protein–protein interaction domain in a repressive transcription complex (11).

The homology between the DMAP interaction domains of DNMT1 and GlcNAc-1-phosphotransferase suggested it was a plausible candidate for mediating the protein–protein interaction with the acid hydrolase substrates. To test this possibility, we prepared a GST-DMAP interaction domain (GST-DMAP) fusion protein and performed pull-down assays with several acid hydrolases and nonlysosomal glycoproteins. The GST-DMAP pulled down the acid hydrolases cathepsin D and α-iduronidase, but not low-density lipoprotein receptor-related protein 9 or heparin-cofactor II, two nonlysosomal proteins (Fig. 1*B*). None of the proteins interacted with the GST control. This result is consistent with the DMAP interaction domain having a role in the binding of acid hydrolases.

Missense Mutation in DMAP Impairs Acid Hydrolase Phosphorylation. We next analyzed the consequences of a Lys732Asn (K732N) missense mutation in the DMAP interaction domain of the *GNPTAB* gene identified in a patient with mucolipidosis II (MLII), also known as I-cell disease (12). In this disorder, GlcNAc-1-phosphotransferase activity is deficient, resulting in impaired phosphorylation of newly synthesized acid hydrolases, which are secreted rather than being targeted to lysosomes (13). Plasmids encoding WT and K732N α/β cDNA with C-terminal V5/His tags were generated and transfected into HEK293 cells. After 48 h, cell lysates were subjected to immunoblotting with an anti-V5 antibody to examine the α/β expression and monitor the proteolytic cleavage of the α/β precursor by the appearance of the mature V5-tagged β subunit. The ratio of the α/β precursor to the β subunit was similar in both cases, indicating that the K732N mutation did not impair

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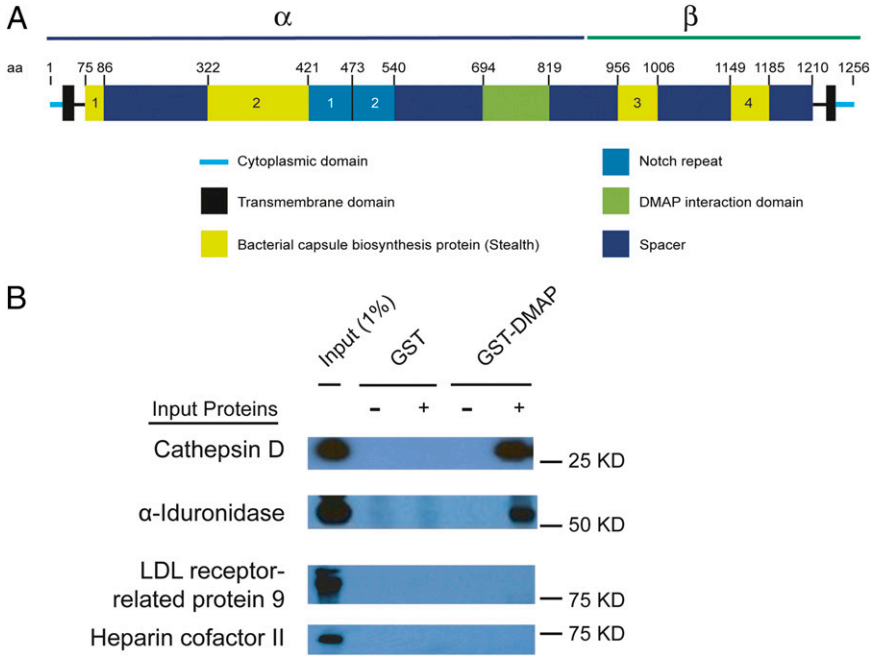

Fig. 1. The DMAP interaction domain exhibits specific binding to lysosomal acid hydrolases. (*A*) Schematic of *Homo sapiens* GlcNAc-1-phosphotransferase α/β subunits. The precursor is cleaved between residues K928 and D929 to give rise to the α and β subunits. (*B*) GST and GST-DMAP (200 μg of each) were immobilized on glutathione-agarose and then incubated in the absence (–) or presence (+) of 10 μg of the indicated proteins in a volume of 50 μL for 2 h at RT. The beads were collected by centrifugation and washed three times, and the bound proteins were released and analyzed by SDS/PAGE and immunoblotting, as described in *Materials and Methods*. Twenty percent of the pellet material was analyzed.

the transport of the α/β precursor from the endoplasmic reticulum to the Golgi, nor the proteolytic cleavage that generates the α and β subunits (Fig. 2*A*).

We also tested whether the K732N mutation might impair the association of the γ subunit with the α/β subunits. The γ subunit of GlcNAc-1-phosphotransferase enhances the catalytic activity of the α/β subunits toward a subset of the acid hydrolases (8), and its loss results in a similar, but attenuated, condition compared with MLII (13). To study this interaction, HEK293 cells were cotransfected with cDNA encoding the γ subunit with a C-terminal Flag tag and either WT or K732N α/β cDNA with C-terminal V5/His tags. Cell lysates were prepared 48 h posttransfection and incubated with Ni-NTA beads to bind the α/β subunits. The bound proteins were then eluted and analyzed by SDS/PAGE and immunoblotting. As shown in Fig. 2*B*, the Flag-tagged γ subunit associated with the beads containing the WT and K732N α/β subunits, whereas none of the γ subunit in the cell extract lacking the α/β subunits bound to the beads. Thus, the K732N mutation does not appear to disrupt the interaction between the α/β and γ subunits.

Confocal immunofluorescence analysis of HeLa cells transfected with the same plasmids revealed that the WT and K732N mutant α/β subunits gave a typical Golgi pattern, exhibiting colocalization with the GM130 *cis*-Golgi marker (Fig. 2*C*). A faint endoplasmic reticulum staining was seen with both constructs, especially in cells with the highest levels of expression. Taken together, these findings indicate that the K732N mutation does not alter the assembly, Golgi localization, processing, or stability of GlcNAc-1-phosphotransferase.

Extracts of HEK293 cells that were either mock-transfected or transfected with WT or K732N plasmids were then assayed for GlcNAc-1-phosphotransferase activity, using the simple sugar α-methyl D-mannoside (αMM) and two acid hydrolases, cathepsin D and α-iduronidase, as acceptors. The αMM serves as a measure of the catalytic function of the enzyme independent of binding to the conformation-dependent protein recognition site. This is reflected by the apparent K_m for αMM being ~40 mM vs. 25 μM for the two acid hydrolases (8). As shown in Fig. 3, the WT and K732N mutant phosphorylated αMM equally. In contrast, the K732N mutant phosphorylated cathepsin D and α-iduronidase only 12–15% as efficiently as the WT enzyme. These data show that the K732N mutant exhibits a selective impairment in the phosphorylation of acid hydrolases that is consistent with a defect in the binding to the protein-recognition domain of these substrates.

K732N Mutant Fails to Rescue MLII Zebrafish. Recently, we generated a morpholino-based model for MLII in the vertebrate organism zebrafish (*Danio rerio*) (14). Embryos depleted of GlcNAc-1-phosphotransferase exhibit decreased mannose phosphorylation of lysosomal acid hydrolases, craniofacial and cardiac defects, and altered development of pectoral fins and otic vesicles. The level of active cathepsin K is strikingly elevated at 3 d postfertilization (dpf) because of abnormal processing and activation (15). This excess cathepsin K activity appears to have a central role in the pathogenesis of the morphologic cartilage defects. All of these abnormalities are prevented by the coinjection of WT *gnptab* mRNA with the morpholino (15). This system provided an opportunity to test the consequences of the K732N mutation in a developing embryo. Fig. 4*A* shows Alcian blue staining of a typical WT embryo and GlcNAc-1-phosphotransferase morphant (MLII) at day 4 dpf. Almost all of the morphant embryos (96%) showed significant dysmorphogenesis of the jaw. Introduction of WT GlcNAc-1-phosphotransferase mRNA resulted in rescue of these structural anomalies in 75% of the embryos, whereas the K732N mRNA failed to correct the abnormalities, with 76% being similar to MLII embryos.

Extracts generated from 3 dpf embryos were also assayed for cathepsin K activity. The extracts of embryos coinjected with WT mRNA and the morpholino exhibited normal decline in cathepsin K activity at day 3. In contrast, extracts of embryos rescued with the K732N mRNA maintained the high levels of cathepsin K activity observed in the MLII morphant (Fig. 4*B*). The findings are consistent with the lack of phenotypic rescue by the K732N mutation (Fig. 4*A*).

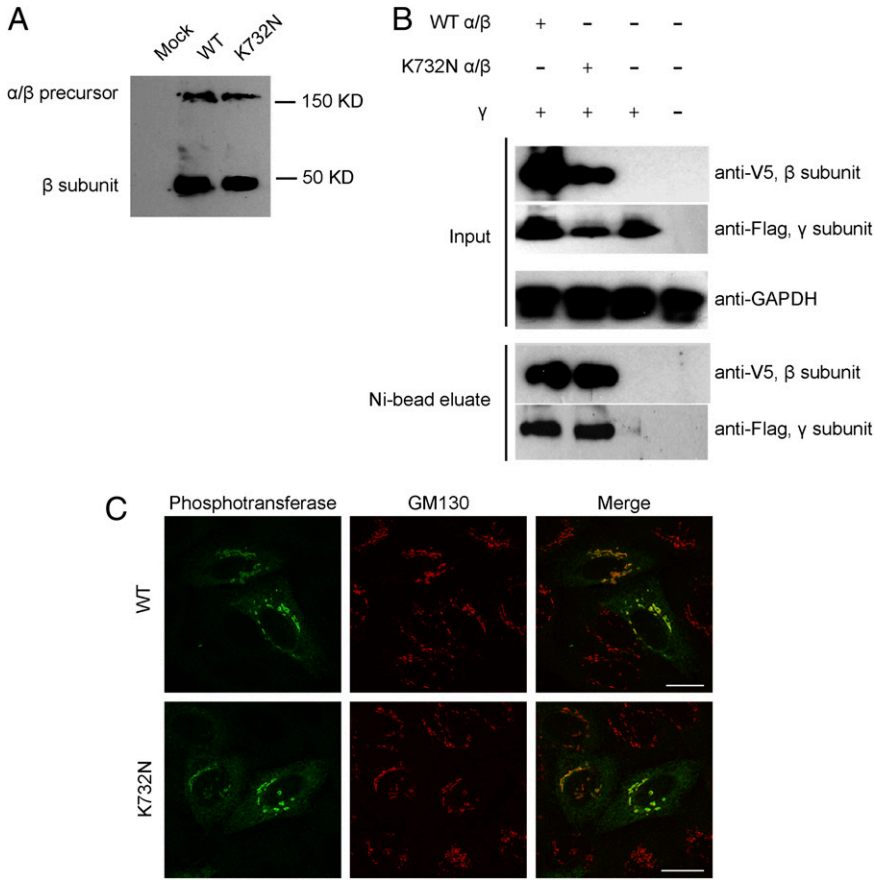

Fig. 2. The K732N mutant exhibits normal expression, interaction with the γ subunit, processing, and localization. (*A*) HEK293 cells were transfected with plasmids encoding WT or K732N mutant α/β cDNAs with a C-terminal His/V5 tag. After 48 h, cell extracts were prepared and subjected to SDS/PAGE and immunoblotting with anti-V5 antibody to detect the α/β precursor and the cleaved β subunit. (*B*) Lysates of HEK293 cells expressing WT or K732N mutant α/β subunits with His/V5 tags plus γ subunits with a Flag tag or γ subunit alone were incubated with Ni-NTA agarose to bind the β subunit. Bound proteins were eluted and subjected to SDS/PAGE and immunoblotting with anti-V5 to detect the β subunit and anti-Flag to detect the γ subunit. (*Upper*) The expression of proteins in the original lysates (Input), with GAPDH serving as a loading control. (*Lower*) The proteins bound and eluted from the Ni-NTA resin (Ni-bead eluate). Nontransfected cells served as an additional control. (*C*) HeLa cells were transfected with WT (*Upper*) or K732N (*Lower*) α/β cDNA in pcDNA6 and fixed with 4% paraformaldehyde/PBS 16 h after transfection. The cells were stained for immunofluorescence microscopy with antibodies to the alpha subunit of phosphotransferase (green) and the *cis*-Golgi marker GM130 (red). (Scale bars, 20 μm.)

An assay of the embryo extracts for GlcNAc-1-phosphotransferase activity toward αMM showed that both the WT and K732N mRNAs increased enzyme activity three- to fourfold over the MLII background (Fig. 4*C*). Aliquots of the various extracts were then applied to cation-independent Man-6-P receptor (CI-MPR) affinity columns, washed with 5 mM Glc-6-P-containing buffer, and eluted with 5 mM Man-6-P to release the bound phosphorylated acid hydrolases. The eluates were then assayed for β-galactosidase activity. As shown in Fig. 4*D*, 12.2 ± 2.6% of the β-galactosidase present in the WT embryo extract bound to the affinity column vs. 3.3 ± 0.7% of the enzyme in the MLII extract. Coinjections of WT mRNA with the morpholino significantly increased the phosphorylation of β-galactosidase (7.9 ± 3.2% binding), whereas the K732N mRNA had no significant effect (4.0 ± 1.0%).

Together, these findings provide additional evidence that although the K732N mutant retains catalytic activity toward the simple sugar αMM, it is impaired in its ability to phosphorylate lysosomal acid hydrolases.

Discussion

A critical step in the Man-6-P targeting pathway is the selective phosphorylation of acid hydrolases by GlcNAc-1-phosphotransferase. We have previously reported that this function is primarily mediated by the α/β subunits of the transferase, although details about the structural elements or domains that mediate the interaction are lacking (8). The data presented in this study demonstrate that the DMAP interaction domain of the α subunit plays a direct role in the binding of acid hydrolase substrates. In accordance, GST-DMAP was found to pull-down several acid hydrolases while failing to interact with nonlysosomal glycoproteins. In addition to these direct binding studies, we were able to take advantage of a missense mutation (K732N) in the DMAP interaction domain of GlcNAc-1-phosphotransferase reported in a patient with MLII. This disorder is characterized by extremely low levels of acid hydrolase phosphorylation (13), indicating that the K732N mutation leads to a major impairment of GlcNAc-1-phosphotransferase activity toward these substrates. Expression studies in HEK293 and HeLa cells showed that the mutation did not alter the processing, interaction with the γ subunit, localization in the Golgi, or stability of the enzyme. Importantly, the mutant enzyme exhibited catalytic activity toward the simple sugar αMM equal to that observed with the WT enzyme. However, its ability to phosphorylate the acid hydrolases cathepsin D and α-iduronidase was greatly impaired, which is consistent with a defect in binding to the protein recognition marker present in these substrates.

Further support for a role of the DMAP interaction domain in the recognition of acid hydrolases comes from experiments with the morpholino-based zebrafish model of MLII. In this system,

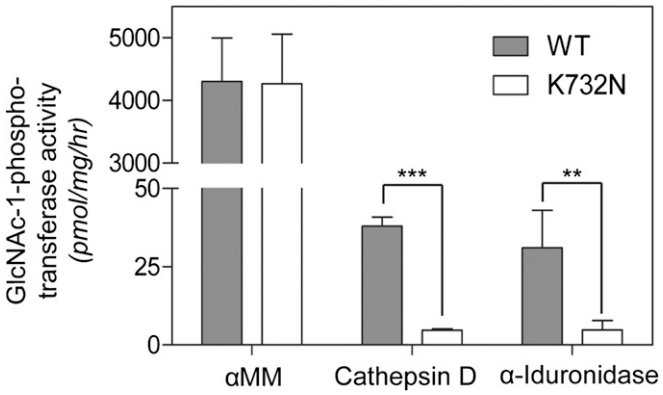

Fig. 3. Activity of WT and K732N mutant phosphotransferase toward various substrates. Lysates of HEK293 cells either mock-transfected or transfected with plasmids encoding WT or a K732N mutant αβ cDNA were assayed for GlcNAc-1-phosphotransferase activity toward αMM (100 mM), cathepsin D (15 μM), and α-iduronidase (15 μM), as described in *Materials and Methods*. The activity of mock-transfected cells has been subtracted. The values represent the average of 6–10 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.005$.

coinjection of WT GlcNAc-1-phosphotransferase mRNA with the morpholino rescued the MLII phenotype and restored β-galactosidase phosphorylation to levels approaching endogenous levels, whereas the K732N mRNA failed to correct these defects. Importantly, extracts of the embryos with either WT or K732N mRNA exhibited similar GlcNAc-1-phosphotransferase activity toward the simple sugar αMM. Together, these findings indicate that the K732N mutation leads to a selective impairment in acid hydrolase phosphorylation.

A number of studies from our laboratory and others have shown that the protein recognition domain of acid hydrolases is a complex conformation-dependent determinant that includes a number of critical lysine residues (16–23). On this basis, it seems likely that the interaction of an acid hydrolase with GlcNAc-1-phosphotransferase would involve multiple contacts between the surfaces of the two proteins. It is surprising, therefore, that a single missense mutation (K732N) has such a drastic effect on the ability of the transferase to phosphorylate acid hydrolases. One possibility is that the mutation causes a local conformational change that impairs the function of the entire DMAP interaction domain without altering the overall folding and catalytic activity of the enzyme. Consistent with this notion is the fact that to date we have been unable to express soluble GST-DMAP containing a K732N or K732A mutation in *Escherichia coli*. Regardless of the precise effects of the K732N mutation on the DMAP interaction domain, our findings support the conclusion that the DMAP interaction domain has a vital role in the recognition of acid hydrolases. However, it is certainly possible that other domains in the α/β subunits, as well as the γ subunit, contribute to this function. Further analysis of the consequences of other missense mutations in the α/β subunits detected in patients with MLII and MLIII on acid hydrolase phosphorylation may provide additional insights into the mechanism whereby GlcNAc-1-phosophotrasferase recognizes acid hydrolases. The combined approach taken in this study, including the use of the morpholino-based model of MLII in zebrafish to test the consequences of missense mutations in GlcNAc-1-phosphotransferase, provides a powerful means for advancing the understanding of this critical enzyme.

Materials and Methods

Materials. UDP-[6-^{3}H]GlcNAc (20 Ci/mmol) was purchased from PerkinElmer Life Sciences. [β-^{32}P]UDP-GlcNAc was prepared as described previously (24).

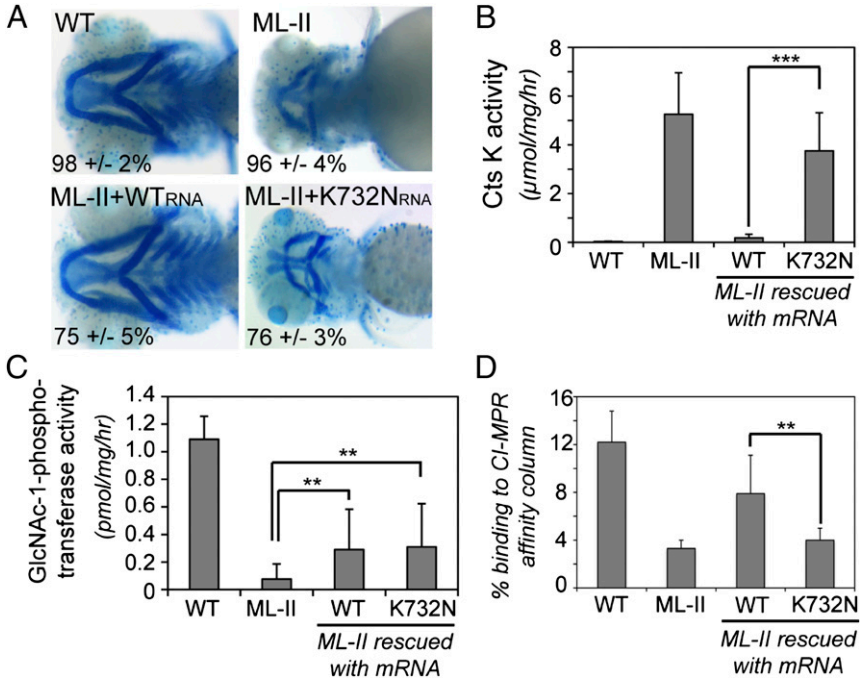

Fig. 4. The K732N mutant fails to rescue MLII zebrafish. (*A*) Embryos were stained at 4 dpf with Alcian blue to reveal cartilage; representative images are shown. The percentage of embryos with a staining pattern consistent with the images shown is presented. Results represent an average of 5 individual experiments, with 30–40 embryos scored per experiment. (*B*) Cathepsin K activity was measured in embryo lysates, using a fluorogenic peptide substrate. Average activity from 8 to 9 independent experiments is shown. Error bars represent SD for all of the graphs. $^{***}P < 0.01$. (*C*) GlcNAc-1-phosphotransferase activity toward αMM was determined in embryo lysates, using the UDP-[^{3}H]GlcNAc substrate. Results represent the average of 3 independent runs. $^{**}P < 0.05$. (*D*) Bars represent the percentage of β-galactosidase activity that is Man-6-P modified in embryo lysates that were fractionated using a CI-MPR affinity column. Averages from 4–5 individual experiments are shown. $^{**}P < 0.05$.

Glutathione agarose and Ni-NTA agarose were purchased from Qiagen. QAE-Sephadex, α-methyl D-mannoside, and the anti-Flag antibody were from Sigma. Anti-V5 antibody was purchased from Life Technologies. Cathepsin K fluorogenic substrate, (Z-Leu-Arg)2-Rhodamine 110, was purchased from Calbiochem. Genecellin transfection reagent was from BioCellChallenge. All other reagents were of the highest quality available and were purchased from Sigma or Fisher.

Cathepsin D was purified as described previously (25). Rabbit anti-cathepsin D antibody was prepared in our laboratory. α-Iduronidase and anti–α-iduronidase antibody were from W. Canfield (Genzyme). Lipoprotein receptor-related protein 9 and heparin-cofactor II, along with antibodies to these proteins, were from B. Doray and D. Tollefsen (Washington University in St. Louis), respectively.

DNA Constructs. Human WT GlcNAc-1-phosphotransferase α/β subunits' cDNA (NM_024312) in pcDNA6/V5-His vector was obtained from W. Canfield (Genzyme). Two point mutations (Ile392Val and Gln901Leu) were corrected. The K732N mutant plasmid was made with primers (5′-GGATACAATTTGTCCAATTCAGCCTTG-CTGAG-3′ and 5′-CTCAGCAAGGCTGAATTGGACAAATTGTATCC-3′), using the quick-change site-directed mutagenesis (Stratagene). The mutant's full sequence was confirmed by DNA sequencing.

Human WT GlcNAc-1-phosphotransferase γ subunit's cDNA (NM_032520) in pCMV vector was purchased from OriGene, and the cDNA sequence was confirmed by sequencing.

Mouse DMAP cDNA (aa694-819, NP_001004164.2) was cloned by primers (5′-CGCCGCGGATCCAAAATCCCCCGGGTAAATATTTC-3′ and 5′-CCGCCGCTCGAGT-CAGCCTAGTGTAGGCTGGGCC-3′) and PCR amplified from mouse brain cDNA library (Clontech). The PCR construct was then cut by BamHI/XhoI and cloned into pGEX 6P-1 vector (GE Healthcare), using general molecular cloning techniques.

GST and GST-DMAP Protein Expression. BL21 cells (Sigma) were transformed with the GST and GST-DMAP pGEX 6P-1 plasmids and grown in 1 L LB with 100 μg/mL ampicillin until OD_{600} was 0.4. The expression of the proteins was induced with 0.2 mg/mL isopropylthio-β-galactoside at room temperature (RT) for 16 h. The cells were collected by centrifugation at 6,000 × *g* for 15 min at 4 °C. The protein was purified on glutathione agarose, following the manufacturer's protocol.

GST Pull-Down. The GST pull-down experiments were performed in buffer A (50 mM Tris·HCl at pH 7.5, 150 mM NaCl, and 0.5% Triton X-100). GST and GST-DMAP (200 μg) were immobilized on 50 μL glutathione agarose for 2 h at RT. The beads with bound proteins were collected by centrifugation at 750 × *g* for 1 min, washed once, and incubated with 10 μg each of the various proteins in a volume of 50 μL for 2 h at RT with tumbling. The samples were then pelleted by centrifugation at 750 g for 1 min, and the beads were washed three times with 1 mL buffer A. The washed pellets were resuspended in 50 μL SDS sample buffer and heated at 100 °C for 5 min. Routinely, 20% of each pellet was loaded on SDS/PAGE, transferred to nitrocellulose membranes, and probed with antibodies to the various proteins.

Cell Culture and Transfection. HEK293 cells were obtained from American Type Culture Collection and cultured in DMEM containing L-glutamine, sodium pyruvate, and 4.5 g/L glucose, supplemented with 10% (vol/vol) FBS (Atlanta Biologicals), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). The cells were grown at 37 °C with 5% CO_2 in 6-well plates until they reached 60–70% confluence. Plasmid DNA (2 μg) was diluted in 200 μL serum-free DMEM, and 8 μL Genecellin transfection reagent was added to the solution. The sample was mixed well and incubated at RT for 15 min. The Genecellin/DNA mixture was then placed onto the cells in drops.

After 48 h, the cells were solubilized with a buffer composed of 0.1 M Tris at pH 8.0, 150 mM NaCl, 1% Triton X-100, and protease inhibitor mixture (Complete, Roche Applied Science) and analyzed for GlcNAc-1-phosphotransferase α/β and γ expression by SDS/PAGE and immunoblot, using anti-V5 and anti-Flag antibodies, respectively.

Immunofluorescence Microscopy. HeLa cells were cultured in DMEM containing L-glutamine, sodium pyruvate, and 4.5 g/L glucose, supplemented with 10% (vol/vol) FBS and penicillin/streptomycin. The day before transfection, the cells were transferred to 12-well plates containing coverslips. The cells were transfected at ∼50% confluence with 700 ng WT or K732N α/β phosphotransferase cDNA in pcDNA6 using Lipofectamine Plus (Life Technologies), according to the manufacturer's protocol. The cells were fixed 16 h after transfection with 4% (wt/vol) paraformaldehyde in PBS for 30 min at RT, washed with PBS, and permeabilized with 0.1% saponin/2% (wt/vol) BSA in PBS (permeabilization buffer) for 10 min. The cells were stained with rabbit anti-phosphotransferase α subunit (gift from W. Canfield) with a 1:1,000 dilution and with mouse anti-GM130 (BD Transduction Laboratories) with a 1:500 dilution for 1 h at RT, washed 3 times for 10 min with permeabilization buffer, and incubated with Alexa Fluor 488-coupled goat anti-rabbit IgG and Alexa Fluor 568-coupled goat anti-mouse IgG (Molecular Probes) for 1 h at RT. Subsequently, the cells were washed 3 times for 10 min with permeabilization buffer, washed quickly with water, and mounted with ProLong Gold antifade reagent (Molecular Probes). The cells were analyzed under a Zeiss LSM 510 Meta confocal microscope using a 63× objective with N.A. 1.4.

GlcNAc-1-Phosphotransferase Assay. The reaction mixtures contained various substrates and the HEK293 cell lysate (100 μg total protein) in 50 mM Tris·HCl at pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 75 μM UDP-[^{3}H]GlcNAc or [β-^{32}P]UDP-GlcNAc (1 μCi), and 2 mg/mL BSA in a final volume of 100 μL. For assays containing 15 μM Cathepsin D or α-iduronidase, the reactions were terminated by addition of 300 μL 1.5% (wt/vol) phosphotungstic acid, 0.75 N HCl, and 100 μL 1 mg/mL BSA. Samples were vortexed and centrifuged at 25,000 × *g* for 10 min at 4 °C. The pellets were washed three times with 1 mL of the acid mixture and resuspended in 1 mL of 50 mM Tris·HCl at pH 11. The incorporated [^{3}H]GlcNAc-P was determined after addition of 10 mL EcoLite scintillation fluid (MP Biomedicals). For assays containing 100 mM αMM substrate, reactions were terminated by the addition of 1 mL of 5 mM EDTA at pH 8.0. The samples were applied to 1-mL columns of QAE-Sephadex equilibrated with 2 mM Tris base at pH 8.0. The columns were washed with 1 mL of 2 mM Tris base three times, and the phosphorylated products were eluted with 1 mL of 30 mM NaCl in 2 mM Tris base three times. The radioactivity in all of the fractions was determined by scintillation counting.

Assay of Complex Formation of the α/β Subunits with the γ Subunit. Human WT and K732N GlcNAc-1-phosphotransferase α/β subunits with C-terminal His/V5 tags in pcDNA6 vectors and human phosphotransferase γ subunit with a C-terminal Flag tag in pCMV vector were coexpressed in HEK293 cells. The cell lysates (200 μg total protein) were incubated with 50 μL Ni-NTA resin overnight at 4 °C with rocking. The resin was washed three times with 1 mL buffer A. The washed resin was resuspended in SDS sample buffer and heated at 100 °C for 5 min, and 20% of the eluates were subjected to SDS/PAGE and then probed with anti-V5 and anti-Flag antibodies in immunoblots.

Zebrafish Strains and Embryo Maintenance. WT zebrafish were obtained from Fish 2U and maintained using standard protocols. Embryos were staged according to the criteria established by Kimmel et al. (26) In some cases, 0.003% 1-phenyl-2-thiourea was added to the growth medium to block pigmentation. Handling and euthanasia of fish for all experiments were carried out in compliance with the policies of the University of Georgia and this protocol was approved by the University of Georgia Institutional Animal Care and Use Committee (permit A2012 07–037-Y1-A0).

Antisense Morpholino Injection and mRNA Rescue. The mouse DMAP interaction domain (694-819, NP_001004164.2) was cloned to replace the zebrafish DMAP interaction domain (691-803, NP_001038233.1) in a plasmid containing WT zebrafish *gnptab* cDNA (NM_001044768.3) in pcDEST vector using primers 5′-GGGAACAAACACACGGTGATGTAGGTACCAAAA-TCCCCCGGGTAAAT-3′/5′-CTCTATGTTGAACAACCGCTCGAGGCCTAGTGTA-GGCTGGGCC-3′ and 5′- ATTTACCCGGGGGATTTTGGTACCTACATCACCGT-GTGTTTGTTCCC-3′/5′- GGCCCAGCCTACACTAGGCCTCGAGCGGTTGTTCAA-CATAGAG-3′ to prepare zebrafish *gnptab*/mouse DMAP chimera. KpnI and XhoI restriction sites were introduced to the 5′ and 3′ ends of the mouse DMAP interaction domain, respectively. K732N mutation was made by primers 5′-GAAAGGATATAACTTGTCCAATTCAGCCCTGCTAAGG-3′ and 5′-CCTTAGCAGGGCTGAATTGGACAAGTTATATCCTTTC-3′, using the quick-change site-directed mutagenesis (Stratagene).

Expression of GlcNAc-1-phosphotransferase (α/β subunit, *gnptab*) was inhibited by injection of morpholino oligonucleotides, as previously described (14). Experiments involving mRNA rescue in the morphant background were performed on embryos sequentially injected with 8 ng of the *gnptab* splice blocking morpholino oligonucleotides, which was previously validated to yield maximal specific inhibition, and 300 pg of the appropriate mRNA. According to titration experiments, 300 pg of either the *gnptab* or *gnptab*/DMAP chimera RNA yielded the highest number of rescued MLII embryos. Rescue was assessed by Alcian blue-stained cartilage and normalized cathepsin K protease values. The Message Machine kit (Ambion) was used to synthesize all full-length mRNAs from PCSII+ constructs containing either a chimera of mouse DMAP and zebrafish *gnptab* (referred to as WT in text) or the *gnptab*/DMAP chimera containing the K732N mutation (referred to as K732N in text). The

PCSII+WT zebrafish gnptab construct was generated as previously described (14) and used to generate both the WT DMAP chimera and the K732N mutant.

Alcian Blue Staining and Quantification of Craniofacial Phenotypes. Embryos were stained with Alcian blue, as described previously (14). Analysis of craniofacial structures was performed using the morphometric parameters outlined in *Results.* Stained embryos were photographed on an Olympus SZ-16 dissecting scope outfitted with Q-capture software and a Retiga 2000R color camera.

Enzyme Assays and CI-MPR Affinity Chromatography. The activity of GlcNAc-1-phosphotransferase and cathepsin K, as well as the percentage of mannose phosphorylated β-galactosidase activity, was determined from the same embryo lysate in each independent experiment. GlcNAc-1-phosphotransferase activity was determined as described earlier, using αMM as substrate and UDP-[^{3}H]GlcNAc as donor. Cathepsin K activity assays were performed using fluorogenic substrates, as previously described (15). CI-MPR affinity chromatography was performed as described previously (14). Activity of β-galactosidase was measured in each column fraction, using 4-methylumbelliferyl-β-galactoside. Reactions were performed in 50 mM citrate buffer at pH 4.5 and 0.5% Triton X-100 containing 3 mM substrate.

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