Structural insights into how GlcNAc-1-phosphotransferase directs lysosomal protein transport

Received for publication, August 19, 2021, and in revised form, February 3, 2022 Published, Papers in Press, February 9, 2022
https://doi.org/10.1016/j.jbc.2022.101702

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Edited by Gerald Hart

GlcNAc-1-phosphotransferase catalyzes the initial step in the formation of the mannose-6-phosphate tag that labels ~60 lysosomal proteins for transport. Mutations in GlcNAc-1-phosphotransferase are known to cause lysosomal storage disorders such as mucolipidoses. However, the molecular mechanism of GlcNAc-1-phosphotransferase activity remains unclear. Mammalian GlcNAc-1-phosphotransferases are αβ2γ2 hexamers in which the core catalytic α- and β-subunits are derived from the GNPTAB (N-acetylglucosamine-1-phosphate transferase subunits alpha and beta) gene. Here, we present the cryo-electron microscopy structure of the Drosophila melanogaster GNPTAB homolog, DmGNPTAB. We identified four conserved regions located far apart in the sequence that fold into the catalytic domain, which exhibits structural similarity to that of the UDP–glucose glycoprotein glucosyltransferase. Comparison with UDP–glucose glycoprotein glucosyltransferase also revealed a putative donor substrate-binding site, and the functional requirements of critical residues in human GNPTAB were validated using GNPTAB-knockout cells. Finally, we show that DmGNPTAB forms a homodimer that is evolutionarily conserved and that disturbing the dimer interface undermines the maturation and activity of human GNPTAB. These results provide important insights into GlcNAc-1-phosphotransferase function and related diseases.

Protein phosphorylation is universally present as a regulatory strategy in eukaryotic cells. Glycan phosphorylation, though not as abundant, also plays essential roles in modulating cellular processes, particularly within the secretory compartments. A canonical glycan phosphorylation event involves ~60 secretory proteins that are destined for lysosomes. Similar to other secretory molecules, these lysosomal proteins are first synthesized in the endoplasmic reticulum and then traverse through the Golgi network. At the Golgi apparatus, these proteins are “phosphorylated” on a terminal mannose residue in their N-linked glycans, resulting in the formation of a mannose 6-phosphate (M6P) tag that is recognized by two specific M6P receptors to direct their lysosomal transport. Interestingly, this essential modification is not performed by an ATP-dependent kinase but is generated by the sequential action of two enzymes: first, the N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) catalyzes the addition of an N-acetylglucosamine-1-phosphate group to the terminal mannose, and the GlcNAc-1-phosphodiester α-N-acetylglucosaminidase then removes the GlcNAc moiety to uncover M6P (1).

Mammalian GlcNAc-1-phosphotransferases are large protein complexes that comprise two α-subunits, two β-subunits, and two γ-subunits (2, 3). The catalytic α- and β-subunits are first synthesized as GNPTAB fusion proteins. GNPTAB has a complex structural organization and includes two transmembrane segments, four conserved regions (CR1–CR4), two Notch repeats (N1 and N2), a DNA methyltransferase-associated protein (DMAP) interaction domain, and several spacer regions (S1–S4) (Fig. 1A). The two transmembrane segments anchor GNPTAB on the Golgi membrane and project most of the molecule into the Golgi lumen. The four CRs are conserved in Stealth proteins, and members of this family can function as hexose-phosphate transferases in bacteria to synthesize cell wall polysaccharides (4). The Notch repeats and DMAP interaction domain mediate the interaction between GlcNAc-1-phosphotransferase and its diverse lysosomal protein substrates (5–7). The spacer regions are also functionally important. For example, the S2 region is responsible for interacting with the γ-subunit (8, 9). S3 contains a recognition site for the site-1 protease, which cleaves GNPTAB into its α- and β-subunits and thereby leads to catalytic activation (10). The S1 spacer facilitates proper processing of GNPTAB by the site-1 protease (11). S4 contains an EF-hand calcium-binding motif, but its function remains unclear. The γ-subunit is encoded by GNPTG and plays a regulatory function to promote the activity of the GlcNAc-1-phosphotransferase holoenzyme toward a subset of substrates (12).

More than 200 mutations in GlcNAc-1-phosphotransferase have been documented in patients with mucolipidoses, a group
of human lysosomal storage disorders that include skeletal and neuronal abnormalities (summarized in (13)). Mutations in GlcNAc-1-phosphotransferase have also been linked to stuttering (14). On the other hand, GlcNAc-1-phosphotransferase might serve as a potential antiviral target because a number of viruses, such as the Ebola virus and the common cold coronaviruses OC43 and 229E, rely on the lysosomal pathway for infection and egress (15, 16). However, the underlying molecular mechanism of GlcNAc-1-phosphotransferase remains insufficiently understood. Here, we sought to characterize the structural architecture of GlcNAc-1-phosphotransferase by cryo-electron microscopy (cryo-EM) and successfully determined the 3.5-Å structure of the Drosophila melanogaster GNPTAB. Our results reveal critical structural features that are conserved in the GNPTAB family. We then generated a GNPTAB-knockout cell line using the CRISPR-Cas9 genome editing technique and validated the importance of residues in human GNPTAB involved in donor substrate binding and dimerization. We also analyzed pathogenic missense mutations and assessed their potential impacts. Together, our results advance the understanding of GlcNAc-1-phosphotransferase and related human diseases.

Results and discussion

Cryo-EM structure of D. melanogaster GNPTAB

We sought to investigate the structural basis of GlcNAc-1-phosphotransferase. Despite intensive attempts, we were unable to obtain the structure of a mammalian GlcNAc-1-phosphotransferase and could not determine the structure of the α/β subcomplex or the GNPTAB precursor. D. melanogaster has a GNPTAB homolog (DmGNPTAB) but lacks a discernable gene encoding GNPTG (4, 17). The DmGNPTAB protein is markedly more compact than its human counterpart (Fig. 1A). Specifically, this protein contains all four CRs and a similar CR3-CR4 spacer but has shorter CR1-CR2 and CR2-CR3 spacers, only one Notch repeat, and lacks the DMAP interaction domain and S1P cleavage site (Figs. 1A and S1). We obtained the luminal portion of DmGNPTAB and analyzed its structure by cryo-EM (Figs. 1B and S2). The structure was determined at an overall resolution of 3.5 Å (Table 1). The center region displayed high resolutions, which allowed us to build the structural model de novo. Approximately half of the protein molecule, including all four CRs and the CR3-CR4 spacer, could be confidently placed. The rest of the molecule, particularly the CR2-CR3 spacer including the Notch module, displayed weak densities and was thus not modeled. The amino and carboxyl termini are located on the same side of a DmGNPTAB monomer, which sheds light on the topology of the full-length protein dimer on the Golgi membrane (Fig. 1B). The membrane localization of mammalian GNPTAB is likely important for its proper processing by membrane-bound S1P within the Golgi apparatus (10).

Two domains can be clearly discerned in the structural model of the DmGNPTAB monomer (Fig. 1C). The large domain, which is referred to as the Stealth domain, features an α/β fold that includes all four Stealth CRs. CR1 contains a β-strand that occupies the center of the Stealth domain. CR2
comprises three β-strands and two α-helices. The three strands sandwich the CR1 strand in a parallel manner to form a β-sheet, whereas the two helices are situated on each side of the sheet. CR3 consists of one strand and three helices. The single CR3 strand runs antiparallel to the four strands described above, and the three helices bundle together with the short helix in CR2. CR4 features a single helix, which packs against the long helix in CR2. The small domain is encoded by the CR3-CR4 spacer, including the calcium-binding EF hand motif, and features a helix bundle to mediate dimerization.

**Structure of the stealth domain**

The four Stealth CRs, which are located far apart in the sequence, fold into a single globular domain. Structural homology search suggests that the Stealth domain displays structural similarities to several GT-A-type glycosyltransferases (18) despite low sequence homology. In particular, the Stealth domain exhibits structural resemblance to UDP-glucose glycoprotein glucosyltransferase (UGGT), particularly at the central β-sheet region (Fig. 2, A and B). UGGT is an ER-resident protein that surveils the folding status of secretory glycoproteins (19). Similar to GlcNAc-1-phosphotransferase, UGGT is a label marker, and the labeling of UGGT also occurs on the terminal mannose of an N-linked glycan. Instead of labeling proteins that are destined for lysosomes, UGGT labels proteins that are incompletely folded; specifically, it recognizes misfolded glycoproteins and transfers a glucose residue to the terminal mannose on the glycan of these proteins, and this modification is recognized by ER chaperones, including calnexin and calreticulin, to facilitate correct folding.

Cryo-EM structures of UGGT homologs from several thermophilic fungi have been determined (20, 21). A structural comparison between the Stealth domain of DmGNPTAB and the catalytic domain of Thermomyces dupontii UGGT (TdUGGT) in complex with UDP-glucose offers a glimpse into the sugar nucleotide-binding site of DmGNPTAB. UDP-glucose binds to the surface pocket of TdUGGT (Fig. 2A). DmGNPTAB has a similar surface pocket, lined by a group of conserved residues, including Thr69 from CR1; Ser156, Ile159, Glu160, Tyr175, Asn177, Asp178, and Asp179 from CR2; His375, Phe378, Arg405, and Gln411 from CR3; Phe546 and Met548 from the CR3-CR4 spacer; and Cys572, Asn574, and Asn576 from CR4 (Fig. 2, B and C). The functional importance of these residues is underscored by the fact that missense mutations of a number of the corresponding human residues have been found in patients (see below). A Ca$^{2+}$ ion facilitates the accommodation of UDP-glucose in TdUGGT and is coordinated by three Asp residues, including Asp1294 and Asp1296 in the Asp-X-Asp signature motif of GT-A glycosyltransferases and Asp1427 (Fig. 2D). A well-conserved Asn177-Asp178-Asp179 motif in CR2 of DmGNPTAB (Fig. S1) aligns with the Asp-X-Asp motif in TdUGGT, whereas Cys572 appears to take the position of TdUGGT-Asp1427. It is thus likely that these residues are also involved in the binding to a divalent cation that assists in positioning the UDP-GlcNAc in DmGNPTAB.

The Notch repeat domain displays weak density and cannot be unambiguously modeled. Nevertheless, it appears to be located in the vicinity of the sugar nucleotide-binding pocket and opposite the side that faces the membrane (Fig. 1B). The Notch domains of human GNPTAB are involved in binding to the lysosomal protein substrates (6, 7). Based on the structural comparison with TdUGGT (Fig. 2B), we envisioned that the donor substrate UDP-GlcNAc is likely accommodated in DmGNPTAB in an orientation similar to that of UDP-glucose in TdUGGT, with the N-acetylglucosamine group holding toward the direction of the Notch domain that engages the cognate acceptor substrates in *Drosophila*.
markedly swollen lysosomes compared with the parental cells (Fig. 3B), which indicated that lysosomal function is severely impaired in these cells. As a result of lysosomal dysfunction, the lysosomal cysteine protease cathepsin B (CatB) was not properly processed, and the mature form of endogenous CatB at 30 kDa was not detected in the lysates of GNPTAB−/− cells (Fig. 3C). This finding was also consistent with previous observations obtained with GNPTAB-deficient HAP1 cells (15).

We then generated alanine substitutions of Asn406, Asp408, and Cys1149, which are equivalent to Asn177, Asp179, and Cys572 in DmGNPTAB that form the putative metal-binding site (Fig. 2D) and examined the abilities of these mutants to rescue CatB maturation. As expected, the expression of WT GNPTAB restored the mature form of CatB (Fig. 3D). In contrast, N406A, D408A, and C1149A displayed reduced activities compared with the WT protein, and D408A appeared to be completely inactive. These results demonstrate the importance of these residues for the function of GlcNAc-1-phosphotransferase, corroborating our structural analyses.

A conserved dimeric architecture

Mammalian GlcNAc-1-phosphotransferases are α2β2γ2 hexameric complexes, and the construction of the hexamer remains poorly understood. In our structure, two DmGNPTAB molecules form a homodimer that resembles two fishes nesting against each other in a head-to-tail orientation (Fig. 1B). The dimer is mainly mediated by the CR3-CR4 spacer and CR4, whose counterparts both reside in the β-subunit of human GlcNAc-1-phosphotransferase (Fig. 1A). A few residues in CR2 also contribute to dimer formation. The dimer interface buries 1500-Å² solvent-accessible surfaces from each molecule and involves a number of invariant residues (Fig. S1), which suggests that the dimerization mechanism observed in this study is likely generally conserved in the GNPTAB family.

To verify the functional relevance of the dimer, we generated two human GNPTAB mutants: G1 (T1019N/D1020G/Q1021S) and G2 (T1035N/R1036G/I1037S). These two mutants were designed to create sites that allow the attachment of N-linked glycans. Thr1019 and Thr1035 in human GNPTAB correspond to Thr438 and Thr454 in DmGNPTAB, both of which are located in the dimer interface (Fig. 4A). The introduction of bulky glycans at these positions would impede dimerization of the human protein. We tagged these mutants with Flag tags at the C terminus, co-expressed them with V5-tagged WT GNPTAB in HEK293T cells, and performed Flag immunoprecipitation. Flag-tagged and V5-tagged WT GNPTAB proteins were efficiently coprecipitated (Fig. 4B), which indicated the formation of GNPTAB dimers in the cells. In contrast, the interactions between G1 or G2 and WT GNPTAB were markedly reduced, which suggested that these two mutants exhibit decreased abilities to dimerize with the WT protein. Furthermore, unlike WT GNPTAB, these two
The CR3-CR4 spacer and CR4 play dominant roles in mediating the formation of the DmGNPTAB homodimer, which likely reflects how the β2 dimer is formed in human GlcNAc-1-phosphotransferase. In addition to the interactions between β-subunits, Cys70 in human GNPTAB is involved in disulfide-linked homodimerization of the α-subunits (8). Cys61 in DmGNPTAB appears to align with Cys70, and in the DmGNPTAB dimer, two Cys61 residues from the two monomers are located in close proximity (Fig. 4A), which suggests that a disulfide bond between the two corresponding Cys70 in human GNPTAB could be readily formed to further stabilize the αβ22 subcomplex. The γ-subunit also forms a disulfide-linked homodimer (3), and only a γ2 dimer can be assembled into the GlcNAc-1-phosphotransferase holoenzyme (22). The γ2 dimer, through its interactions with the S2 spacers in the two α-subunits, supplies another layer of interaction to architect the αβ22y2 hexamer.

**Cryo-EM structure of GlcNAc-1-phosphotransferase**

Our results provide molecular insights into the pathogenesis of human mucolipidoses caused by GNPTAB mutations. More than 200 mutations in GNPTAB, including at least 65 missense mutations, have been detected in patients with various forms of mucolipidoses (13). The effects of some of these mutations have been characterized biochemically, but the structure of human GNPTAB has remained elusive despite its long research history. The structure of DmGNPTAB reveals a dimeric framework that sheds light on the structural core of human GlcNAc-1-phosphotransferase and can be used to further assess the molecular impacts of disease-causing mutations (Fig. 5). For example, the human GNPTAB residues Ser385, Glu389, Asp407, Asp408, His956, Arg986, and Asn1153 correspond to Ser156, Glu160, Asp178, Asp179, His375, Arg405, and Asn576 in DmGNPTAB (Fig. 5A), all of which are found among the sugar nucleotide-binding pockets described above (Fig. 2C). Thus, their mutations likely affected the binding of UDP-GlcNAc, consistent with the findings from previous biochemical studies showing that missense mutations involving these sites, such as S385L, E389K, D407A, D408N, H956Y, R986C, and N1153, resulted in markedly decreased enzyme activities (7, 23, 24). We also showed that D408A was unable to restore the maturation of CatB in GNPTAB−/− cells (Fig. 3D). Another set of mutations, including W81L, R334Q, and R334L, S399F, I403T, and D1018G, displayed defects in exit from the ER, which indicated protein misfolding (7). These sites were also conserved in DmGNPTAB (Trp70, Arg105, Ser170, and Asp435; Fig. 5A). Trp70 and Leu174 are both present in the interior of the structure and are intimately packed with surrounding hydrophobic residues. Arg105 forms a bidentate interaction with Glu607 to support Pro601, which is involved in dimerization. Ser170 likely forms hydrogen bond interactions with Asp65 and Arg115, and the corresponding human residues, Asp76 and Arg115, are also mutated in some patients (Fig. 5A). Similarly, some of the other mutations also lead to structural disturbance of human GlcNAc-1-phosphotransferase, as can be rationalized by the DmGNPTAB structure.
In summary, we have elucidated the cryo-EM structure of the *D. melanogaster* GNPTAB homolog, which allows extrapolation of the core structure of human GlcNAc-1-phosphotransferase. One deficiency of our study is the lack of further mechanistic insight into the function of human GlcNAc-1-phosphotransferase. In particular, the most critical issue regarding the molecular mechanism of GlcNAc-1-phosphotransferase, namely, its specific targeting of lysosomal proteins among thousands of glycoproteins that traverse the Golgi apparatus, remains to be addressed. The molecular interaction between the α/β- and γ-subunits also remains to be structurally characterized. Nevertheless, our structural data offer a valuable model to advance the understanding of human GNPTAB and also provide deeper insights into the pathogenesis of human mucolipidoses caused by GNPTAB mutations.

**Experimental procedures**

**Protein expression and purification**

The *D. melanogaster* GNPTAB gene (CG8027) was cloned from a fly cDNA library. The DNA fragment encoding DmGNPTAB residues 50 to 630 was cloned into the psMBP2 vector (25), which facilitates its expression in insect cells as a secreted His6-MBP fusion protein with a tobacco etch virus protease cleavage site. Bacmids were generated in DH10Bac cells using the Bac-to-Bac system (Invitrogen). Sf21 insect cells grown in SIM SF medium (Sino Biological Inc) were used to generate and amplify the baculoviruses. For protein production, Hi5 cells grown in SIM HF medium (Sino Biological Inc) were infected at a density of 1.5 to 2.0 × 10^6 cells/ml. Forty-eight hours later, conditioned media were collected by centrifugation at 2000g for 30 min. The media were then concentrated using a Hydrosart Ultrafilter (Sartorius) and transferred into binding buffer containing 25 mM Tris-HCl, pH 8.0, and 150 mM NaCl. The recombinant protein was then isolated using Ni-NTA resin (GE Healthcare) and eluted with a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 500 mM imidazole. After tobacco etch virus protein digestion for 10 h at 4°C to remove the N-terminal His6-MBP tag, the protein mixture was transferred into a buffer containing 25 mM Tris-HCl, pH 8.0, and 50 mM NaCl using a Centricon with a cutoff of 10 kDa (Millipore). The untagged proteins were then purified by anion exchange chromatography (Resource Q) and eluted using a 50 to 1000 mM NaCl salt gradient in 25 mM Tris-HCl, pH 8.0, followed by size exclusion chromatography (Superdex Increase 200) and elution in 25 mM Hepes, pH 7.5, and 150 mM NaCl.

**Cryo-EM data collection, model building, and structure analyses**

Four-microliter aliquots of purified DmGNPTAB at 0.5 mg/ml were applied onto glow-discharged Quantifoil holey-carbon grids (R1.2/1.3, 300 mesh, gold), blotted at 4°C in 100% humidity, and plunged into liquid ethane with a Vitrobot Mark IV (FEI). The cryogrids were screened with a 200-kV Talos Arctica microscope. Data collection was performed with a 300-kV Titan Krios G3 microscope equipped with a Gatan GIF Quantum K2 Summit direct electron detector using SerialEM
The generation and characterization of GNPTAB were performed using the DALI server (32). Figures were used as landmarks during the structural modeling process (30). Bulky aromatic residues and N-linked glycosylation sites were used as landmarks during the structural modeling process. A total of 131,301 particles were then selected for 3D refinement, which resulted in a map with an overall resolution of 3.53 Å after Bayesian polishing and contrast transfer function refinement. The resolution was estimated using the gold-standard Fourier shell correlation 0.143 criteria.

The structure of DmGNPTAB was built de novo in Coot (30). Bulky aromatic residues and N-linked glycosylation sites were used as landmarks during the structural modeling process. Structure refinement was performed using real-space refinement in Phenix (v1.18) (31). A structural homology search was performed using the DALI server (32). Figures were prepared with ESPript (33), PyMOL (Schrödinger), and UCSF Chimera (34).

**Generation and characterization of GNPTAB−/− HeLa cells**

HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a 5% CO2 incubator. GNPTAB−/− cells were generated using CRISPR/Cas9 technology. The guide RNA, 5'-ACAAAAACATG GTATTGATCT-3', which targets exon 2 of GNPTAB, was cloned into the pSpCas9(BB)-2A-GFP vector (Addgene, 48,138). Three micrograms of the plasmid were then transfected into HeLa cells using Lipofectamine 2000 (Thermo Scientific). Two days after transfection, GFP-positive cells were sorted into single clones using an Astraqs EQ cell sorter (Beckman Coulter). Single clones were cultured in 96-well plates for 2 weeks. The genome type of the knockout cells was determined by DNA sequencing.

For the visualization of lysosomes, parental and GNPTAB−/− HeLa cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 min, permeabilized with PBS containing 0.1% saponin and 2% bovine serum albumin, and then incubated with anti-LAMP1 antibody (Santa Cruz, sc-20011, 1:50) at 4 °C overnight. The next day, the cells were washed with PBS and incubated with Alexa Fluor 488 donkey anti-mouse antibody (Invitrogen, A21202, 1:150) for 1 h at 25 °C. The coverslips containing the cells were then re-washed with PBS, incubated with DAPI for the visualization of nuclei, and examined with a Delta Vision microscope. The images were analyzed using Velocity (v6.1.1) software.

To examine the maturation of CatB, WT and mutant GNPTAB-Flag plasmids were transfected into GNPTAB−/− HeLa cells as indicated. Seventy-two hours later, the cells were harvested, washed with PBS, lysed in lysis buffer (PBS supplemented with 0.5% Triton X-100, 1% protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C, and analyzed by Western blotting.

**Immunoprecipitation and Western blotting**

The human GNPTAB gene was cloned into modified pcDNA 3.1 vectors that encode a C-terminal Flag or V5 tag. Mutations were generated using a PCR-based method and verified by sequencing. For the immunoprecipitation experiments, V5-tagged GNPTAB was cotransfected with Flag-tagged GNPTAB into HEK293T cells in 10-cm dishes using polyethyleneimine. Forty-eight hours later, the cells were harvested, washed with PBS, and lysed in lysis buffer. The lysates were then centrifuged for 15 min at 13,000g, and the supernatant was incubated with Flag M2 beads (Sigma, A2220) for 2 h at 4 °C. The beads were washed three times with lysis buffer. The immunoprecipitated proteins were eluted from the beads using the 3x Flag peptide (NJPeptide, NJP50002) and analyzed by Western blotting.

For the Western blot analyses, protein samples separated by SDS-PAGE were transferred to nitrocellulose membranes, and the membranes were then blocked with 4% nonfat milk for 30 min at 25 °C and incubated with primary antibody overnight at 4 °C. The next day, the membranes were incubated with HRP-conjugated secondary antibodies in 4% nonfat milk for 1 h at room temperature. Detection was performed by enhanced chemiluminescence using an Amersham Imager 800. The primary antibodies used for immunoblotting were anti-Flag (ABclonal, AE005, 1:1000), anti-Flag (MBL, PM020, 1:1000), anti-V5 (Santa Cruz, sc-81594, 1:1000), anti-V5 (Millipore, AB3792, 1:1000), anti-CatB (Cell Signaling, 31,718, 1:1000), and anti-GAPDH (TransGen, HC301-02, 1:5000). The secondary antibodies were goat anti-mouse (TransGen, HS201-01, 1:5000) and goat anti-rabbit (TransGen, HS101-01, 1:5000).

**Data availability**

The cryo-EM map and atomic coordinates of DmGNPTAB have been deposited in the EMDB and PDB with accession codes EMD-30910 and 7DXI, respectively.

**Supporting information**—This article contains supporting information.

**Acknowledgments**—We thank the Core Facilities at the School of Life Sciences, Peking University, for help with negative-staining EM; the Cryo-EM Platform of Peking University for help with data collection; the High-performance Computing Platform of Peking University for help with computation; and the National Center for Protein Sciences at Peking University for assistance with cell sorting.

**Author contributions**—S. D., G. W., Z. Z., and C. M. investigation; N. G. and J. X. supervision; J. X. conceptualization; J. X. writing—original draft; J. X. funding acquisition.

**Funding and additional information**—The work was supported by the National Key Research and Development Program of China.
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(2017YFA0505200 to J. X., 2019YFA0508904 to N. G.), the National Science Foundation of China (31822014 to J. X., 31725007 and 31630087 to N. G.), and the Qidong-SLS Innovation Fund to J. X. and N. G.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CatB, cathepsin B; cryo-EM, cryo-electron microscopy; CR1 to CR4, conserved regions in the Stealth proteins; DMAP, DNA methyltransferase-associated protein interaction domain; M6P, mannose 6-phosphate; N1 and N2, Notch repeats; S1 to S4, spacer regions; TdUGGT, Therma myces dupontii UGGT; UGGT, UDP-glucose glycoprotein glucosyltransferase.

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