

Molecular determinants of Neu5Ac binding to a tripartite ATP independent periplasmic (TRAP) transporter

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This **valuable** work provides novel insights into the substrate binding mechanism of a tripartite ATP-independent periplasmic (TRAP) transporter, which may be helpful for the development of specific inhibitors. The structural analysis is **convincing**, but additional work will be required to establish the transport mechanism as well as well as binding sites for all ligands. This study will be of interest to the membrane transport and bacterial biochemistry communities.

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Abstract

N-Acetylneuraminic acid (Neu5Ac) is a negatively charged nine-carbon amino-sugar that is often the peripheral sugar in human cell-surface glycoconjugates. Some bacteria scavenge, import, and metabolize Neu5Ac or redeploy it on their cell surfaces for immune evasion. The import of Neu5Ac by many bacteria is mediated by tripartite ATP-independent periplasmic (TRAP) transporters. We have previously reported the structures of SiaQM, a membrane-embedded component of the *Haemophilus influenzae* TRAP transport system, (Currie *et al.*, 2024). However, none of the published structures contain Neu5Ac bound to SiaQM. This information is critical for defining the transport mechanism and for further structure-activity relationship studies. Here, we report the structures of *Fusobacterium nucleatum* SiaQM with and without Neu5Ac. Both structures are in an inward (cytoplasmic side) facing

conformation. The Neu5Ac-bound structure reveals the interactions of Neu5Ac with the transporter and its relationship with the Na⁺ binding sites. Two of the Na⁺-binding sites are similar to those described previously. We identify a third metal-binding site that is further away and buried in the elevator domain. Ser300 and Ser345 interact with the C1-carboxylate group of Neu5Ac. Proteoliposome-based transport assays showed that Ser300-Neu5Ac interaction is critical for transport, whereas Ser345 is dispensable. Neu5Ac primarily interacts with residues in the elevator domain of the protein, thereby supporting the elevator with an operator mechanism. The residues interacting with Neu5Ac are conserved, providing fundamental information required to design inhibitors against this class of proteins.

Introduction

Sialic acids are nine-carbon amino sugars common on the surface of mammalian cells and on secreted molecules. *N*-Acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are the most prevalent forms in mammals (Lewis and Lewis, 2012). A dynamic interplay between genetics, environmental cues, and cell signaling orchestrates the intricate regulation of sialic acid synthesis in mammals. These sugars frequently occupy terminal positions in glycolipids and glycoproteins and play pivotal roles in cell-cell interactions such as signaling, adhesion, and recognition (Chen and Varki, 2010). Notably, a frameshift mutation disrupts the CMP-Neu5Ac hydrolase (CMAH) gene and leads to the loss of enzymatic activity, resulting in Neu5Ac being the sole outermost sugar in humans. Conversely, primates possessing a functional CMP-Neu5Ac hydrolase, Neu5Gc is typically found as the outermost sugar (Chou *et al.*, 1998).

This evolutionary divergence has profound consequences for the interactions between humans and pathogenic agents, including bacteria, parasites, and viruses (Varki, 2009). While commensal bacteria harness Neu5Ac as a carbon source, pathogenic bacteria, such as *Haemophilus influenzae* (Hi) and *Fusobacterium nucleatum* (Fn), evolved the ability to add Neu5Ac as the outermost sugar in their cell surface glycol-conjugates and use molecular mimicry to evade the immune system (Bell *et al.*, 2023; Severi *et al.*, 2007). Sialic acid is abundant in many niches [e.g., Neu5Ac in human serum is 1.6–2.2 mM (Sillanauke *et al.*, 1999)]. However, the concentration of free sialic acid is often low (approximately 0.2% of the total), and much of the sialic acid is conjugated to other macromolecules on the cell surface and is, therefore, not immediately available. Secreted sialidases from bacteria cleave Neu5Ac from mucins and other biomolecules within their niche (Sillanauke *et al.*, 1999). Cleaved Neu5Ac is transported into the periplasm of Gram-negative bacteria via porin-like β -barrel proteins. NanC from *E. coli* is the best-studied porin specific for sialic acids (Wirth *et al.*, 2009).

Our groups have made significant strides in elucidating the structural intricacies of the proteins involved in sequestration, uptake, catabolism, and incorporation of Neu5Ac by bacteria (Bose *et al.*, 2019; Coombes *et al.*, 2020; Currie *et al.*, 2021; Davies *et al.*, 2019; Horne *et al.*, 2020; Kumar *et al.*, 2018; Manjunath *et al.*, 2018; North *et al.*, 2016; Setty *et al.*, 2018). Bacteria rely on specialized Neu5Ac transporters within their cytosolic membranes, and four distinct classes of Neu5Ac transporters have been identified: sodium solute symporters (SSS) (North *et al.*, 2018), major facilitator superfamily (MFS), ATP-binding cassette (ABC), and tripartite ATP-independent periplasmic (TRAP) transporters (Davies *et al.*, 2023; Peter *et al.*, 2022). TRAP transporters that manifest as two- or three-component systems consisting of a substrate-binding protein (SiaP) and transmembrane protein (SiaQM) (Rosa *et al.*, 2018) are of particular interest due to their absence in eukaryotes. Deleting the sialic acid transporter abolishes Neu5Ac uptake, rendering bacteria incapable of incorporating Neu5Ac into lipopolysaccharides. Without a SiaQM transporter, bacteria form defective biofilms, have a lower cell density, and experience higher cell death (Allen *et al.*, 2005). Additionally, amino acids, C4-dicarboxylates, aromatic substrates and alpha-keto acids are also transported by TRAP transporters (Vetting *et al.*, 2015).

The first structures of SiaP were obtained from *H. influenzae* in an unliganded form and bound to Neu5Ac (Johnston *et al.*, 2008 [DOI](#); Müller *et al.*, 2006 [DOI](#)). The binding of Neu5Ac to SiaP results in the closure of the two domains of the protein and the mechanism has been described as a “Venus fly trap” mechanism, a common phenomenon observed in many ligand-binding proteins (Felder *et al.*, 1999 [DOI](#)). Structural and thermodynamic analyses of SiaP from *F. nucleatum*, *V. cholerae*, and *P. multocida* have revealed a conserved binding site, dissociation constant of Neu5Ac in the nanomolar range, and enthalpically driven substrate binding (Setty *et al.*, 2018 [DOI](#)). Using smFRET on VcSiaP, Peter and co-workers showed that conformational switching is strictly substrate-induced and that binding of the substrate stabilizes the interactions between the two domains (Peter *et al.*, 2021 [DOI](#)). All the SiaP structures show the presence of a conserved Arginine that binds to the C1-carboxylate of Neu5Ac, and this Arg residue is critical as the high electrostatic affinity may be important to have a strong binding affinity that sequesters the small amounts that reach the bacterial periplasmic space (Glaenger *et al.*, 2017 [DOI](#)).

The second component of the TRAP transporter is the transmembrane protein SiaQM, which is characterized by two distinct domains: the Q-domain and M-domain. Two SiaQM transporter structures have been reported by cryo-EM, one from *H. influenzae* (HiSiaQM) ranging from 2.95–4.70 Å resolution and another from *Protobacterium profundum* (PpSiaQM) at 2.97 Å resolution (Currie *et al.*, 2024 [DOI](#); Davies *et al.*, 2023 [DOI](#); Peter *et al.*, 2022 [DOI](#)).

The first structure of HiSiaQM (4.7 Å resolution) demonstrated that it is composed of 15 transmembrane helices (TM) and two helical hairpins. HiSiaQM likely functions as a monomeric unit, although a dimeric form has recently been reported (Currie *et al.*, 2024 [DOI](#); Peter *et al.*, 2022 [DOI](#)). Similarly, PpSiaQM is composed of 16 TM helices (Davies *et al.*, 2023 [DOI](#)). Based on these structures, an elevator-type mechanism has been proposed for TRAP transporters to move substrates from the periplasm to the cytoplasm (Peter *et al.*, 2024 [DOI](#)). Apart from the dimeric HiSiaQM structure, the other two structures determined used a megabody bound to SiaQM for cryo-EM analysis. In both cases, the megabody was bound to the extracellular side of the QM complex, featuring a deep open cavity on the cytosolic side (Supplementary Figure 1). This observation suggested that the transporter was captured in an inward-facing conformation, although the higher resolution dimeric HiSiaQM structure, even without a fiducial megabody, is also in the inward-facing conformation. Notably, the transport of Neu5Ac by TRAP transporters requires at least two sodium ions (Currie *et al.*, 2024 [DOI](#); Davies *et al.*, 2023 [DOI](#); Mulligan *et al.*, 2012 [DOI](#), 2009 [DOI](#)).

In sialic acid-rich environments such as the gut and saliva, bacterial virulence often correlates with their capacity to utilize Neu5Ac as a carbon source (Almagro-Moreno and Boyd, 2010 [DOI](#); Corfield, 2015 [DOI](#); Haines-Menges *et al.*, 2015 [DOI](#)). Although inhibitors targeting viral sialidases (neuraminidases) have been developed as antiviral agents (e.g., oseltamivir, zanamivir, and peramivir) (Glanz *et al.*, 2018 [DOI](#)), no drug currently targets bacterial infections by inhibiting sialic acid sequestration, uptake, catabolism, or incorporation. Human analogs of TRAP transporters are notably absent, underscoring their potential as promising therapeutic targets for combatting bacterial infections.

Both HiSiaQM and PpSiaQM structures lack information on the Neu5Ac binding site, which was identified based on modeling studies that relied on the ligand-bound structure of VcINDY (Kinz-Thompson *et al.*, 2022 [DOI](#)). Moreover, only the structures of SiaQM in the elevator-down conformation (inward-facing) have been reported, and further conformations along the transport cycle remains to be elucidated. The conformation of Neu5Ac bound to the transport domain may provide clues as to how it is received from the substrate-binding protein. In this study, we present the cryo-EM structures of SiaQM from *F. nucleatum*, both in its unliganded form and Neu5Ac bound form. The unliganded structure has density for two Na⁺-binding sites, whereas the liganded form has density for two Na⁺-binding sites and an additional metal-binding site.

Results and Discussion

Construct design, isolation, and strategy for structural determination

The TRAP transporter from *F. nucleatum* (*FnSiaQM*) was tagged with green fluorescent protein (GFP) at the C-terminus and expressed in BL21 (DE3) cells for protein expression and detergent stabilization trials. Detergent scouting was performed to identify the preferred detergent for *FnSiaQM* purification using fluorescence detection size-exclusion chromatography (Kawate and Gouaux, 2006 [\[1\]](#)). n-Dodecyl- β -D-maltopyranoside (DDM) was chosen as it solubilized and stabilized the protein better than other detergents. *FnSiaQM* was re-cloned into the pBAD vector with N-terminal 6X-histidine followed by Strep-tag II for large-scale purification and expressed in TOP10 (Invitrogen) cells. The purified *FnSiaQM* protein in the detergent micelles eluted as a monodisperse peak in size exclusion chromatography (Supplementary Figure 2). Subsequently, *FnSiaQM* was further reconstituted in MSP1D1 nanodiscs, along with *E. coli* polar lipids.

Initially, we attempted to determine the *FnSiaQM* structure using X-ray crystallography. After failing to obtain well-diffracting crystals, we switched to single-particle electron cryo-microscopy (cryo-EM). To achieve a reasonable size and better particle alignment, we raised nanobodies against *FnSiaQM* in Alpaca (Center for Molecular Medicine, University of Kentucky College of Medicine). Two high-affinity binders (T4 and T7) were identified and tested for complex formation using *FnSiaQM*. Hexa-histidine-tag based affinity chromatography was used to purify the nanobody from the host bacterial periplasm, producing a monodisperse peak in the size-exclusion chromatogram. Both nanobodies were added to *FnSiaQM* in DDM micelles and tested for complex formation. Although both nanobodies bound to the transporter, the T4 nanobody was selected over T7 because of its higher expression. The structures of the purified *FnSiaQM*-nanobody (T4) complexes in nanodiscs with and without Neu5Ac bound were determined using cryo-EM.

Structure of the *FnSiaQM*-nanobody complex

The nanodisc reconstituted *FnSiaQM*, and the bound nanobody is a monomeric complex in the cryo-EM structure. The overall global resolutions of the both the unliganded *FnSiaQM*-nanobody complex and the Neu5Ac bound form is ~ 3.2 Å. The maps were of good quality and allowed us to build all the TM helices of *FnSiaQM* unambiguously and both the unliganded and the Neu5Ac bound form clearly allow us to trace the protein and the nanobody (Figure 1A, 1B, 1C, 1D [\[1\]](#)). The best resolution for both structures are observed in the interior of the *SiaQM* protein (Supplementary Figure 3C, 3D). The details of the structure quality and refinement parameters are shown in Table 1 [\[1\]](#), and a flow diagram for structure determination and resolution statistics is shown in Supplementary Figure 3 A and B. Owing to their flexibility, the N-terminal 6X histidine and Strep II tags on *FnSiaQM*-nanobody complex were not visible in the cryo-EM maps. Similarly, the last few amino acids at the C-terminus were not built because of poor density. The density of the nanodisc was visible but of insufficient quality for model building. The density of the bound Neu5Ac is clear and in the higher-resolution region. The observed density around the ligand binding site for the two maps clearly indicate minimal structural changes around the binding pocket (Figure 1C, 1D, inset [\[1\]](#)). As the name suggests, many TRAP transporters comprise three units: a substrate-binding protein (*SiaP*) and two membrane-embedded transporter units (*SiaQ* and *SiaM*) (Severi *et al.*, 2007 [\[2\]](#)). In *FnSiaQM*, the two transporter units are fused by a long connecting helix, similar to other TRAP transporters such as *H. influenzae* TRAP (*HiSiaQM*) (Currie *et al.*, 2024 [\[3\]](#); Peter *et al.*, 2022 [\[4\]](#)). The Q-domain of *FnSiaQM* consists of the first four long helices, which are tilted in the membrane plane. This tilting creates a large contact surface area between the Q-and M-domains, which is dominated by buried hydrophobic residues. A small connecting

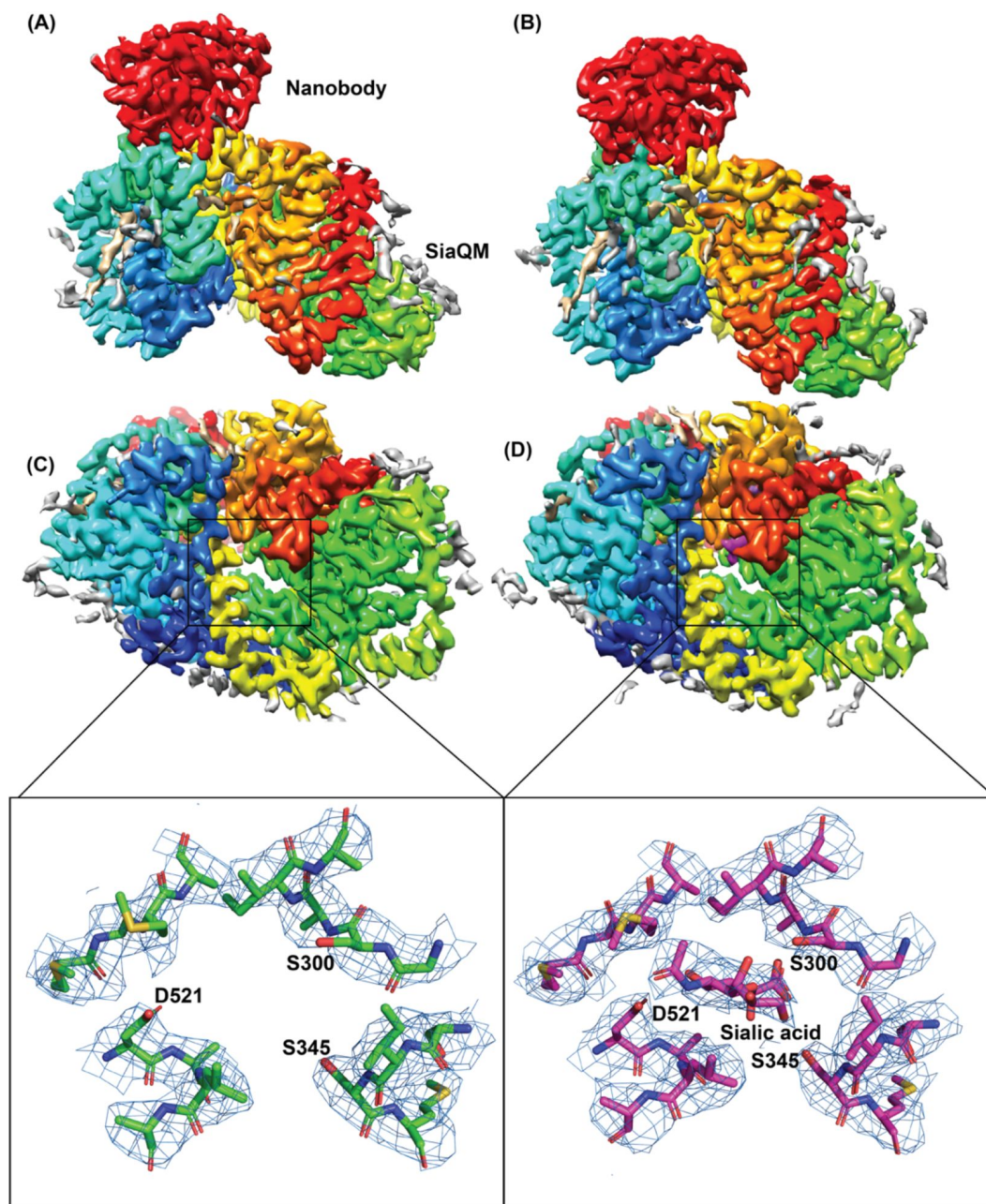


Figure 1

Architecture of FnSiaQM with nanobody. **(A and B)** Cryo-EM maps of FnSiaQM unliganded and Neu5Ac bound at 3.2 and 3.17 Å, respectively. The TM domain of FnSiaQM is colored using the rainbow model (N-terminus in blue and C-terminus in red). The nanobody density is colored in red. The density for modeled lipids is colored in tan and the unmodelled density in gray. The figures were made with Chimera at thresholds of 1.2 and 1.3 for the unliganded and Neu5Ac-bound maps. **(C and D)** The cytoplasmic view of apo and Neu5Ac bound FnSiaQM, respectively. Color coding is the same as in panels A and B. The density corresponding to Neu5Ac and sodium ions are in purple. The substrate binding sites of apo and Neu5Ac bound FnSiaQM are shown with key residues labeled. The density (blue mesh) around these atoms was made in Pymol with 2 and 1.5 σ for the apo and the Neu5Ac structures, respectively, with a carve radius of 2 Å.

helix lies perpendicular to the cell membrane plane and does not contact either domain (**Figure 2**). The lack of interaction between this connecting helix and the two domains suggests that it may be redundant for the transporter function. For example, *PpSiaQM* contains two separate polypeptides and does not require a connecting helix for its function (Davies *et al.*, 2023).

While there is no direct observation, the possible mode of function of the transporter suggests that the N-terminus of *FnSiaQM* lies on the cytoplasmic side, whereas its C-terminus lies on the periplasm side of the membrane. The nanobody is bound to the periplasmic side of *FnSiaQM*, making no contact with the cytoplasmic side of the protein (**Figure 1A** and **1B**). The M-domain is located towards the C-terminus and comprises the bulk of the transporter. It consists of 10 transmembrane helices with two hairpins that do not cross the entire membrane.

Superposition of the reported structures from *HiSiaQM* and *PpSiaQM* demonstrate that all are in inward (cytoplasmic) open and elevator-down conformations (Supplementary Figure 1). There are two conserved Na⁺ ion binding sites in these proteins (Currie *et al.*, 2024; Davies *et al.*, 2023; Peter *et al.*, 2022). Structurally, the M-domain can be divided into an outer variable scaffold domain and a centrally conserved transport domain. The scaffold domain is composed of six TM helices and serves as a support for the transport domain. It also interacts with the Q-domain to form a structurally rigid domain. The Q and scaffold domains are the least conserved among the *SiaQM* domains, suggesting that they are not directly involved in substrate transport (**Figure 2** and Supplementary Figure 4).

To determine whether substrate binding resulted in conformational changes, we solved the structure of the ligand-bound form of *FnSiaQM*. The superposition of the two structures revealed a high degree of similarity with an RMSD of 0.3 Å over all Ca atoms (**Figure 2A**). A comparison of the binding site residues revealed that they are in a similar conformation. The transporter has a large cytoplasmic facing open cavity, suggesting that the unliganded as well as Neu5Ac bound structures are in an inward-open conformations. The nomenclature used for labeling the secondary structures uses a description of the published *HiSiaQM* structure with similar architecture (Currie *et al.*, 2024). The elevator part of the domain has one helix-loop-helix motif called HP1 (HPin), which is directed towards the cytoplasmic side. A structurally homologous helix-loop-helix domain, HP2 (HPout), is present on the periplasmic side. The arrangement of TM helices have been described in detail in *HiSiaQM* and *PpSiaQM* structures (Davies *et al.*, 2023; Peter *et al.*, 2022). Interestingly, this domain has two-fold inverted symmetry, as found in other TRAP transporters. The entire domain is populated with highly conserved residues, suggesting that it may play a direct role in substrate transport (Supplementary Figure 4). A single molecule of Neu5Ac is bound to the aperture formed by HP1 and HP2 in the central core transport domain (**Figure 2B**). The Neu5Ac binding site has a large solvent-exposed vestibule towards the cytoplasmic side, while its periplasmic side is sealed off. Cryo-EM map shows the presence of multiple densities that could be modeled as lipids, possibly preventing the substrate from leaving the transporter. However, the densities are not well defined to model them as specific lipids, hence they have not been modeled. We describe this as the “inward-facing open state” with the substrate-bound. While, both the HP1 and HP2 loops have been hypothesized to be involved in gating, in the human neutral amino acid transporter (ASCT2), (which also uses the elevator mechanism), only the HP2 loops have been shown to undergo conformational changes to enable substrate binding and release (Garaeva *et al.*, 2019). Hence, it is suggested that there is a single gate that controls substrate binding. Superposition of the *PpSiaQM* and *HiSiaQM* structures do not reveal any change in these loop structures upon substrate binding. For TRAP transporters, the substrate is delivered to the QM protein by the P protein; hence, these loop changes may not play a role in ligand binding or release. This may support the idea that there is minimal substrate specificity within *SiaQM* and that it will transport the cargo delivered by *SiaP*, which is more selective.

Data collection and processing	Apo form	Neu5Ac-bound
EMDB	38926	38925
PDB	8Y4X	8Y4W
Magnification	75000	75000
Voltage (kV)	300	300
Electron dose (e ⁻ /Å ³)	27.7	24.67
Defocus Range (nm)	1800-3000	1600-2800
Pixel size (Å)	1.07	1.07
Symmetry imposed	C1	C1
Initial particle images (no.)	385668	653554
Final particle images (no.)	141272	225006
Map resolution (Å)	3.20	3.16
FSC threshold	0.143	0.143
Map resolution range (Å)	2.7-5.5	2.6-4.1
Refinement		
Model composition		
Non-hydrogen atoms	5801	5873
Protein residues	732	732
Ligand	2 Na ⁺ ; 1 Lipid	3 Na ⁺ ; 2 Lipid; 1 Neu5Ac
B-factor (Å ²)		
Protein (mean)	46.72	43.60
Ligand (mean)	45.88	56.25
R.m.s deviations		
Bond lengths (Å)	0.005	0.004
Bond angles (°)	0.531	0.512
Validation		
MolProbity score	1.76	1.54
Clashscore	5.57	5.07
CaBARM outliers (%)	1.52	1.52
Rotamer outliers (%)	1.77	1.13
Ramachandran plot (%) (Favored/Allowed/Disallowed)	(96.02/3.98/0)	(96.43/3.57/0)

Table 1.

Details of cryo-EM data collection, processing, refinement and built model.

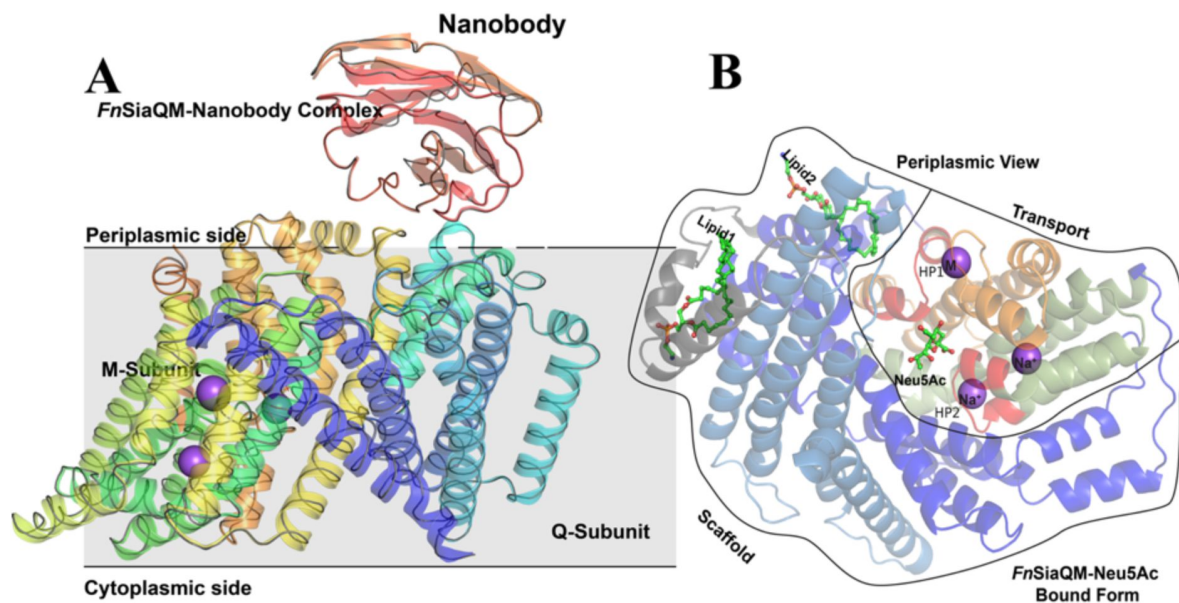


Figure 2

***FnSiaQM*-Nanobody complex with Neu5Ac bound form.**

(A) A cartoon representation of the *FnSiaQM*-Nanobody complex structure. The *SiaQM* polypeptide has been colored in rainbow with the N-terminus starting in blue and the C-terminus ends in orange. The nanobody is shown in cartoon representation and in red color. Two of the modeled sodium sites are shown as purple spheres. A ribbon representation of the unliganded structure of *FnSiaQM* is superposed in grey. The superposition reveals that the overall structures are similar. (B) Cartoon representation of the *FnSiaQM* structure bound to Neu5Ac. In shades of blue are the helices that form the scaffold domain. In gray is the connecting domain. In olive, orange, and red is the elevator domain. HP1 and HP2, the two helix-loop-helix motifs, are in red and marked. The positions of the known Na⁺ ions and metal ion (M) are in spheres, and the position of Neu5Ac is shown in ball and stick.

Sodium ion binding site

The transport of molecules across the membrane by TRAP transporters depends on Na^+ transport (Currie *et al.*, 2024 [↗](#); Davies *et al.*, 2023 [↗](#); Mulligan *et al.*, 2012 [↗](#), 2009 [↗](#)). We observed cryo-EM density at conserved Na^+ binding sites and modeled two Na^+ ions in the unliganded- and ligand-bound forms (**Figure 3** [↗](#)). The two sites that are present in the unliganded form are also conserved in other reported SiaQM structures (Currie *et al.*, 2024 [↗](#); Davies *et al.*, 2023 [↗](#)) [**Figure 2B** [↗](#), Supplementary Figure 5), compared with **Figure 5** [↗](#) in (Currie *et al.*, 2024 [↗](#)). The Na1 site interacts with residues at the HP1 site and helix 5 (**Figure 3A** [↗](#), Supplementary Figure 6A). The Na2 site combines residues at the HP2 site and a short stretch that splits helix 11 into two parts (**Figure 3A** [↗](#), Supplementary Figure 6B). These two sites have been previously well described (Currie *et al.*, 2024 [↗](#)).

Surprisingly, we observed density for an additional metal binding site. This site is distinctly located towards the cytoplasmic side of the transporter and away from the Neu5Ac binding site. It interacts with helices that form HP1 and the next helix 5a (**Figure 3A** [↗](#)). Interestingly, a similar site was observed in the SSS Neu5Ac transporter, in which the third site was located away from the other Na^+ binding sites (Wahlgren *et al.*, 2018 [↗](#)). Peter *et al.*, proposed that Asp304 coordinates with this Na^+ ion, showing loss-of-function when mutated to an alanine residue (Peter *et al.*, 2022 [↗](#)). While it is tempting to label it as a third Na^+ -binding site, the metal–ligand distances are longer (Supplementary Figure 6C). Hence, we conservatively designated this site as a metal-binding site. The coordinated movement of different Na^+ ions is expected to provide the energy to create conformational changes that lead to the movement of Neu5Ac from the periplasm to the cytoplasm. While we found two Na^+ -binding sites and a third metal-binding site, it is not clear how the movement of these sites can cause the conformational changes required for the motion of the elevator along with the ligand. It is also unclear what the function of the third metal-binding site is beyond the mutagenesis data, which suggests that the lack of this site results in a loss of function.

Lipid binding to FnSiaQM

We have modelled two phosphatidylethanolamine (PE) lipids in the maps of unliganded FnSiaQM (Supplementary Figure 7A and 7B). The FnSiaQM nanodisc reconstitution procedure included the addition of *E. coli* polar lipids, where PE is the most abundant lipid. One of the lipids binds between the connector helix and the Q-domain (**Figure 2B, Lipid1** [↗](#)) and is present in both the unliganded and Neu5Ac bound structure of FnSiaQM. This lipid is present at a similar position in the HiSiaQM structure (Currie *et al.*, 2024 [↗](#)). However, we observe a second lipid molecule (**Figure 2B, Lipid2** [↗](#)) that is bound between the stator and elevator domains only in the Neu5Ac bound structure. It is tempting to hypothesize that this lipid molecule is displaced during the movement of the elevator domain; however, this requires further investigation. A similar observation was also made in the transporter GltPh that invokes the elevator mechanism (Wang and Boudker, 2020 [↗](#)).

Activity of FnSiaQM

To demonstrate that the purified protein is active and transports Neu5Ac across the membrane, we performed transport assays. FnSiaQM was reconstituted in proteoliposomes containing intraliposomal potassium. Valinomycin was then incorporated into these proteoliposomes to establish an inner-negative membrane potential. Periplasmic binding protein (FnSiaP) and radioactive Neu5Ac were added to the proteoliposomes to initiate transport (**Figure 4A** [↗](#)). In this experimental setup, a significant accumulation of radiolabeled Neu5Ac was measured in the presence of extraliposomal Na^+ -gluconate when the artificial membrane potential was imposed by the addition of valinomycin (**Figure 4B, black squares** [↗](#)). When ethanol was used as a control for valinomycin, a much lower accumulation of radiolabeled Neu5Ac was observed (**Figure 4B, open squares** [↗](#)). Importantly, transport was negligible when Na^+ was absent in the extra-liposomal

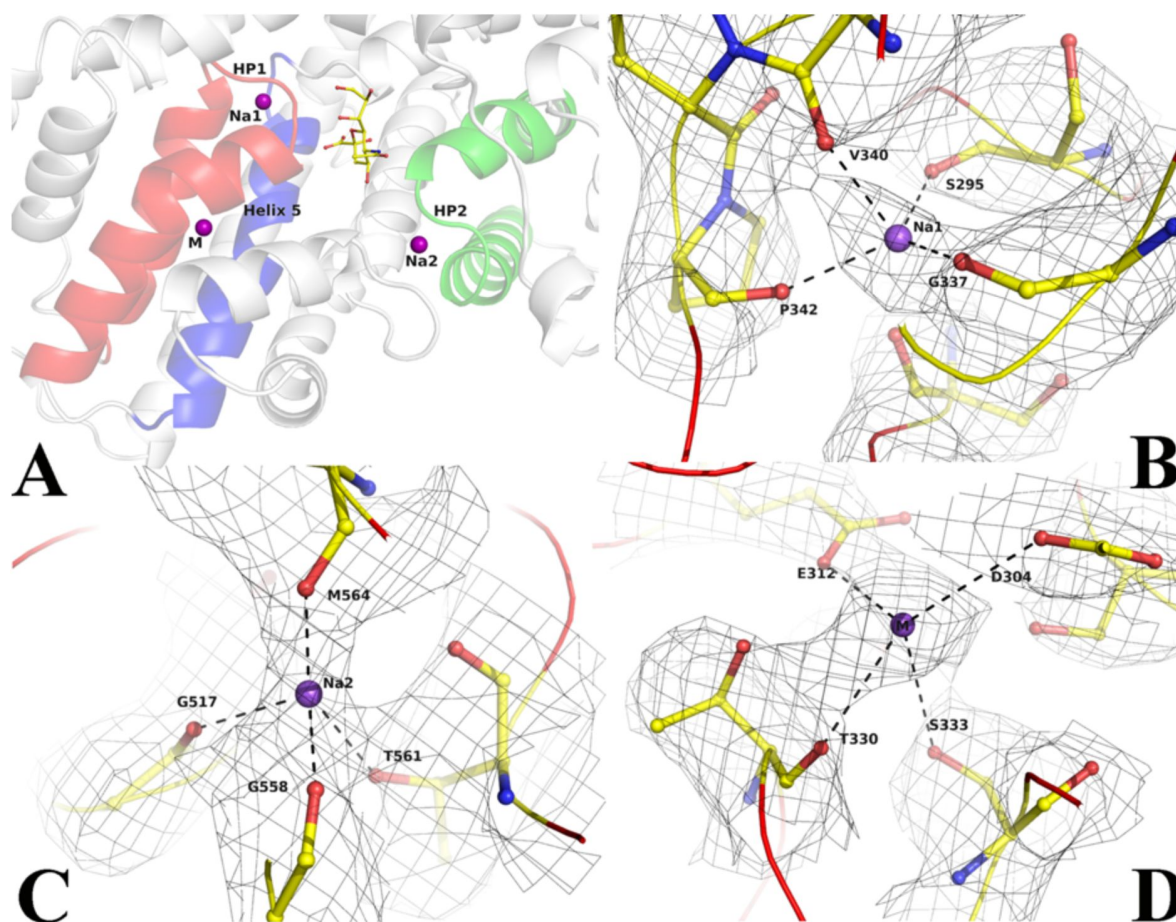


Figure 3

Ion binding sites in *FnSiaQM*

(A) Close-up view of the Neu5Ac and the two Na⁺ and metal binding sites. In red is the HP1 helix-loop-helix. In green is the HP2 helix-loop-helix. In blue is helix 5 A, the purple spheres are the two Na⁺ ion binding sites and metal binding site (M), and the bound Neu5Ac is shown in ball and stick. (B-D) Density and interaction details of the Na1, Na2, and M sites, respectively. The figures are made with a contour of 1.0 r.m.s. in PyMol.

environment or when K^+ was absent in the intraliposomal environment. Transport was negligible when soluble *FnSiaP* was omitted from the assay, further demonstrating the requirement of periplasmic binding proteins (**Figure 4B, stars** [↗](#)). The energetics of transport is similar to that of other SiaPQM that have been characterized (Mulligan *et al.*, 2012 [↗](#)).

Architecture of Neu5Ac binding site

While the structures of the unliganded and Neu5Ac bound *FnSiaQM* are in similar inward-open conformation, the density of Neu5Ac is unambiguous in the liganded form (**Figure 1D (inset)** [↗](#), **5A** [↗](#)). The overall architecture of the Neu5Ac binding site is similar to that of citrate/malate/fumarate in the di/tricarboxylate transporter of *V. cholerae* (VcINDY), but the residues involved in providing specificity are different (Kinz-Thompson *et al.*, 2022 [↗](#); Mancusso *et al.*, 2012 [↗](#); Nie *et al.*, 2017 [↗](#); Sauer *et al.*, 2022 [↗](#)). Neu5Ac binds to the transport domain without direct interactions with the residues in the scaffold domain. The majority of the interactions are with residues in the HP1 and HP2 loops of the transport domain (**Figure 5B** [↗](#)). Asp521 (HP2), Ser300 (HP1), and Ser345 (helix 5) interact with the substrate through their side chains, except for one interaction between the main chain amino group of residue 301 and the C1-carboxylate oxygen of Neu5Ac. Mutation of the residue equivalent to Asp521 has been shown to result in loss of transport (Peter *et al.*, 2022 [↗](#)). To evaluate the role of residues Ser-300 and Ser-345, we mutated them to alanine and performed the transport assays.

The data clearly showed that the Ser300Ala mutant was inactive, whereas the Ser345 mutation did not affect *FnSiaQM* functionality (**Figure 5C** [↗](#)). This suggests that the interaction of Neu5Ac with the residues in HP1 and HP2 is critical for transport. The carboxylate oxygens of the C1 atom of Neu5Ac interact with Ser300 and Ser345 and the main chain of Ala301 (**Figure 5B** [↗](#)). Ser 345 OG is 3.5Å away from the C1-carboxylate oxygen – a distance that would result in a weak interaction between the two groups. It is, therefore, not surprising that the mutation into Ala did not affect transport. The space created by the mutation can be occupied by a water molecule. The N5 atom of the *N*-acetyl group of the sugar interacts with Asp521. These interactions are conserved even if Neu5Ac is converted into Neu5Gc, as the addition of an extra hydroxyl group at the C11 position does not break this interaction, and there is sufficient cavity space for modifications in C11 (**Figure 5D** [↗](#)). Cavity Plus (Wang *et al.*, 2023 [↗](#)) estimated the cavity to be 1875 Å³ with a druggability score of 3631, suggesting that the environment of the binding site is highly suited for drug binding (Wang *et al.*, 2023 [↗](#)).

No direct measurement of K_d of Neu5Ac binding to the SiaQM region of the transporter is available. We infer from the interactions it makes and the fact that high concentrations of Neu5Ac are required to obtain a complex (30 mM), the affinity is unlikely to be high. Neu5Ac binds with nanomolar affinity ($K_d = 45$ nM) to *FnSiaP* (**Figure 6A** [↗](#)) and several electrostatic interactions stabilize this binding (Gangi Setty *et al.*, 2014 [↗](#)). The binding of Neu5Ac to the SSS-type transporter, which also transports Neu5Ac across the cytoplasmic membrane, was also stabilized by several electrostatic interactions (**Figure 6B** [↗](#)). The polar groups bind to both the C1-carboxylate side of the molecule and the C8-C9 carbonyls, suggesting that *Proteus mirabilis* Neu5Ac transporter (SSS type) evolved specifically to transport nine-carbon sugars such as Neu5Ac (Wahlgren *et al.*, 2018 [↗](#)). Interestingly, even the dicarboxylate transporter from *V. cholerae* (VcINDY) binds to its ligand via electrostatic interactions with both carboxylate groups (**Figure 6C** [↗](#)) (Kinz-Thompson *et al.*, 2022 [↗](#); Mancusso *et al.*, 2012 [↗](#); Nie *et al.*, 2017 [↗](#); Sauer *et al.*, 2022 [↗](#)). The high affinity of the substrate-binding component (*FnSiaP*) to Neu5Ac is physiologically relevant because it sequesters Neu5Ac in a volume where the concentration of Neu5Ac is very low. In contrast, as Neu5Ac is delivered to the SiaQM component by the SiaP component, the affinity could be lower, an argument also made by Peter *et al.* in their recent work (Peter *et al.*, 2024 [↗](#)). The corollary of this argument is that the specificity of the transport system

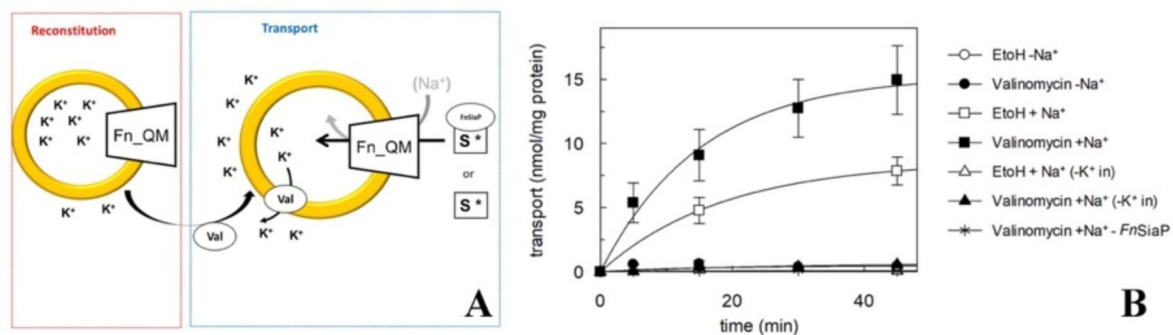


Figure 4

Proteoliposome transporter assays for *FnSiaQM*.

(A) Schematic diagram showing the experimental setup. First, *FnSiaQM* is incorporated into proteoliposome in the presence of internal K^+ . Then, valinomycin (val) is added to induce the efflux of K^+ down its concentration gradient, imposing an artificial membrane potential. To start transport measurement, *FnSiaP*, [^3H]-Neu5Ac and Na^+ -gluconate are added in the extraliposomal environment. **(B)** Time course of Neu5Ac uptake into proteoliposomes reconstituted with *FnSiaQM*. In black circle, black square, white triangles, black triangle, asterisk, are with conditions, where valinomycin was added to facilitate K^+ movement before transport. Ethanol was added instead of valinomycin as a control in the white circle, white square, and white triangle. In white square, black square, white triangle, black triangle, 10 mM Na^+ -gluconate was added together with 5 μM [^3H]-Neu5Ac and 0.5 μM *FnSiaP*; in white triangle and black triangle proteoliposomes are prepared without internal K^+ ; in black asterisk transport is measured in the presence of 10 mM Na^+ -gluconate, 5 μM [^3H]-Neu5Ac and in the absence of *FnSiaP*. Uptake data were fitted in a first-order rate equation for time course plots. Data are means $\pm$ s.d. of three independent experiments.

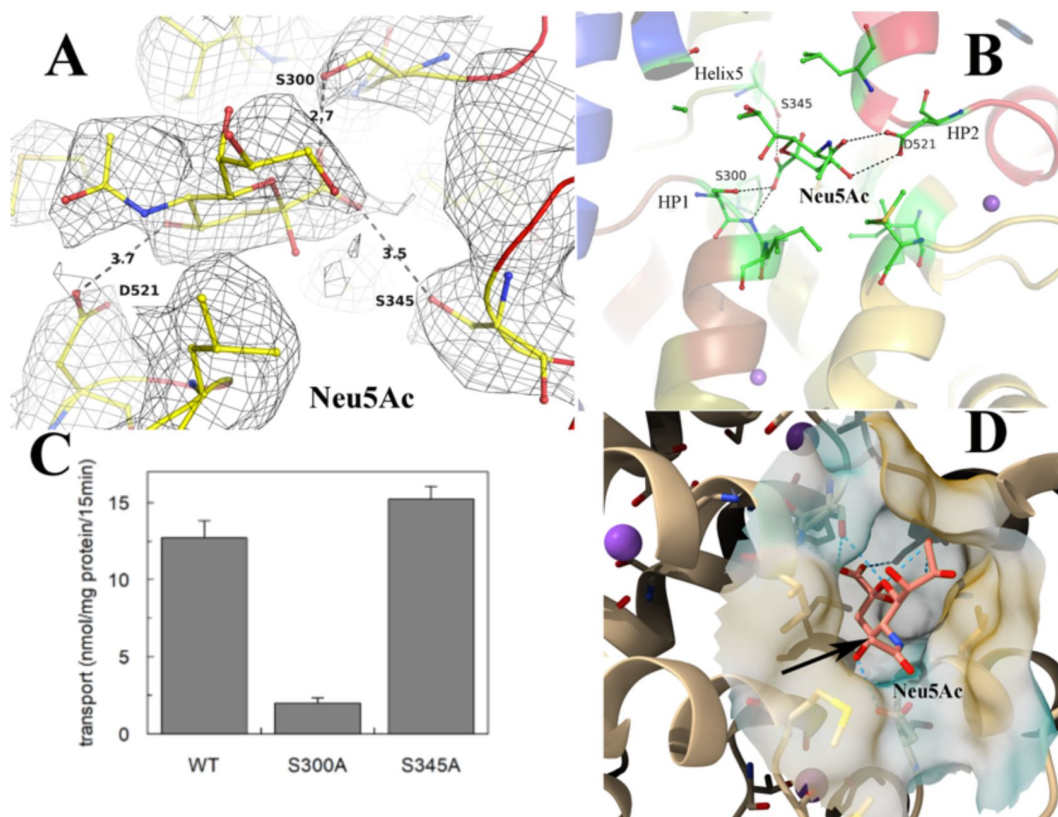


Figure 5

Validation of Neu5Ac binding pocket in *FnSiaQM* transporter.

(A) The fit of the modeled Neu5Ac into the density, contoured to $0.9 \times \text{r.m.s.}$ The figure also shows the fit to the density of the residues that interact with Neu5Ac and the distances to key residues discussed in the manuscript. (B) The interactions of Neu5Ac with side chains of interacting residues. Ser300 O γ is 2.8 Å from the C1-carboxylate oxygen of Neu5Ac, while the main chain NH of residue 301 is 2.6 Å away. The other close polar side chain is that of Ser345 γ , which is 3.3 Å away. The Neu5Ac O10 is 2.9 Å from Asp521 O δ . (C) Transport of Neu5Ac in 15 min. Transport was started by adding 5 μM [^3H]-Neu5Ac, 10 mM Na $^+$ -gluconate, and 0.5 μM *FnSiaP* to proteoliposomes in the presence of valinomycin (expected membrane potential, $\Delta\psi$, -117.1 mV). Data are expressed as nmol/mg prot/15 min $\pm$ s.d. of three independent experiments. (D) The cavity of Neu5Ac is exposed from the cytoplasmic side. The cavity is large and extends into the cytoplasmic side. The black arrow shows the methyl group with an extra hydroxyl in Neu5Gc.

(or the choice of molecules that can be transported) is likely to be determined by the substrate-binding component. There is probably very little selectivity in the SiaQM component, which is also reflected by fewer interactions.

Conclusion

This is the first report of the structure of a SiaQM from the TRAP-type transporter of Gram-negative bacteria with bound Neu5Ac. The structure shows no direct interaction between Neu5Ac and Na^+ ions. The affinity of Neu5Ac for periplasmic sialic acid-binding proteins (SiaP) is in the nanomolar range, with many polar interactions ($K_d=45$ nM) (Gangi Setty *et al.*, 2014 [↗](#)). However, relatively fewer polar interactions stabilize Neu5Ac binding to the open binding pocket of SiaQM, and the affinity is probably poor. We used APBS to calculate the electrostatic charge distribution on SiaQM (Supplementary Figure 7C) (Jurrus *et al.*, 2018 [↗](#)). A view of the structure of Neu5Ac bound SiaQM from the periplasmic side towards the bound Neu5Ac shows that the sugar is bound in the cavity, the entrance of which is repulsive to the binding of a negatively charged sugar like Neu5Ac. This likely prevents Neu5Ac from binding to the SiaQM from the cytoplasmic side. The structure of the protein in the outward-facing conformation is unknown, which will reveal how Neu5Ac is transferred from SiaP to SiaQM. While mechanistic questions require further investigation, the precise definition of the binding pocket provided in this work is a starting point for structure-based drug discovery.

Experimental procedures

Construct design

The gene sequence encoding the TRAP transporter from *F. nucleatum* (NCBI Reference Sequence: WP_005902322.1) was synthesized and cloned into a pBAD vector by GeneArt. A Strep-tag was inserted in addition to the 6X-His Tag at the N-terminus to enhance the protein purity. It was also cloned into pWarf (+) vector for fluorescence-detection size-exclusion chromatography (Hsieh *et al.*, 2010 [↗](#)).

Protein purification of FnSiaQM

Expression trials and detergent scouting were performed as described by Hsieh *et al.*, 2010 [↗](#). For large-scale production, the FnSiaQM construct in pBAD was introduced into *E. coli* strain TOP10 cells. A single colony from the transformed plate was inoculated into 150 mL of terrific broth (TB) containing 100 $\mu\text{g/mL}$ ampicillin. The culture was then incubated overnight at 30 °C in a shaker incubator. The following day, 10 mL of the primary culture was transferred to a culture flask containing 1 L Terrific broth supplemented with ampicillin and incubated at 30 °C in an orbital shaker. The cultures were induced with 0.02% arabinose at an optical density (OD) of 1.5 at 600 nm and allowed to grow for an additional 18 h. Cells were harvested by centrifugation at 4000 $\times$ g for 20 min at 4 °C. The resulting cell pellet was rapidly frozen in liquid nitrogen and stored at -80 °C for future use. The cell pellet was resuspended in lysis buffer (100 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM dithiothreitol (DTT)) supplemented with lysozyme, DNase, and cComplete EDTA-free inhibitor cocktail (Roche). Lysis was achieved by two passes through a Constant cell disruptor at 20 and 28 kPsi, respectively. Subsequently, cell debris and unbroken cells were pelleted by centrifugation at 10,000 $\times$ g for 30 min at 4 °C. The supernatant from the low-speed centrifugation was subjected to ultracentrifugation at 150,000 $\times$ g at 4 °C for 1 h to pellet the cell membranes. The membranes were then resuspended in solubilizing buffer (1% n-dodecyl- β -D-maltoside (DDM), 50 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole, 3 mM β -ME) at a 1:10 w/v ratio and solubilized at 4 °C for two hours. The insoluble membrane components were separated by ultracentrifugation for 30 min at 100,000 $\times$ g, and the resulting supernatant was loaded onto a 5 mL His-Trap (Cytiva)

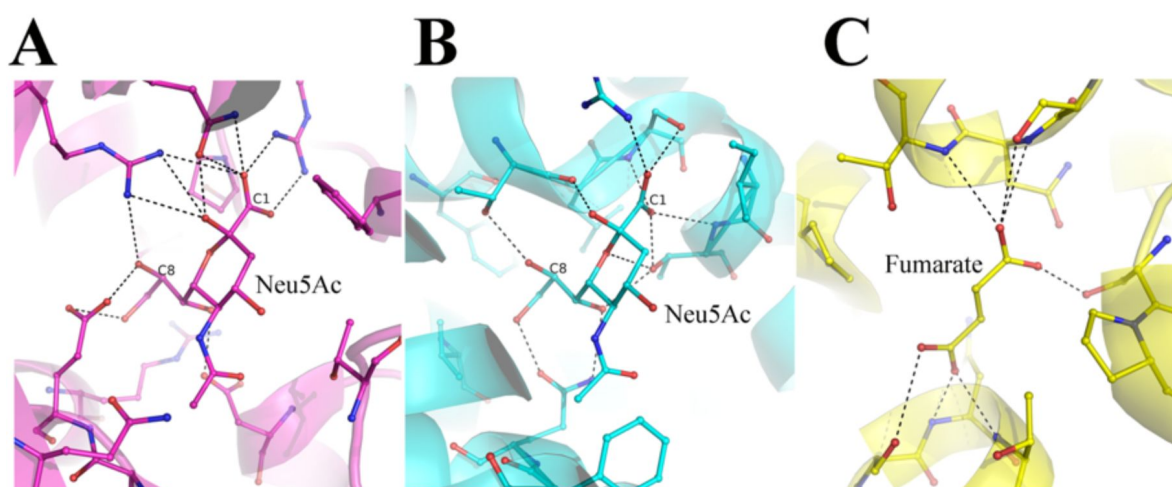


Figure 6

Comparison of Neu5Ac binding pocket

(**A**) The interactions of Neu5Ac with the substrate binding protein SiaP from *F. nucleatum* (PDB-ID 4MNP). The C1 and the C8 carbon atoms are labeled to show the different ends of the nine-carbon sugar. (**B**) the interaction of Neu5Ac with the SSS-type Neu5Ac transporter (PDB-ID 5NV9). (**C**) The interaction of fumarate with the dicarboxylate transporter VcINDY (PDB-ID 6OKZ).

column pre-equilibrated with buffer A (50 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole, 3 mM β -ME, 0.02% DDM). The resin was extensively washed with buffer A until a stable UV absorbance was achieved in the chromatogram. The *FnSiaQM* protein was eluted using an elution buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 150 mM imidazole, 3 mM β -ME, and 0.02% DDM). The eluted fractions were pooled and used for strep-tag purification. A 5 mL Strep-Trap HP column (Cytiva) was used for subsequent purification. The column was pre-equilibrated with a buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM imidazole, 3 mM β -ME, and 0.02% DDM). After loading the sample onto the column, the column was washed with 10 CV buffer (100 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole, 3 mM β -ME, and 0.02% DDM). The *FnSiaQM* protein was eluted in the presence of 10 mM desthiobiotin, 100 mM Tris (pH 8.0), 150 mM NaCl, 3 mM β -ME, and 0.02% DDM. The eluted fraction was concentrated using a 100 kDa Amicon filter and subsequently injected into a size-exclusion column (Superdex200 16/300 increase) in buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 0.02% DDM). Size exclusion chromatography yielded a monodisperse peak for *FnSiaQM* protein (Supplementary Figure 2).

Generation, isolation and purification of *FnSiaQM*-specific nanobodies

Generation of the nanobodies was performed by the University of Kentucky Protein Core, following previously established protocols (Chow *et al.*, 2019 [DOI](#)). Alpacas were subcutaneously injected with 100 μ g of recombinant *FnSiaQM* in DDM once a week for six weeks. Peripheral blood lymphocytes were isolated from alpaca blood and used to construct a bacteriophage display cDNA library. Two rounds of phage display against the recombinant *FnSiaQM* identified two potentially VHH-positive clones. Positive clones were confirmed by sequencing and were analyzed for nanobody components. These two nanobodies were cloned into the pMES4 vector and *E. coli* strain BL21 (DE3) was used for their expression. A single colony was inoculated into 100 mL LB medium and cultured overnight. These overnight cultures were diluted in 1 L of LB media in a 1:100 ratio. Expression was induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) when the O.D.₆₀₀ reached 1.0, followed by further incubation at 28 °C for 16 h for protein production. Cultures were then centrifuged at 4000 x g for 20 min at 4 °C, and periplasmic extracts were prepared using the osmotic shock method with 20% sucrose. The periplasmic extract was dialyzed to remove sucrose and filtered for affinity chromatography. This filtered fraction was loaded onto a 5 mL His-Trap (Cytiva) column pre-equilibrated with buffer A (50 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole, and 3 mM β -ME). The column was extensively washed with 20 CV of buffer B until stable UV absorbance was observed in the chromatogram. The nanobodies were eluted using an elution buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 150 mM imidazole, and 3 mM β -ME). The eluted fractions were pooled and concentrated using a 10 kDa Amicon (Sigma) filter and subsequently injected into a Superdex75 column. Both nanobodies exhibited monodisperse peaks during the size-exclusion chromatography. The peak fractions were pooled and stored at 4 °C.

Complex formation of *FnSiaQM*-nanobody (T4)-nanodisc (MSP1D1)

MSP1D1 was expressed and purified following established protocols (Denisov and Sligar, 2016 [DOI](#)). The 6X-His tag was cleaved from the affinity-purified MSP1D1 using TEV protease and the resulting product was concentrated to 5 mg/mL. For nanodisc formation, nickel affinity-eluted *FnSiaQM*, MSP1D1, and *E. coli* polar lipids were combined in a molar ratio of 1:6:180 and incubated on ice for 15 min. Nanodisc formation was initiated by adding washed Bio-Beads (200 mg dry weight per 1 mL of the protein mixture; Bio-Rad), and the mixture was rotated for two hours at 4 °C. The protein mixture was subsequently separated from the Bio-Beads using a fine needle. A two-molar excess of the T4-Nanobody was added at this step to the *FnSiaQM*-nanodiscs. A Strep-Tag chromatography step removed the empty nanodiscs and excess T4-nanobodies. The *FnSiaQM*-reconstituted nanodisc with the T4 nanobody were then separated from the aggregates using a Superdex200 16/300 increase size exclusion column. The fractions corresponding to the

peaks were concentrated to 2.5 mg/mL and used for cryo-EM analysis. To acquire the Neu5Ac-bound structure, 30 mM Neu5Ac was incorporated in the gel filtration buffer (100 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM DTT).

Cryo-EM sample preparation and data collection

The *FnSiaQM*-T4 complex at a concentration of 2.5 mg/mL was immediately applied onto UltrAuFoil R0.6/1.0 grids (300 mesh) previously subjected to glow discharge for 150 s at 25 mA. Excess fluid was removed by blotting using a VitroBot Mark IV (ThermoFisher Scientific) apparatus, and the grids were rapidly vitrified by plunging them into liquid ethane that was pre-cooled with liquid nitrogen. Subsequently, the grids were screened at a magnification of 59,000 × using a Titan Krios microscope G3i (ThermoFisher Scientific) operating at 300 kV. Data were collected at a magnification of 75,000 ×, corresponding to a pixel size of 1.07 Å using a Falcon 3 detector in counting mode.

Data processing of unliganded form

A total of 960 movies were collected for the unliganded structure. All processing was performed using the CryoSPARC software suite (Punjani *et al.*, 2017 [DOI](#)). After patch motion correction and CTF correction, the summed images were examined and 941 micrographs were selected for particle picking. The initial particle picking (blob picker) was performed using 315 images. This was followed by 2D classification, the selection of 2D classes, and *ab initio* reconstruction into two classes. One class clearly showed the presence of the nanodisc, the protein inside, and the bound nanobody. This was refined with 49,659 particles to a resolution of 6.7 Å resolution. This 3-D map was used to create templates, template-based particle picking was performed on all 941 images, and 385,668 particles were picked. Multiple rounds of 2-D classification and selection yielded 141,272 good particles. These particles were used for *ab initio* reconstruction in cryoSPARC (Punjani *et al.*, 2017 [DOI](#)), followed by homogenous and non-uniform refinement (Punjani, 2020 [DOI](#)). The final map resulted in an overall resolution of 3.2 Å resolution (Fourier Shell Correlation cutoff 0.143). The B-factor estimated from the Guinier plot is 125.2 Å².

Data processing of Neu5Ac-bound form

A total of 2341 images were collected. After manually inspecting the micrographs, 1752 images were selected for further processing. The template generated from the unliganded maps was used for the particle picking. After multiple rounds of 2D classification and particle pruning, 653,554 particles were used to create six *ab initio* classes. Four of these classes appeared identical (225,006 particles) and were refined to better than 4.5 Å using homogeneous refinement. The final map was constructed using non-uniform refinement protocols in Cryo-SPARC. The map has an overall resolution of 3.17 Å at the FSC 0.143 threshold. The B-factor estimated from the Guinier plot is 135.5 Å².

Model building and model refinement

The starting model was constructed using AlphaFold2 software (Evans *et al.*, 2022 [DOI](#)). Subsequently, this model was docked within the cryo-EM map of *FnSiaQM* unliganded form of *FnSiaQM*. A series of iterative rounds of model building using Coot (Emsley and Cowtan, 2004 [DOI](#)) and refinement using Phenix (Adams *et al.*, 2010 [DOI](#)) were performed to complete the model. These steps were essential for enhancing the accuracy and quality of the structural model and ensuring its congruence with the experimental cryo-EM data. The refined unliganded model was used as the starting model for the substrate-bound map, and sugars and ions were modeled. All the figures were either made in UCSF Chimera or PyMOL (DeLano, 2002 [DOI](#); Pettersen *et al.*, 2004 [DOI](#)). The final details of the data collection, processing, and results of model building and refinement are summarized in **Table 1** [DOI](#).

Reconstitution of FnSiaQM into proteoliposomes

Purified FnSiaQM was reconstituted using a detergent removal method performed in batches following previously described procedures (Wahlgren *et al.*, 2018⁴). In summary, 50 µg FnSiaQM was combined with 120 µL of 10% C₁₂E₈ detergent and 100 µL of 10% egg yolk phospholipids (w/v) in the form of sonicated liposomes. To this mixture, 50 mM K⁺-gluconate and 20 mM HEPES/Tris (pH 7.0) were added to a final volume of 700 µL. The reconstitution blend was then exposed to 0.5 g of Amberlite XAD-4 resin while continuously stirred at 1200 rev/min at 23 °C for 40 min.

Transport measurements and transport assay

Following reconstitution, 600 µL of proteoliposomes were loaded onto a Sephadex G-75 column (0.7 cm diameter × 15 cm height) pre-equilibrated with 20 mM HEPES/Tris (pH 7.0) containing 100 mM sucrose to balance the internal osmolarity. Valinomycin (0.75 µg/mg phospholipid) prepared in ethanol was introduced into the eluted proteoliposomes to create a K⁺ diffusion potential. Following a 10 s incubation with valinomycin, transport was initiated by adding 5 µM [³H]-Neu5Ac, 0.5 µM FnSiaP, and 10 mM Na⁺-gluconate to 100 µL of liposomes. The initial transport rate was determined by halting the reaction after 15 min, which fell within the initial linear range of [³H]-Neu5Ac uptake into the proteoliposomes, as established through the time-course experiments.

The transport assay was concluded by loading each proteoliposome sample (100 µL) onto a Sephadex G-75 column (0.6 cm diameter × 8 cm height) to eliminate external radioactivity. The experimental values were corrected by subtracting the control, i.e. the radioactivity taken up in liposomes reconstituted in the absence of protein. The radioactivity associated with the control samples, i.e. empty liposomes was less than 10% with respect to proteoliposomes. Proteoliposomes were eluted using 1 mL of 50 mM NaCl and the collected eluate was mixed with 4 mL of scintillation mixture, followed by vortexing and counting. Data analysis was conducted using Grafit software (version 5.0.13) using the first-order equation for time-course analysis. As specified in the figure legend, all measurements are presented as mean ± s.d. from at least three independent experiments.

Data availability

The cryo-EM maps and the coordinates of the Neu5Ac bound and the unliganded transporter have been deposited in the EMDB (ID 38926 & 38925), and in the Protein Data Bank (8Y4X & 8Y4W) respectively.

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Additional information

Author contributions

PG expressed and purified all the protein samples for this study. KD was involved in the production of nanobodies and protein purification. With the help of KRV, PG optimized grid preparation and data collection in cryo-EM. PG, RS, and KRV performed the computational work

on the cryo-EM data. PG, KRV, and RS designed experiments. MS and CI performed the proteoliposome assays and interpreted the data. RF, RCJD, and RS conceived of the project. All the authors contributed to the writing and revision of the manuscript.

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Conflict of interest

The authors have no conflicts of interest.

Ethics Statement

No human subjects were involved in the study.

Abbreviations

- Neu5Ac: *N*-acetylneuraminic acid
- TRAP: Tripartite ATP-independent periplasmic
- Cryo-EM: Cryo-Electron Microscopy
- DDM: n-dodecyl- β -D-maltopyranoside
- SiaP: Neu5Ac TRAP substrate-binding protein
- SiaQM: Neu5Ac TRAP membrane transporter

References

- Adams PD *et al.* (2010) **PHENIX: a comprehensive Python-based system for macromolecular structure solution** *Acta Crystallographica Section D*
- Allen S, Zaleski A, Johnston JW, Gibson BW, Apicella MA (2005) **Novel sialic acid transporter of *Haemophilus influenzae*** *Infection and immunity* **73**:5291–300 <https://doi.org/10.1128/IAI.73.9.5291-5300.2005>
- Almagro-Moreno S, Boyd EF (2010) **Bacterial catabolism of nonulosonic (sialic) acid and fitness in the gut** *Gut Microbes* **1**:45–50 <https://doi.org/10.4161/gmic.1.1.10386>
- Bell A, Severi E, Owen CD, Latousakis D, Juge N (2023) **Biochemical and structural basis of sialic acid utilization by gut microbes** *Journal of Biological Chemistry* **299** <https://doi.org/10.1016/j.jbc.2023.102989>
- Bose S, Purkait D, Joseph D, Nayak V, Subramanian R (2019) **Structural and functional characterization of CMP-N-acetylneuraminase synthetase from *Vibrio cholerae*** *Acta Crystallographica Section D: Structural Biology* **75**:564–577 <https://doi.org/10.1107/S2059798319006831>
- Chen X, Varki A (2010) **Advances in the biology and chemistry of sialic acids** *ACS chemical biology* **5**:163–176
- Chou HH, Takematsu H, Diaz S, Iber J, Nickerson E, Wright KL, Muchmore EA, Nelson DL, Warren ST, Varki A (1998) **A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence** *Proceedings of the National Academy of Sciences of the United States of America* **95**:11751–11756 <https://doi.org/10.1073/pnas.95.20.11751>
- Chow KM, Whiteheart SW, Smiley JR, Sharma S, Boaz K, Coleman MJ, Maynard A, Hersh LB, Vander Kooi CW (2019) **Immunization of alpacas (*Lama pacos*) with protein antigens and production of antigen-specific single domain antibodies** *JoVE (Journal of Visualized Experiments) e* **58471**
- Coombes D *et al.* (2020) **The basis for non-canonical ROK family function in the N-acetylmannosamine kinase from the pathogen *Staphylococcus aureus*** *Journal of Biological Chemistry* **295**:3301–3315 <https://doi.org/10.1074/jbc.RA119.010526>
- Corfield AP (2015) **Mucins: A biologically relevant glycan barrier in mucosal protection** *Biochimica et Biophysica Acta (BBA) - General Subjects* **1850**:236–252 <https://doi.org/10.1016/j.bbagen.2014.05.003>
- Currie MJ *et al.* (2024) **Structural and biophysical analysis of a *Haemophilus influenzae* tripartite ATP-independent periplasmic (TRAP) transporter** *eLife* **12** <https://doi.org/10.7554/eLife.92307>
- Currie MJ, Manjunath L, Horne CR, Rendle PM, Subramanian R, Friemann R, Fairbanks AJ, Muscroft-Taylor AC, North RA, Dobson RCJ (2021) **N-acetylmannosamine-6-phosphate 2-epimerase uses a novel substrate-assisted mechanism to catalyze amino sugar epimerization** *Journal of Biological Chemistry* **297** <https://doi.org/10.1016/j.jbc.2021.101113>

- Davies JS, Coombes D, Horne CR, Pearce FG, Friemann R, North RA, Dobson RC (2019) **Functional and solution structure studies of amino sugar deacetylase and deaminase enzymes from *Staphylococcus aureus*** *FEBS letters* **593**:52–66
- Davies JS *et al.* (2023) **Structure and mechanism of a tripartite ATP-independent periplasmic TRAP transporter** *Nat Commun* **14** <https://doi.org/10.1038/s41467-023-36590-1>
- DeLano WL (2002) **The PyMOL molecular graphics system**
- Denisov IG, Sligar SG (2016) **Nanodiscs for structural and functional studies of membrane proteins** *Nat Struct Mol Biol* **23**:481–486 <https://doi.org/10.1038/nsmb.3195>
- Emsley P, Cowtan K (2004) **Coot: model-building tools for molecular graphics** *Acta crystallographica section D: biological crystallography* **60**:2126–2132
- Evans R *et al.* (2022) **Protein complex prediction with AlphaFold-Multimer** *bioRxiv* <https://doi.org/10.1101/2021.10.04.463034>
- Felder CB, Graul RC, Lee AY, Merkle H-P, Sadee W (1999) **The venus flytrap of periplasmic binding proteins: An ancient protein module present in multiple drug receptors** *AAPS PharmSci* **1**:7–26 <https://doi.org/10.1208/ps010202>
- Gangi Setty T, Cho C, Govindappa S, Apicella MA, Ramaswamy S (2014) **Bacterial periplasmic sialic acid-binding proteins exhibit a conserved binding site** *Acta Cryst D* **70**:1801–1811 <https://doi.org/10.1107/S139900471400830X>
- Garaeva AA, Guskov A, Slotboom DJ, Paulino C (2019) **A one-gate elevator mechanism for the human neutral amino acid transporter ASCT2** *Nat Commun* **10** <https://doi.org/10.1038/s41467-019-11363-x>
- Glaenger J, Peter MF, Thomas GH, Hagelueken G (2017) **PELDOR Spectroscopy Reveals Two Defined States of a Sialic Acid TRAP Transporter SBP in Solution** *Biophys J* **112**:109–120 <https://doi.org/10.1016/j.bpj.2016.12.010>
- Glanz VY, Myasoedova VA, Grechko AV, Orekhov AN (2018) **Inhibition of sialidase activity as a therapeutic approach** *Drug Des Devel Ther* **12**:3431–3437 <https://doi.org/10.2147/DDDT.S176220>
- Haines-Menges BL, Whitaker WB, Lubin JB, Boyd EF (2015) **Host Sialic Acids: A Delicacy for the Pathogen with Discerning Taste** *Metabolism and Bacterial Pathogenesis* **3**:321–342 <https://doi.org/10.1128/microbiolspec.MBP-0005-2014>
- Horne CR, Kind L, Davies JS, Dobson RC (2020) **On the structure and function of *Escherichia coli* YjhC: an oxidoreductase involved in bacterial sialic acid metabolism. Proteins: Structure Function, and Bioinformatics** **88**:654–668
- Hsieh JM, Besserer GM, Madej MG, Bui H-Q, Kwon S, Abramson J (2010) **Bridging the gap: a GFP-based strategy for overexpression and purification of membrane proteins with intra and extracellular C-termini** *Protein Science* **19**:868–880

- Johnston JW, Coussens NP, Allen S, Houtman JCD, Turner KH, Zaleski A, Ramaswamy S, Gibson BW, Apicella MA (2008) **Characterization of the N-Acetyl-5-neuraminic Acid-binding Site of the Extracytoplasmic Solute Receptor (SiaP) of Nontypeable Haemophilus influenzae Strain 2019 *** *Journal of Biological Chemistry* **283**:855–865 <https://doi.org/10.1074/jbc.M706603200>
- Jurrus E *et al.* (2018) **Improvements to the APBS biomolecular solvation software suite** *Protein Science* **27**:112–128 <https://doi.org/10.1002/pro.3280>
- Kawate T, Gouaux E (2006) **Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins** *Structure (London, England : 1993)* **14**:673–81 <https://doi.org/10.1016/j.str.2006.01.013>
- Kinz-Thompson CD *et al.* (2022) **Elevator mechanism dynamics in a sodium-coupled dicarboxylate transporter** *bioRxiv* <https://doi.org/10.1101/2022.05.01.490196>
- Kumar JP, Rao H, Nayak V, Ramaswamy S (2018) **Crystal structures and kinetics of N-acetylneuraminate lyase from Fusobacterium nucleatum** *Acta Crystallographica Section F: Structural Biology Communications* **74**:725–732 <https://doi.org/10.1107/S2053230X18012992>
- Lewis AL, Lewis WG (2012) **Host sialoglycans and bacterial sialidases: a mucosal perspective** *Cellular Microbiology* **14**:1174–1182 <https://doi.org/10.1111/j.1462-5822.2012.01807.x>
- Mancusso R, Gregorio GG, Liu Q, Wang D-N (2012) **Structure and mechanism of a bacterial sodium-dependent dicarboxylate transporter** *Nature* **491**:622–626 <https://doi.org/10.1038/nature11542>
- Manjunath L, Guntupalli SR, Currie MJ, North RA, Dobson RCJ, Nayak V, Subramanian R (2018) **Crystal structures and kinetic analyses of N-acetylmannosamine-6-phosphate 2-epimerases from Fusobacterium nucleatum and Vibrio cholerae** *Acta Crystallographica Section F Structural Biology Communications* **74**:431–440 <https://doi.org/10.1107/S2053230X18008543>
- Müller A, Severi E, Mulligan C, Watts AG, Kelly DJ, Wilson KS, Wilkinson AJ, Thomas GH (2006) **Conservation of Structure and Mechanism in Primary and Secondary Transporters Exemplified by SiaP, a Sialic Acid Binding Virulence Factor from Haemophilus influenzae*** *Journal of Biological Chemistry* **281**:22212–22222 <https://doi.org/10.1074/jbc.M603463200>
- Mulligan C, Geertsma ER, Severi E, Kelly DJ, Poolman B, Thomas GH (2009) **The substrate-binding protein imposes directionality on an electrochemical sodium gradient-driven TRAP transporter** *Proceedings of the National Academy of Sciences of the United States of America* **106**:1778–83 <https://doi.org/10.1073/pnas.0809979106>
- Mulligan C, Leech AP, Kelly DJ, Thomas GH (2012) **The membrane proteins SiaQ and SiaM form an essential stoichiometric complex in the sialic acid tripartite ATP-independent periplasmic (TRAP) transporter SiaPQM (VC1777-1779) from Vibrio cholerae** *The Journal of biological chemistry* **287**:3598–3608 <https://doi.org/10.1074/jbc.M111.281030>
- Nie R, Stark S, Symersky J, Kaplan RS, Lu M (2017) **Structure and function of the divalent anion/Na⁺ symporter from Vibrio cholerae and a humanized variant** *Nat Commun* **8** <https://doi.org/10.1038/ncomms15009>

North RA *et al.* (2018) **The Sodium Sialic Acid Symporter From *Staphylococcus aureus* Has Altered Substrate Specificity** *Frontiers in Chemistry* **6** <https://doi.org/10.3389/fchem.2018.00233>

North RA, Watson AJ, Pearce FG, Muscroft-Taylor AC, Friemann R, Fairbanks AJ, Dobson RC (2016) **Structure and inhibition of N-acetylneuraminase lyase from methicillin-resistant *Staphylococcus aureus*** *FEBS letters* **590**:4414–4428

Peter MF, Gebhardt C, Glaenger J, Schneberger N, de Boer M, Thomas GH, Cordes T, Hagelueken G (2021) **Triggering Closure of a Sialic Acid TRAP Transporter Substrate Binding Protein through Binding of Natural or Artificial Substrates** *Journal of Molecular Biology* **433** <https://doi.org/10.1016/j.jmb.2020.166756>

Peter MF *et al.* (2022) **Structural and mechanistic analysis of a tripartite ATP-independent periplasmic TRAP transporter** *Nat Commun* **13** <https://doi.org/10.1038/s41467-022-31907-y>

Peter MF, Ruland JA, Kim Y, Hendricks P, Schneberger N, Siebrasse JP, Thomas GH, Kubitscheck U, Hagelueken G (2024) **Conformational coupling of the sialic acid TRAP transporter HiSiaQM with its substrate binding protein HiSiaP** *Nat Commun* **15** <https://doi.org/10.1038/s41467-023-44327-3>

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) **UCSF Chimera—a visualization system for exploratory research and analysis** *Journal of computational chemistry* **25**:1605–1612

Punjani A (2020) **Algorithmic Advances in Single Particle Cryo-EM Data Processing Using CryoSPARC** *Microscopy and Microanalysis* **26**:2322–2323 <https://doi.org/10.1017/S1431927620021194>

Punjani A, Rubinstein JL, Fleet DJ, Brubaker MA (2017) **cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination** *Nature Methods* **14**:290–296 <https://doi.org/10.1038/nmeth.4169>

Rosa LT, Bianconi ME, Thomas GH, Kelly DJ (2018) **Tripartite ATP-Independent Periplasmic (TRAP) Transporters and Tripartite Tricarboxylate Transporters (TTT): From Uptake to Pathogenicity** *Front Cell Infect Microbiol* **8** <https://doi.org/10.3389/fcimb.2018.00033>

Sauer DB, Marden JJ, Sudar JC, Song J, Mulligan C, Wang D-N (2022) **Structural basis of ion – substrate coupling in the Na⁺-dependent dicarboxylate transporter VcINDY** *Nat Commun* **13** <https://doi.org/10.1038/s41467-022-30406-4>

Setty TG, Mowers JC, Hobbs AG, Maiya SP, Syed S, Munson RS, Apicella MA, Subramanian R (2018) **Molecular characterization of the interaction of sialic acid with the periplasmic binding protein from *Haemophilus ducreyi*** *Journal of Biological Chemistry* **293**:20073–20084 <https://doi.org/10.1074/jbc.RA118.005151>

Severi E, Hood DW, Thomas GH (2007) **Sialic acid utilization by bacterial pathogens** *Microbiology* **153**:2817–2822 <https://doi.org/10.1099/mic.0.2007/009480-0>

Sillanauke Pönö, Jääskeläinen (1999) **Occurrence of sialic acids in healthy humans and different disorders** *European Journal of Clinical Investigation* **29**:413–425 <https://doi.org/10.1046/j.1365-2362.1999.00485.x>

- Varki A (2009) **Multiple changes in sialic acid biology during human evolution** *Glycoconj J* **26**:231–245 <https://doi.org/10.1007/s10719-008-9183-z>
- Vetting MW *et al.* (2015) **Experimental Strategies for Functional Annotation and Metabolism Discovery: Targeted Screening of Solute Binding Proteins and Unbiased Panning of Metabolomes** *Biochemistry* **54**:909–931 <https://doi.org/10.1021/bi501388y>
- Wahlgren WY *et al.* (2018) **Substrate-bound outward-open structure of a Na⁺-coupled sialic acid symporter reveals a new Na⁺ site** *Nature Communications* **9** <https://doi.org/10.1038/s41467-018-04045-7>
- Wang S, Xie J, Pei J, Lai L (2023) **CavityPlus 2022 Update: An Integrated Platform for Comprehensive Protein Cavity Detection and Property Analyses with User-friendly Tools and Cavity Databases.** *Journal of Molecular Biology Computation Resources for Molecular Biology* **435** <https://doi.org/10.1016/j.jmb.2023.168141>
- Wang X, Boudker O (2020) **Large domain movements through the lipid bilayer mediate substrate release and inhibition of glutamate transporters** *eLife* **9** <https://doi.org/10.7554/eLife.58417>
- Wirth C, Condemine G, Boiteux C, Bernèche S, Schirmer T, Peneff CM (2009) **NanC Crystal Structure, a Model for Outer-Membrane Channels of the Acidic Sugar-Specific KdgM Porin Family** *Journal of Molecular Biology* **394**:718–731 <https://doi.org/10.1016/j.jmb.2009.09.054>

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Reviewer #1 (Public review):

Summary:

This manuscript reports the substrate-bound structure of SiaQM from *F. nucleatum*, which is the membrane component of a Neu5Ac-specific Tripartite ATP-dependent Periplasmic (TRAP) transporter. Until recently, there was no experimentally derived structural information regarding the membrane components of TRAP transporter, limiting our understanding of the transport mechanism. Since 2022, there have been 3 different studies reporting the structures of the membrane components of Neu5Ac-specific TRAP transporters. While it was possible to narrow down the binding site location by comparing the structures to proteins of the same fold, a structure with substrate bound has been missing. In this work, the authors report the Na⁺-bound state and the Na⁺ plus Neu5Ac state of FnSiaQM, revealing information regarding substrate coordination. In previous studies, 2 Na⁺ ion sites were identified. Here, the authors also tentatively assign a 3rd Na⁺ site. The authors reconstitute the transporter to assess the effects of mutating the binding site residues they identified in their structures. Of the 2 positions tested, only one of them appears to be critical to substrate binding.

Strengths:

The main strength of this work is the capture of the substrate bound state of SiaQM, which provides insight into an important part of the transport cycle.

Weaknesses:

The main weakness is the lack of experimental validation of the structural findings. The authors identified the Neu5Ac binding site, but only test 2 residues for their involvement in substrate interactions, which is quite limited. However, comparison with previous mutagenesis studies on homologues supports the location of the Neu5Ac binding site. The authors tentatively identified a 3rd Na⁺ binding site, which if true would be an impactful finding, but this site was not sufficiently experimentally tested for its contribution to Na⁺ dependent transport. This lack of experimental validation prevents the authors from unequivocally assigning this site as a Na⁺ binding site. However, the reporting of these new data is important as it will facilitate follow up studies by the authors or other researchers.

Comments on revisions:

Overall, the authors have done a good job of addressing the reviewers' comments. It's good to know that the authors are working on the characterisation of the potential metal binding site mutants - characterising just a few of these will provide much needed experimental support for this potential Na⁺ site.

The new MD simulations provide some additional support for the new Na⁺ site and could be included. However, as the authors know, direct experimental characterisation of mutants is the ideal evidence of the Na⁺ site.

Aside from the characterisation of mutants, which seems to be held up by technical issues, the only remaining issue is the comparison of the Na⁺- and Na⁺/Neu5Ac-bound states with ASCT2.

It still does not make sense to me why the authors are not directly comparing their Na⁺ only and Na⁺/Neu5Ac states with the structures of VcINDY in the Na⁺-only and Na⁺/succinate bound states. These VcINDY structures also revealed no conformational changes in the HP loops upon binding succinate, as the authors see for SiaQM. Therefore, this comparison is very supportive. It is understood that the similarity to the DASS structure is mentioned on p.17, but it is also interesting and useful to note that TRAP and DASS transporters also share a lack of substrate-induced local conformational changes, to the extent these things have been measured.

<https://doi.org/10.7554/eLife.98158.2.sa2>

Reviewer #3 (Public review):

The manuscript by Goyal et al report substrate-bound and substrate-free structures of a tripartite ATP independent periplasmic (TRAP) transporter from a previously uncharacterized homolog, *F. nucleatum*. This is one of most mechanistically fascinating transporter families, by means of its QM domain (the domain reported in his manuscript) operating as a monomeric 'elevator', and its P domain functioning as a substrate-binding 'operator' that is required to deliver the substrate to the QM domain; together, this is termed an 'elevator with an operator' mechanism. Remarkably, previous structures had not demonstrated the substrate Neu5Ac bound. In addition, they confirm the previously reported Na⁺ binding sites, and report a new metal binding site in the transporter, which seems to be mechanistically relevant. Finally, they mutate the substrate binding site and use proteoliposomal uptake assays to show the mechanistic relevance of the proposed substrate binding residues.

Strengths:

The structures are of good quality, the presentation of the structural data has improved, the functional data is robust, the text is well-written, and the authors are appropriately careful

with their interpretations. Determination of a substrate bound structure is an important achievement and fills an important gap in the 'elevator with an operator' mechanism.

Weaknesses:

Although the possibility of the third metal site is compelling, I do not feel it is appropriate to model in a publicly deposited PDB structure without directly confirming experimentally. The authors do not extensively test the binding sites due to technical limitations of producing relevant mutants; however, their model is consistent with genetic assays of previously characterized orthologs, which will be of benefit to the field. Finally, some clarifications of EM processing would be useful to readers, and it would be nice to have a figure visualizing the unmodeled lipid densities - this would be important to contextualize to their proposed mechanism.

<https://doi.org/10.7554/eLife.98158.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

*This manuscript reports the substrate-bound structure of SiaQM from *F. nucleatum*, which is the membrane component of a Neu5Ac-specific Tripartite ATP-dependent Periplasmic (TRAP) transporter. Until recently, there was no experimentally derived structural information regarding the membrane components of the TRAP transporter, limiting our understanding of the transport mechanism. Since 2022, there have been 3 different studies reporting the structures of the membrane components of Neu5Ac-specific TRAP transporters. While it was possible to narrow down the binding site location by comparing the structures to proteins of the same fold, a structure with substrate bound has been missing. In this work, the authors report the Na⁺-bound state and the Na⁺ plus Neu5Ac state of FnSiaQM, revealing information regarding substrate coordination. In previous studies, 2 Na⁺ ion sites were identified. Here, the authors also tentatively assign a 3rd Na⁺ site. The authors reconstitute the transporter to assess the effects of mutating the binding site residues they identified in their structures. Of the 2 positions tested, only one of them appears to be critical to substrate binding.*

Strengths:

The main strength of this work is the capture of the substrate-bound state of SiaQM, which provides insight into an important part of the transport cycle.

Weaknesses:

The main weakness is the lack of experimental validation of the structural findings. The authors identified the Neu5Ac binding site, but only tested 2 residues for their involvement in substrate interactions, which was very limited. The authors tentatively identified a 3rd Na⁺ binding site, which if true would be an impactful finding, but this site was not tested for its contribution to Na⁺ dependent transport, and the authors themselves report that the structural evidence is not wholly convincing. This lack of experimental validation undermines the confidence of the findings. However, the reporting of these new data is important as it will facilitate follow-up studies by the authors or other researchers.

The main concern, also mentioned by other reviewers, is the lack of mutational data and functional studies on the identified binding sites. Two other structures of TRAP transporters

have been determined, one from *Haemophilus influenzae* (Hi) and the other from *Photobacterium profundum* (Pp). We will refer to the references in this paper as [1], Peter et al. as [2], and Davies et al. as [3]. The table below lists all the mutations made in the Neu5Ac binding site, including direct polar interactions between Neu5Ac and the side chains, as well as the newly identified metal sites.

The structure of *Fusobacterium nucleatum* (Fn) that we have reported shows a significant sequence identity with the previously reported Hi structure. When we superimpose the Pp and Fn structures, we observe that nearly all the residues that bind to the Neu5Ac and the third metal site are conserved. This suggests that mutagenesis and functional studies from other research can be related to the structure presented in our work.

The table below shows that all three residues that directly interact with Neu5Ac have been tested by site-directed mutagenesis for their role in Neu5Ac transport. Both D521 and S300 are critical for transport, while S345 is not. We do not believe that a mutation of D521A in Fn, followed by transport studies, will provide any new information.

However, Peter et al. have mutated only one of the 5 residues near the newly identified metal binding site, which resulted in no transport. The rest of the residues have not been functionally tested. We propose to mutate these residues into Ala, express and purify the proteins, and then carry out transport assays on those that show expression. We will include this information in the revised manuscript.

Author response table 1.

Site description	Residues in FnSiaQM	Residues in Hi SiaQM	Equivalent Residues in Pp SiaQM	Mutation and results
	[1]	Peter et al., 2022 [2]	Davis et al., 2023 [3]	
Neu5Ac Binding Site	S300			S300A -Mutation resulted in no transport
	S345			S345A - Transport is not affected. This is also not conserved in Pp
	D521	D521	E329	Mutation of D521A in Hi and E329A in Pp resulted in no transport. [2][3]
New metal Site				
	D304	D304	D112	D304A mutation shows no transport. [2]. Their model, however suggested that this binds to substrate.
	E312	E312	E120	No Data
	S291	S291	S99	No Data
	T330	T330	T138	No Data
	S333	S333	S141	No Data

Reviewer #2 (Public Review):

*In this exciting new paper from the Ramaswamy group at Purdue, the authors provide a new structure of the membrane domains of a tripartite ATP-independent periplasmic (TRAP) transporter for the important sugar acid, N-acetylneuraminic acid or sialic acid (Neu5Ac). While there have been a number of other structures in the last couple of years (the first for any TRAP-T) this is the first to trap the structure with Neu5Ac bound to the membrane domains. This is an important breakthrough as in this system the ligand is delivered by a substrate-binding protein (SBP), in this case, called SiaP, where Neu5Ac binding is well studied but the 'hand over' to the membrane component is not clear. The structure of the membrane domains, SiaQM, revealed strong similarities to other SBP-independent Na⁺-dependent carriers that use an elevator mechanism and have defined Na⁺ and ligand binding sites. Here they solve the cryo-EM structure of the protein from the bacterial oral pathogen *Fusobacterium nucleatum* and identify a potential third (and theoretically predicted) Na⁺ binding site but also locate for the first time the Neu5Ac binding site. While this sits in a region of the protein that one might expect it to sit, based on comparison to other transporters like VcINDY, it provides the first molecular details of the binding site architecture and identifies a key role for Ser300 in the transport process, which their structure suggests coordinates the carboxylate group of Neu5Ac. The work also uses biochemical methods to confirm the transporter from *F. nucleatum* is active and similar to those used by selected other human and animal pathogens and now provides a framework for the design of inhibitors of these systems.*

The strengths of the paper lie in the locating of Neu5Ac bound to SiaQM, providing important new information on how TRAP transporters function. The complementary biochemical analysis also confirms that this is not an atypical system and that the results are likely true for all sialic acid-specific TRAP systems.

The main weakness is the lack of follow-up on the identified binding site in terms of structure-function analysis. While Ser300 is shown to be important, only one other residue is mutated and a much more extensive analysis of the newly identified binding site would have been useful.

Please see the comments above.

Reviewer #3 (Public Review):

*The manuscript by Goyal et al reports substrate-bound and substrate-free structures of a tripartite ATP-independent periplasmic (TRAP) transporter from a previously uncharacterized homolog, *F. nucleatum*. This is one of the most mechanistically fascinating transporter families, by means of its QM domain (the domain reported in his manuscript) operating as a monomeric 'elevator', and its P domain functioning as a substrate-binding 'operator' that is required to deliver the substrate to the QM domain; together, this is termed an 'elevator with an operator' mechanism. Remarkably, previous structures had not demonstrated the substrate Neu5Ac bound. In addition, they confirm the previously reported Na⁺ binding sites and report a new metal binding site in the transporter, which seems to be mechanistically relevant. Finally, they mutate the substrate binding site and use proteoliposomal uptake assays to show the mechanistic relevance of the proposed substrate binding residues.*

The structures are of good quality, the functional data is robust, the text is well-written, and the authors are appropriately careful with their interpretations. Determination of a substrate-bound structure is an important achievement and fills an important gap in the 'elevator with an operator' mechanism. Nevertheless, I have concerns with the data presentation, which in its current state does not intuitively demonstrate the discussed

findings. Furthermore, the structural analysis appears limited, and even slight improvements in data processing and resulting resolution would greatly improve the authors' claims. I have several suggestions to hopefully improve the clarity and quality of the manuscript.

We appreciate your feedback and will make the necessary modifications to the manuscript incorporating most of the suggestions. We will submit the revised version once the experiments are completed. We are also working on improving the quality of the figures and have made several attempts to enhance the resolution using CryoSPARC or RELION, but without success. We will continue to explore newer methods in an effort to achieve higher resolution and to model more lipids, particularly in the binding pocket.

Reviewing Editor (Recommendations for the Authors):

After discussing the reviews, the reviewers and reviewing editor have agreed on a list of the most important suggested revisions for the authors, which, if satisfactorily addressed, would improve the assessment of the work. These suggested revisions are listed below. We also include the full Recommendations For The Authors from each of the individual reviewers.

(1) The authors tentatively identified a 3rd Na⁺ binding site, which if true would be an impactful finding, but this site was not tested for its contribution to Na⁺ dependent transport, and the authors themselves report that the structural evidence is not wholly convincing. Additional mutagenesis and activity experiments to test the contribution of this site to transport would strengthen the manuscript. Measuring Na⁺ concentration-response relations and calculating Hill slopes in WT vs. an M site mutant would be a good experiment. Given the lack of functional data and poor density, it does not seem appropriate to build the M site sodium in the PDB model.

The density is well defined to suggest a metal bound (waters would not be clearly defined at this resolution). While our modeling of the site as a Na⁺ is arbitrary, this was done to satisfy the refinement programs where we have a known scatterer modeled. We could model this density with other metals, but unlike crystallographic refinement, real-space refinement of cryoEM maps does not produce a difference map that might allow us to identify the metal but not conclusively. The density of the maps is good (we have added better figures to demonstrate this). We tried making multiple mutations to test for activity – unfortunately, we are still struggling to express proteins with mutations in this site in sufficient quantities to carry out transport assays.

In the absence of being able to do the experiments, we did MD simulations (carried out by Senwei Quan and Jane Allison at University of Auckland). Our results are shown below – we are not certain without further studies that these should be included in the current paper (we will add them as authors if the editor feels that this evidence is critical).

Author response table 2.

<i>Simulations</i>	<i>Ions-Binding site 1</i>	<i>Ions-Binding site 2</i>	<i>Ions-binding site 3</i>	<i>Neu5Ac</i>
<i>with K+</i>	stay stably	left at around 35ns	K+ left at ~358ns	left at ~110ns
<i>with Na+</i>	stay stably	left at around 470ns	Na+stay stably	left at ~248ns
<i>with Ca2+</i>	stay stably	stay stably	Ca2+ left at ~88ns	left at ~58ns
<i>NO ions at binding site 3</i>	stay stably	left at around 295ns	NA	left at 148 ns

We are showing this for review to suggest that K⁺, Ca²⁺, and Na⁺ were tried, and only Na⁺ stays stably in the binding pocket. The rest of the results will also have to be explained, which would change the focus of the paper.

We also provided the sequence to AlphaFold3 and asked it to identify the possible metal binding sites—when the input was Na⁺, it found all three binding sites.

Summary: Both our experimental data and computational studies suggest the observed metal binding site is real but at the moment, it is not possible to refine the structure and put an unidentified metal. Computational studies suggest that this is a high-probability Na⁺ site.

Demonstration of cooperativity between the Na⁺ site and transport require carrying out these experiments with mutations in these sites in a concentration-dependent manner. Unfortunately, our inability to produce well-expressed and purified proteins with mutations in a short time frame failed.

(2) The authors identified the Neu5Ac binding site but only tested 2 residues for their involvement in substrate interactions, which was very limited. Given that the major highlight of this paper is the identification of the Neu5Ac binding site, it would strengthen the manuscript if the authors provided a more extensive series of mutagenesis experiments - testing at least the effect of D521A would be important. One inconsistency is Ser345 mutagenesis not affecting transport, and the authors should further discuss in the text why they think that is.

D521A has been tested in *H. influenzae*, and this mutation results in loss of transport. This residue is highly conserved and occupies the same position. We expect the result to remain the same.

We have added a few extra lines to discuss Serine 345: “Ser 345 OG is 3.5Å away from the C1-carboxylate oxygen – a distance that would result in a weak interaction between the two groups. It is, therefore, not surprising that the mutation into Ala did not affect transport. The space created by the mutation can be occupied by a water molecule.”

(3) The purification and assessment of the stability of the protein are described in text alone with no accompanying data. It would be beneficial to include these data (e.g. in the Supplementary info) as it allows the reader to evaluate the protein quality.

This is now added as Supplementary Figure 2.

(4) The structural figures throughout the paper could benefit from more clarity to better support the conclusions. Specific critiques are listed below:

- Figure 1: since the unbound map has a similar reported resolution, displaying the unbound structure's substrate binding site with the same contour would clearly demonstrate that the appearance of this density is substrate-dependent.
- Figure 1: the atomic fit of the ligand to the density, and the suggested coordination by side chain and backbone residues, would be useful in this figure.
- Figure 1: I think it would be more intuitive to compare apo and bound structures with the same local resolution scale.

We have remade Figure 1 “Architecture of FnSiaQM with nanobody. (A and B) Cryo-EM maps of FnSiaQM unliganded and sialic acid bound at 3.2 and 3.17 Å, respectively. The TM domain of FnSiaQM is colored using the rainbow model (N-terminus in blue and C-terminus in red). The nanobody density is colored in purely in red. The density for modeled lipids is colored in tan and the unmodelled density in gray. The figures were made with Chimera at thresholds of 1.2 and 1.3 for the unliganded and sialic acid-bound maps. (C and D) The cytoplasmic view of apo and sialic acid bound FnSiaQM, respectively. Color coding is the same as in panels A and B. The density corresponding to sialic acid and sodium ions are in purple. The substrate binding sites of apo and sialic acid bound FnSiaQM are shown with key residues labeled. The density (blue mesh) around these atoms was made in Pymol with 2 and 1.5 s for the apo and the sialic acid, respectively, with a carve radius of 2 Å.”

The local resolution maps have been moved to Supplementary Figure 3.

- Figure 3, Figure 5a: The mesh structures throughout the manuscript are blocky and very difficult to look at and interpret, especially for the ion binding sites, which are currently suggestive of but not definitively ion densities. Either using transparent surfaces, higher triangle counts, or smoothing the surface might help this.

We have made Figure 3 again with higher triangle counts. We tried all three suggestions and this provided the best figure. We have replaced Figure 5A with density for Neu5Ac and residues around it.

- Figure 5A: It would be important to show the densities of the entire binding pocket, especially coordinating side chains, to show the reader what is and isn't demonstrated by this structure.
- It's not clear how Figure 5D is supposed to show that the cavity can accommodate Neu5Gc, as suggested by the text - please make the discussed cavity clearer in the Figure.

We have now marked with an arrow the Methyl Carbon where the hydroxyl group is added. We have mentioned that in the legend. It is open to the periplasmic side of the cavity.

- Supplementary Figure 4: Please label coordinating residue sites.

Labels have been added to Supplementary Figure 6 which was earlier Supplementary Figure 4.

- (5) Intro section: the authors should introduce the work on HiSiaP around the role of the R147 residue in high-affinity Neu5Ac binding, which coordinates the carboxylate of Neu5Ac, and which is a generally conserved mechanism for organic acid binding in other TRAP transporters. This context will help magnify their discovery later that in the

membrane domains, it is a key serine and not an arginine that coordinates the carboxylate group (probably as the local concentration of Neu5Ac is high and tight binding site is not desirable for rapid transport, which is mentioned in the discussion).

Thank you for pointing this out. We have added a new sentence to the introduction.

“All the SiaP structures show the presence of a conserved Arginine that binds to the C1-carboxylate of Neu5Ac, and this Arg residue is critical as the high electrostatic affinity may be important to have a strong binding affinity that sequesters the small amounts that reach the bacterial periplasmic space (Glaenger *et al.*, 2017).”

(6) TRAP transporters exist for many organic compounds and not just sialic acid, which might be nice to make the reader aware of.

We initially did not do this as this is an advance paper and this was discussed in the earlier paper (Currie *et al.*, 2024). However, we have now added a sentence to the introduction. “Additionally, amino acids, C4-dicarboxylates, aromatic substrates and alpha-keto acids are also transported by TRAP transporters (Vetting *et al.*, 2015). “

(7) On p. 12, the authors describe the Neu5Ac binding site as a large solvent-exposed vestibule, having previously described the substrate-bound state as occluded. These descriptions should be adjusted to make clear which structure is being referenced. The clarity of this would be substantially improved if the authors included a figure that showed this occlusion - currently none of the structure figures clearly demonstrate what the authors are referring to. There are several conspicuous unmodeled densities proximal to the substrate, reminiscent of lipids (in between transport and scaffold domain) and possibly waters/ions. Given this, it is really surprising that the substrate binding site is described as "solvent-exposed" since the larger molecules seem to occlude the pocket. The authors should further process their dataset and discuss the implications of these surrounding densities.

We have processed the data sets carefully both with cryosparc and relion and the resolution described here is same with both software with the cryosparc maps slightly better in terms of interpretability of peripheral helices and described in the manuscript. The current sample (FnTRAP) with the nanobody is a relatively stable sample (in our experience with other similar proteins) as evident from the number of images and particles to achieve a decent resolution and thus the workflow is straightforward and simple. There are number of non-protein densities, which in principle can be modelled but we have chosen a conservative approach not to model these extra densities (except for the two lipids, few ions) due to limit of the resolution. It is possible that increasing the number of particles will result in an increase in resolution but from the estimated B-factor (125 or 135 Å² for unliganded and liganded), this will certainly require lot of more images with no guarantee of increased resolution.

The question of outward open Vs outward occluded is a valid point. We have now modified this in the manuscript. “The Neu5Ac binding site has a large solvent-exposed vestibule towards the cytoplasmic side, while its periplasmic side is sealed off. Cryo-EM map shows the presence of multiple densities that could be modeled as lipids, possibly preventing the substrate from leaving the transporter. However, the densities are not well defined to model them as specific lipids, hence they have not been modeled. We describe this as the “inward-facing open state” with the substrate-bound.”

(8) On p.15, the activity of FnSiaPQM in liposomes is reported, although the impetus for this study is not clear. Presumably, the reason for its inclusion is to ensure that the structurally characterized protein is active. It would be useful to say this at the start of

the section if this is the case. This study nicely shows that the energetics and requirements of transport are identical to all the previous studies on Neu5Ac TRAP transporters - it would be good to acknowledge this somewhere in this section as well.

These changes have been incorporated. We have added a line to say why we did this and added as the last line that this is similar to other SiaPQM's characterized.

(9) Figure 5C. The authors show the transport activity with and without valinomycin. The authors do not explain the rationale for testing and reporting both conditions for these mutants; an explanation is required, or the data should be simplified. The expected membrane potential induced by valinomycin should be mentioned in the legend.

We have simplified Figure 5C and added the expected membrane potential value.

(10) The authors state that the S300A mutant is inactive. However, unless the authors also measured the background binding/transport of radiolabelled substrate in the absence of protein, then the accuracy of this statement is not clear because Figure 5C does indicate some activity for S300A, albeit much lower than WT. This is an important point in light of the authors' suggestion that the membrane protein does not need a binding site of high affinity or stringent selectivity.

We thank the reviewer for pointing this out we have now added a line in the experimental protocols "The experimental values were corrected by subtracting the control, i.e. the radioactivity taken up in liposomes reconstituted in the absence of protein. The radioactivity associated with the control samples, i.e. empty liposomes was less than 10% with respect to proteoliposomes."

(11) There are several issues and important omissions in the work cited:

- It is not normal practice to cite a reference in the abstract and the citation is only to the second structure of HiSiaQM, which does not fairly reflect previous work in the field by only referring to their own work. Also throughout the article, it is normal practice with in-text citations to order them chronologically, i.e. earliest first. Please update this.

This article was submitted as an "Research advance article". The instructions specifically say that "Research advance article should cite the article in eLife this paper advances. Hence the citation of the "second structure of HiSiaQM". In fact, in the manuscript we explicitly say "The first structure of _Hi_SiaQM (4.7 Å resolution) demonstrated that it is composed of 15 transmembrane helices and two helical hairpins." We are following the policy laid out.

Zotero organizes multiple references in alphabetical order, we did not choose to do it that way – the suggestion of bias is not true. The final version of the accepted paper will have numbers, and this argument will automatically be corrected.

- Intro: please cite the primary papers discovering other families of sialic acid transporters.

- Intro: When introducing information on the binding site, dissociation constant of Neu5Ac, and thermodynamics of ligand binding to SiaP, the authors should also include references to the work done by others in addition to their own work.

The Setty *et al.* paper was the first to demonstrate that the two-component systems are distinct, and that the binding protein of the TRAP system binds enthalpically while the binding protein of the ABC system binds entropically (SiaP vs SatA). As the reviewer points out, this is significant because it highlights how the Arg binding to the carboxylate, which is

the enthalpic driver in this case and contributes to the difference between sugar binding to SiaP and SatA. Many studies have published binding affinities of molecules to SiaP, but this paper offers valuable insight into the differences between these systems. We have cited a number of the SiaP papers from other groups, including acknowledging the first structure of SiaP from *H. influenzae* by Muller *et al.*, in 2006.

- p.5 "TRAP transporters are postulated to employ an elevator-type mechanism...". This postulation has been experimentally tested and published, so should be discussed and referenced (Peter *et al.* 2024. <https://doi.org/10.1038/s41467-023-44327-3>).

We have now corrected this error. We removed "are postulated to" and added the reference.

- p.5 "Notably, the transport of Neu5Ac by TRAP transporters requires at least two sodium ions (Davies *et al.*, 2023)." The requirement for at least 2 Na⁺ ions for Neu5Ac transport was first demonstrated in Mulligan *et al.* PNAS 2009, so should also be cited (for completion, so should Mulligan *et al.* JBC. 2012 and Currie *et al.* elife 2023, which have also shown this requirement is a commonality amongst all Neu5Ac TRAP transporters).

Added.

- P.12, Mulligan *et al.* JBC, 2012 should be added to the citations in the first sentence.

Added.

- p.19 "Interestingly, even the dicarboxylate transporter from *V. cholerae* (VcINDY) binds to its ligand via electrostatic interactions with both carboxylate groups". Other references are more appropriate than the one used to support this statement.

Also added references for Mancusso *et al.*, 2012, Nie *et al.*, 2017 and Sauer *et al.*, 2022 here.

- p.19. "The structure of the protein in the outward-facing conformation is unknown". The authors do not discuss the mechanistic findings from Peter *et al* 2024 Nat Comm here. The work described in that paper revealed an experimentally verified model of the OFS of HiSiaQM, so really needs to be included.

This is not an experimentally determined 3D structure. They have shown the possible existence of this by microscopy, but the structure is not determined. The work mentioned is a wonderful piece of work, but it does not report the three-dimensional structure of the protein in the outward-facing conformation to allow us to understand the nature of the molecular interactions.

- The reference to Kinz-Thompson *et al* 2022 on p. 6 is not appropriate - neither the HiSiaQM papers nor the PpSiaQM paper makes reference to this work when identifying the binding site. More suitable references are used, for example, Mancusso *et al* 2012, Nie *et al* 2017 and Sauer *et al* 2022; this should be reported accurately.

Added the suggested references. We think the paper (Kinz-Thompson *et al* 2022) is relevant and have also kept that reference.

- Garaeva *et al* report the opposite of what the authors mention - "In the human neutral amino acid transporter (ASCT2), which also uses the elevator mechanism, the HP1 and HP2 loops have been proposed to undergo conformational changes to enable substrate

binding and release (Garaeva et al., 2019)." In fact, this paper suggested a one-gate model of transport (HP2), where HP1 seems uninvolved in gating.

The Reviewer is correct. We were wrong and not clear. The entire paragraph has been rewritten.

"While, both the HP1 and HP2 loops have been hypothesized to be involved in gating, in the human neutral amino acid transporter (ASCT2), (which also uses the elevator mechanism), only the HP2 loops have been shown to undergo conformational changes to enable substrate binding and release (Garaeva et al., 2019). Hence, it is suggested that there is a single gate that controls substrate binding. Superposition of the _Pp_SiaQM and _Hi_SiaQM structures do not reveal any change in these loop structures upon substrate binding. For TRAP transporters, the substrate is delivered to the QM protein by the P protein; hence, these loop changes may not play a role in ligand binding or release. This may support the idea that there is minimal substrate specificity within SiaQM and that it will transport the cargo delivered by SiaP, which is more selective."

- p.19 "suggesting that SSS transporters have probably evolved to transport nine-carbon sugars such as Neu5Ac (Wahlgren et al, 2018)." Surely this goes without saying since Wahlgren et al 2018 demonstrated that SiaT, an SSS, could transport sialic acid? It's unclear why this was included here - perhaps it needs to be rewritten to make the point more clearly, but as it stands, this statement appears self-evident. Furthermore, these proteins can transport all kinds of molecules (see TCDB 2.A.21). This statement needs to be clarified.

This was a comparison to other Neu5Ac binding sites in other Neu5Ac transporters. We have modified the sentence. "The polar groups bind to both the C1-carboxylate side of the molecule and the C8-C9 carbonyls, suggesting that *Proteus mirabilis* Neu5Ac transporter (SSS type) evolved specifically to transport nine-carbon sugars such as Neu5Ac (Wahlgren et al., 2018)". These were arguments we were making to suggest that the lack of tight binding could also mean reduced specificity.

- The authors reconstitute the FnSiaQM and measure transport with SiaP, which resembles closely what is known for both HiSiaPQM, VcSiaPQM, which is not cited (<https://doi.org/10.1074/jbc.M111.281030>).

- Regarding lipids between transport and scaffold domains: there is precedent for such lipids in the elevator transporter GltPh, Wang, and Boudker (eLife 2020) proposed similar displacements during transport and would be appropriate to cite here.

We have now cited the reference to the Mulligan et al., 2012 paper. We also added a sentence on the findings of GltPh paper by Wang and Boudker. Thank you for pointing this out.

(12) p.9 "TRAP transporters, as their name suggests, comprise three units: a substrate-binding protein (SiaP) and two membrane-embedded transporter units (SiaQ and SiaM) (Severi et al., 2007)." This is somewhat odd phrasing because the existence of fused membrane components has been well-documented for a long time. The addition of "Many" at the start of the sentence fixes this.

Added Many.

(13) On p.12 the authors compare the ligand-induced conformational changes of FnSiaQM with ASCT2, citing Garaeva et al, 2019. This comparison does not make sense considering TRAP transporters and ASCT2 do not share a common fold. A far superior comparison is with DASS transporters, which actually do have the same fold as TRAP

transporters. And, importantly, the Na⁺ and substrate-induced conformational changes have been investigated for DASS transporters revealing a unique mechanism likely shared by TRAP transporters (Sauer et al, Nat Comm, 2022). The text on p.12 should be adjusted to replace the ASCT comparison with a VcINDY comparison.

The purpose of citing the ASCT2 paper was only concerning the HP1 and HP2 gates. The authors show that HP2 changes conformation only. Comparing the two FnSiaQM structures – with and without ligand, we see no change in either the HP1 or the HP2 loops. On Page 17, when we describe the structure, we do specifically mention that the overall architecture is similar to VcINDY and the DASS transporters.

(14) p.12 "For TRAP transporters, the substrate is delivered to the QM protein by the SiaP^P protein;" "SiaP protein" should be "P protein"

Corrected.

(15) p.18. "periplasmic membrane" should be "cytoplasmic membrane".

Corrected.

(16) p.19. "This prevents Neu5Ac from binding..." There is no evidence for this so this needs to be softened, e.g. "This likely prevents Neu5Ac from..."

Agree – Modified.

(17) Figure 2B is rather small, cramped, and difficult to see. We suggest that the authors make that panel larger, or include it as a stand-alone supplementary figure.

We have moved this figure into a supplementary figure as suggested by the reviewer.

(18) The authors describe the Neu5Ac binding site in SiaQM. It would be helpful if the authors provided a figure in support of the statement that the Neu5Ac binding site architecture is similar to dicarboxylate in VcINDY (especially as Neu5Ac is a monocarboxylate).

The Neu5Ac binding site is NOT similar to the VcINDY binding site. But, we understand the origin of the comment. We have now changed the sentence: “The overall architecture of the Neu5Ac binding site is similar to that of citrate/malate/fumarate in the di/tricarboxylate transporter of *V. cholerae* (Vc_INDY), but the residues involved in providing specificity are different (Kinz-Thompson *et al.*, 2022; Mancusso *et al.*, 2012; Nie *et al.*, 2017; Sauer *et al.*, 2022). Neu5Ac binds to the transport domain without direct interactions with the residues in the scaffold domain. The majority of the interactions are with residues in the HP1 and HP2 loops of the transport domain (Figure 5B). Asp521 (HP2), Ser300 (HP1), and Ser345 (helix 5) interact with the substrate through their side chains, except for one interaction between the main chain amino group of residue 301 and the C1-carboxylate oxygen of Neu5Ac. Mutation of the residue equivalent to Asp521 has been shown to result in loss of transport (Peter *et al.*, 2022). To evaluate the role of residues Ser-300 and Ser-345, we mutated them to alanine and performed the transport assays.”

(19) When comparing the binding modes of Neu5Ac to different proteins in Figure 6, it would be helpful to include the structure in this paper as well.

The Neu5Ac binding site is present in figure 5. We would prefer not to show it again in Figure 6.

Additionally, there is a clear binding mode of Neu5Ac in Figure 1 as well.

(20) The manuscript would benefit from a more detailed comparison between Na⁺-bound (described as apo) and Na⁺/Neu5Ac structures, especially the prospective gates. If this transporter behaves anything like the archetypical ion-coupled glutamate transporters, some structural changes in the gates might be expected to facilitate transport domain movement when the substrate is loaded, but not when only Na⁺ is bound. It would be important to discuss and visualize these changes.

We have described in the manuscript that there is NO change in the HP1 and HP2 gates between the unliganded structure and the Neu5Ac bound structure. The major difference we observe is the ordering of the third metal binding site.

A figure comparing the substrate binding pockets between the different high-resolution structures would also be informative. Do the bonding distances between ligands and side chains significantly change between homologs?

This is the only Neu5Ac bound structure. Since the specificity to the substrate comes from the variability of the residues that interact it, we do not believe that this figure would not add much value.

(21) A supplementary figure (or an inset to Figure 2) showing pairwise percent identity between different characterized QM transporters would be useful.

We have now added a Supplementary Figure 4 showing the comparison of the three QM sequences whose structures have been determined.

(22) There is relatively minimal EM processing. More rigorous processing would require relatively little effort and could boost resolution, making this a vastly improved manuscript with a much more confident interpretation of structures.

We described the overall workflow. The processing was rigorous. After obtaining the first maps, we created templates with the structure and did template-based picking. We then did several rounds of 2D classification followed by homogenous refinement, Non-Uniform Refinement. We then made masks and carried out local refinement. We then got the best maps and did a 3D classification. Refined the 3D classes independently. Then, we regrouped them based on how similar they were. We then went back and picked particles again (we used different methods of particle picking, but template-based picking resulted in the final set of particles used) and went through the whole process again. At the end of the refinement, we carried out global and local CTF refinement followed by reference-based motion correction. The final refinement was then done with the Bayesian polished particles. The final refinement was local refinement with a mask over only the transporter and the nano-body. After the reviews came, we tried multi-body refinement in Relion5. It did not improve resolution. We have expanded the legend to supplementary Figure 2 (without listing all the different things we tried). The best resolution we obtained for the structure was 3.1 Å. However, it is important to note that the local resolution of the map around the ligand is good.

We realized this is not easy to depict in a local resolution map. So, we wrote a script to take every atom, then take a radius of 5 Å (again we tried different radii and used the optimal one; we are preparing a manuscript to describe this), take all the local resolution values within the 5 Å sphere and average it and add it as B-factor that atom. We have moved the local resolution map figure to the supplement and replaced Figure 1 with a Cartoon, where the color represents the local resolution in which the atom is.

(23) Calling the structure without Neu5Ac bound an "apo" structure is confusing since it indeed has the ligand Na⁺ present and bound. "Na⁺" and "Na⁺/Neu5Ac" structures would be more appropriate.

Changed all "apo" to "unliganded".

<https://doi.org/10.7554/eLife.98158.2.sa0>