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Supporting Information

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Supporting Information

for

Detection of Ligand Binding to Nucleotide Sugar Transport Proteins by STD NMR Spectroscopy

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Additional results

The binding epitope of CMP-Neu5Ac and CMP when bound to CST present in GeF-I preparations was determined by calculating relative STD NMR effects. A detailed 1H and STD NMR spectrum of GeF-I:CMP-Neu5Ac complex is shown in Figure S1.

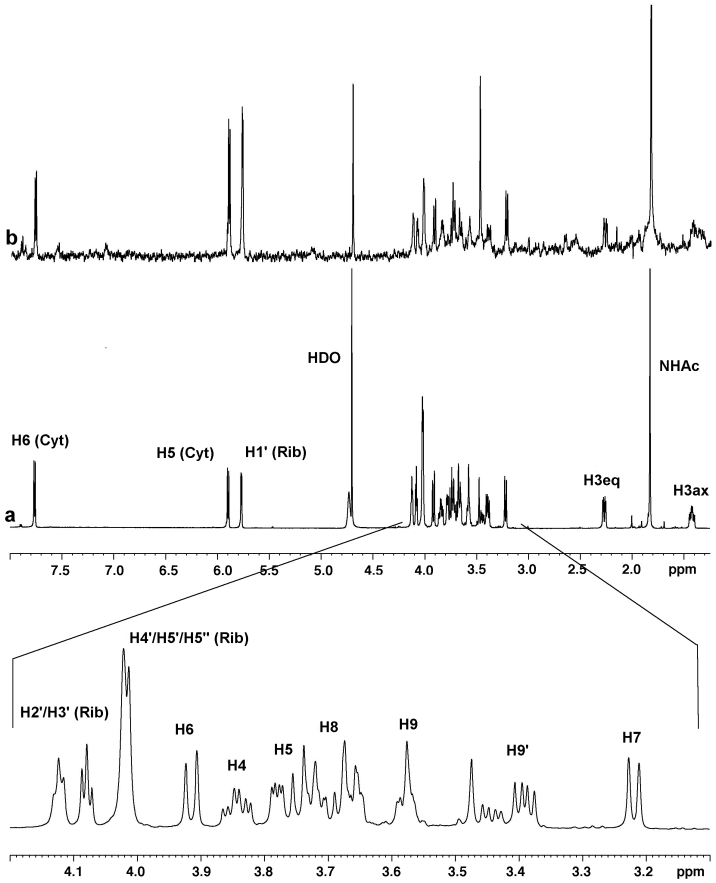

Figure S1. A) ^{1}H and B) STD NMR spectrum of GeF-I:CMP-Neu5Ac with all proton signals assigned (bottom panel).

To remove the signal intensities resulting from the binding of CMP to endogenous proteins, the STD NMR spectrum for the GeF-wt:CMP complex (Figure S2c) was subtracted from the GeF-I:CMP complex (Figure S2b) resulting in a Saturation Transfer Double Difference (STDD) NMR spectrum (Figure S2d). Relative STD NMR effects were calculated according to the formula $A_{STD} = (I_0 \times I_{sat}) / I_0 = I_{STD} / I_0$. All STD effects are given relative to the STD effect of H1' (Rib; 100%). Interestingly, the relative STD NMR effects of CMP-Neu5Ac and CMP for the nucleotide moiety are comparable suggesting that both ligands are bound similarly to CST. In both ligand complexes the highest amount of saturation received from the protein was at the H1' ribose proton (Figure S3).

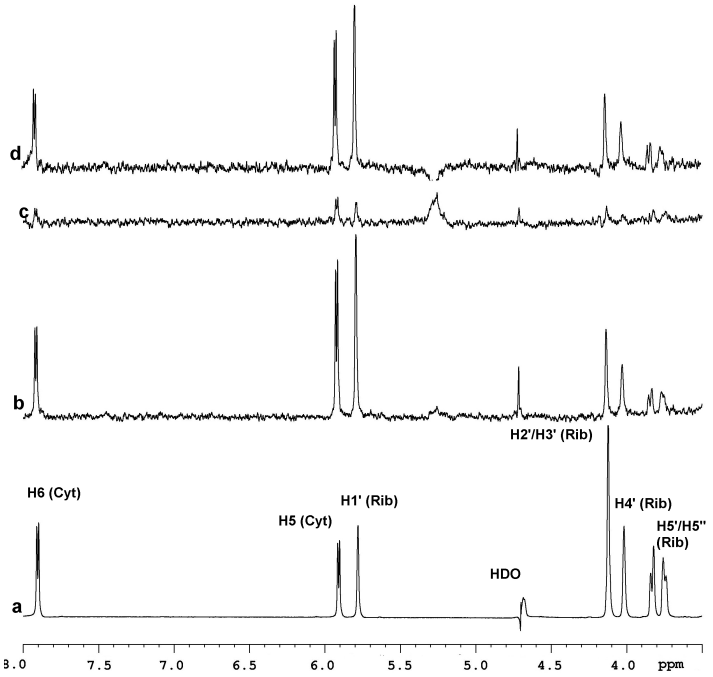

Figure S2. A) ^{1}H, STD NMR spectra of Gef-I:CMP (B) and GeF-wt:CMP (C), and D) the STDD NMR spectrum generated by substracting (C) from (B).

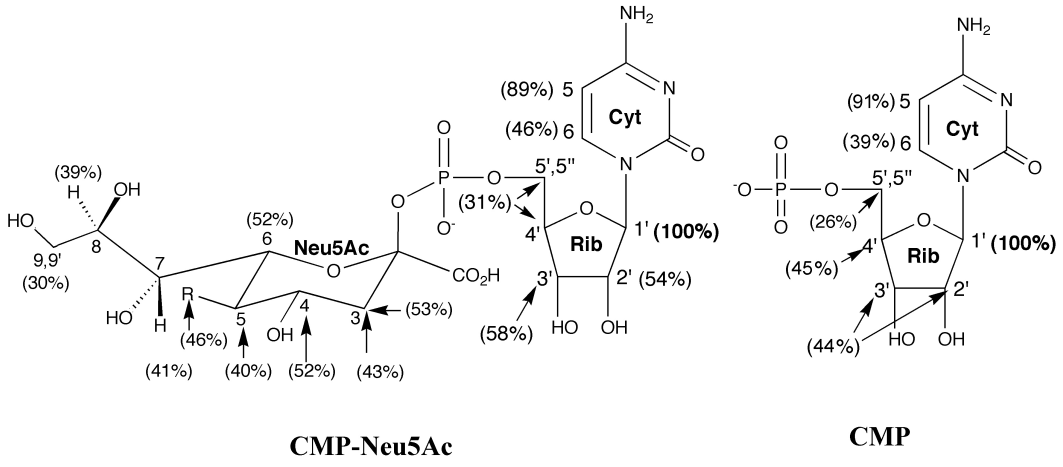

Figure S3. Relative STD NMR values of CMP-Neu5Ac and CMP. The STD NMR spectrum of the GeF-I:CMP-Neu5Ac complex was used to calculate the relative STD NMR effects for CMP-Neu5Ac. A STDD spectrum (GeF-I:CMP – GeF-wt:CMP) was used to calculate relative STD NMR effects for CMP. All STD NMR effects were calculated according to the formula $A_{STD} = (I_0 \times I_{sat})/I_0 = I_{STD}/I_0$ using the H1' (Rib) proton to 100%.

The authenticity of the observed STD NMR effects was investigated by pre-treating GeF-I preparations with trypsin for 2 h. The acquisition of an STD NMR spectrum (Figure S4) shows an almost complete reduction in the STD NMR signals for CMP-

Neu5Ac. This result clearly shows that the binding of CMP-Neu5Ac to GeF-I is protein specific.

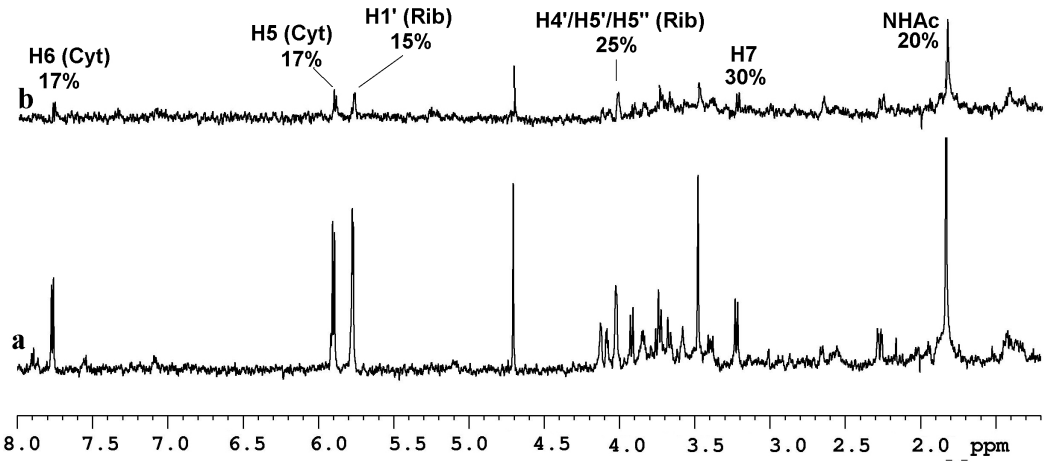

Figure S4: STD NMR spectrum of GeF-I:CMP-Neu5Ac A) before and B) after pretreatment with trypsin. Percentages describe residual STD NMR effects compared to the untreated GeF-I preparation.

To assess the potential of STD NMR to monitor interactions of nucleotide sugars with endogenous NSTs associated with the *P. pastoris* GeF-wt preparation, STD NMR spectra using a variety of different sugar nucleotides as binding ligands were acquired.

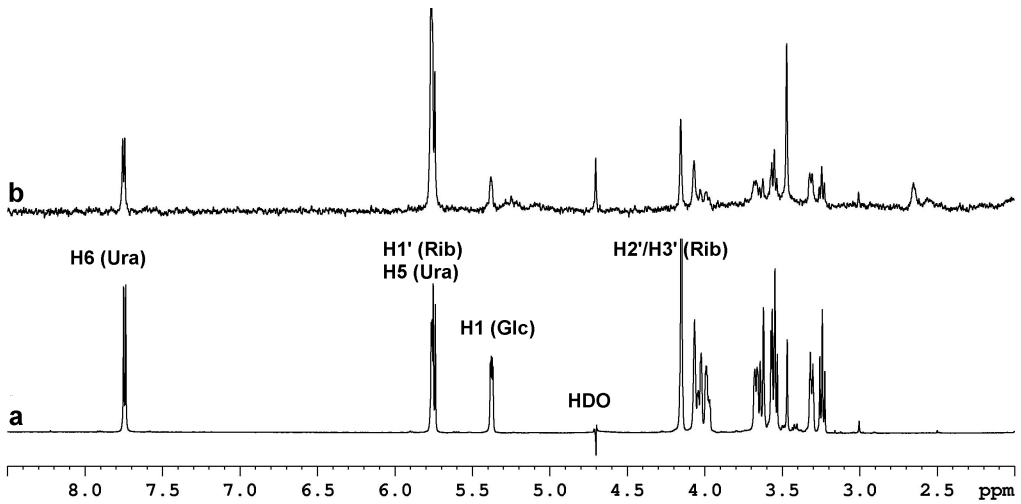

Figure S5. A) ^{1}H and B) STD NMR spectra of GeF-wt:UDP-Glc complex using 3 s saturation time.

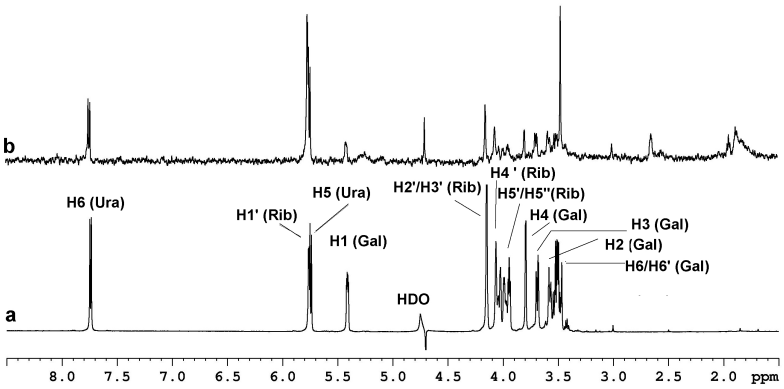

Figure S6. A) ^{1}H and B) STD NMR spectra of GeF-wt:UDP-Gal complex using 3 s saturation time.

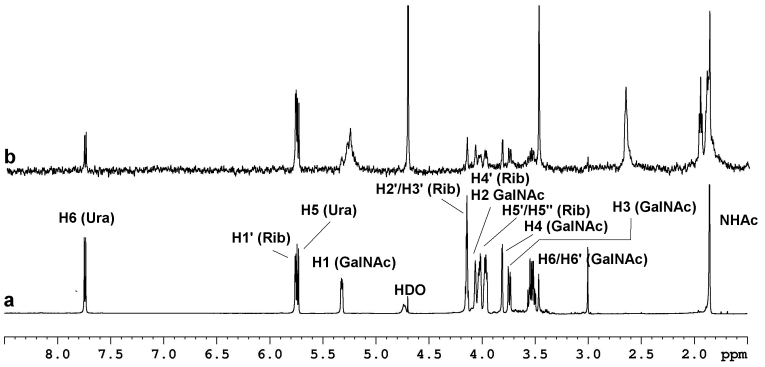

Figure S7. A) ^{1}H and B) STD NMR spectra of GeF-wt:UDP-GalNAc complex using 3 s saturation time

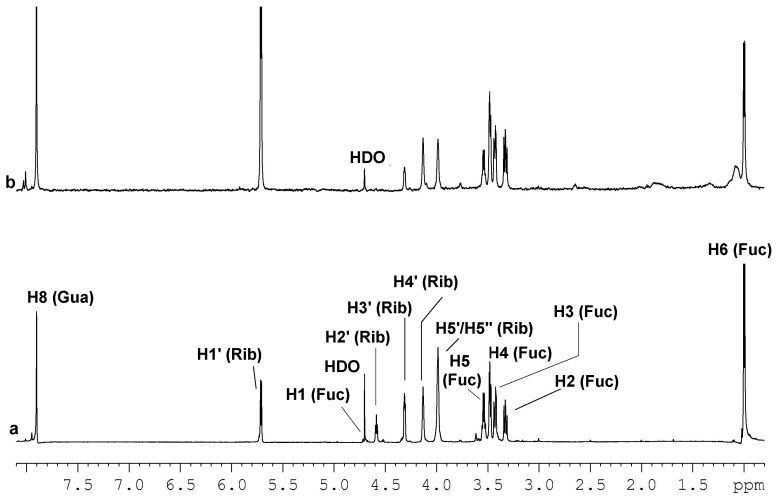

Figure S8. A) ^{1}H and B) STD NMR spectra of GeF-wt:GDP-Fuc complex using 3 s saturation time.

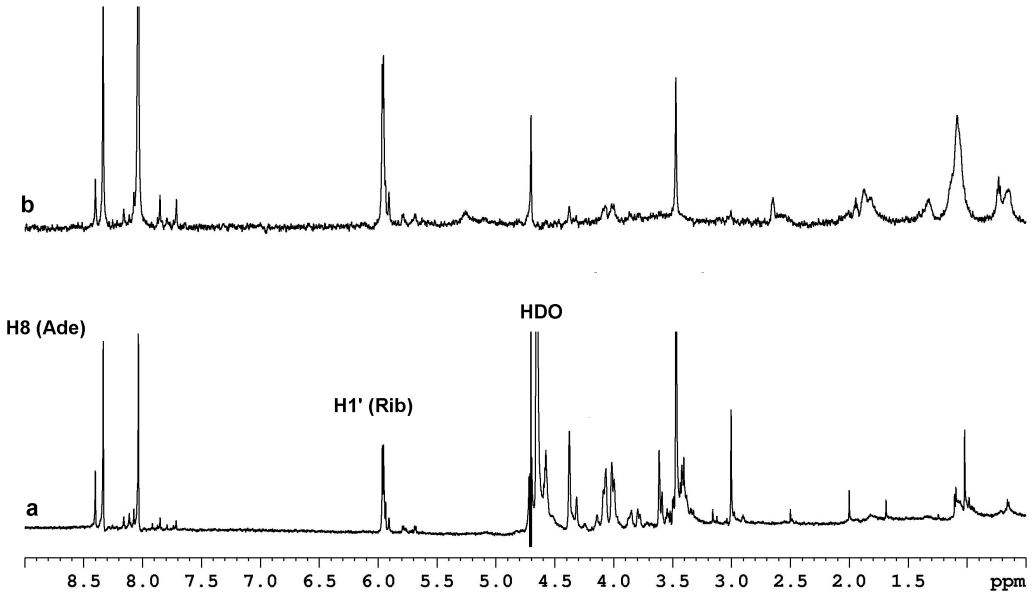

Figure S9. ^{1}H (a) and STD (b) NMR spectra of GeF-wt:PAPS complex using 3 s saturation time.

The specificity of the UDP-Gal binding to GeF-wt was investigated by performing competition STD NMR experiment in the presence of equimolar amounts of CMP (Figure S10).

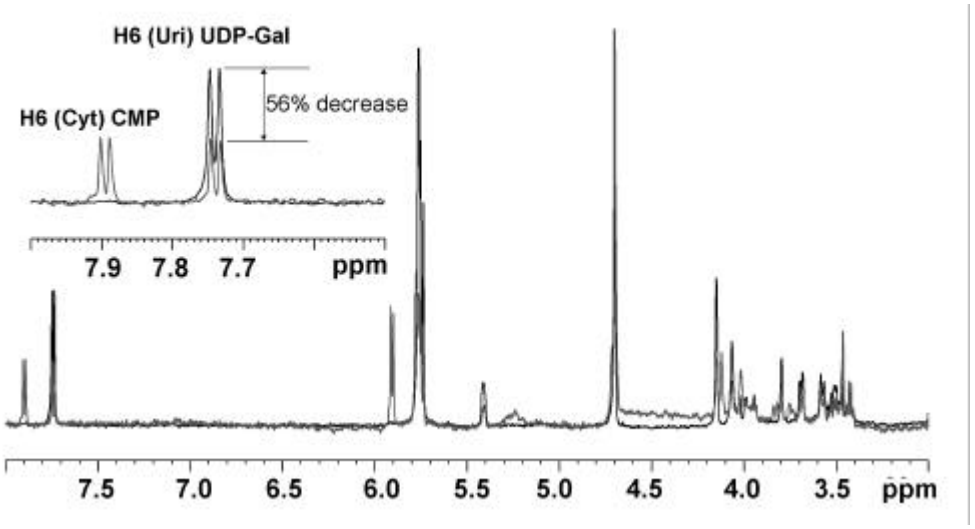

Figure S10. STD NMR spectra of GeF-wt:UDP-Gal complex (black line) following the addition of equimolar amounts of CMP (grey line). The decrease of the H6 (Uri) STD NMR signal of UDP-Gal in the competition STD NMR experiment clearly suggests that CMP displaces UDP-Gal from a nucleotide sugar transport protein present at endogenous level in the GeF-wt preparation.

Experimental Section

Expression plasmid construction: PCR amplification of the mouse CST cDNA from pTrcME8HA (kindly provided by Prof. Rita Gerardy-Schahn, Medical School Hannover, Germany) was performed using a proof reading DNA polymerase and gene specific primers. The forward and reverse primers introduced an EcoRI and a NotI site respectively, thus allowing directional and in-frame insertion of the amplified sequence into pPICZ-B (Invitrogen). The resulting plasmid pPICZ-B-CST thus permitted the intracellular expression of the mouse CST with a C-terminal His_6 tag. The integrity of pPICZ-B-CST was confirmed by DNA sequencing.

Protein expression in Pichia pastoris KM71H: Preparation of media and electrocompetent *P. pastoris* KM71H cells, and *P. pastoris* transformation and transformant selection were performed as described by the manufacturer (Invitrogen). Briefly, 5 µg of pPICZ-B-CST was linearized at the 5' AOX1 locus using PmeI that would subsequently permit homologous recombination into the *P. pastoris* genome. Following electroporation at 1500 V (*t* 5ms), the transformation reaction was plated on YPDS (yeast extract peptone dextrose agar) plates supplemented with 500 µg/mL of zeocin, and incubated at 28ºC for up to 5 days. Integration of linearized plasmid into the *P. pastoris* genome was verified by PCR using plasmid and gene specific primers.

Single colonies of PCR-verified transformants were used to inoculate 10 mL overnight cultures of BMGY (buffered glycerol-complex media) in 100 mL buffled flasks and incubated overnight at 20ºC, at 220 rpm. Overnight cultures were then transfer to a 4 L buffled flask containing 1 L of BMGY and further incubated at 20°C, at 220 rpm until an OD_{600} of between 2 and 6 was reached. Cells were subsequently collected by centrifugation at 1500 *g* for 5 min and re-suspended in 200 mL of BMMY (buffered methanol-complex media) to induce protein expression. 24 h post induction, the cells were collected by centrifugation (1500 *g* for 5 min), washed once with ice-cold 10 mM sodium azide and immediately used.

*Preparation Golgi-enriched fraction***:** Untransformed, methanol uninduced and induced *P. pastoris* KM71H cells were used to prepare Golgi-enriched fractions. Cells were spheroplasted using 0.5 mg zymolyase T100 (MP Biomedicals) per gram of wet cell pellet. Spheroplasting was monitored spectrophotometrically by measuring the decrease in absorbance at 800 nm upon the addition of 10 µL of cell suspension to 990 µL of 1% SDS. Cells were incubated with zymolyase until 75-80% sepheroplast-

ing was achieved. Spheroplasts were collected by centrifugation at 1000 *g* for 5 min and resuspended in ice-cold lysis buffer (10 mM HEPES/Tris pH 7.4, 1 mM EDTA, 0.8 M sorbitol supplemented with 1 mM PMSF and Complete EDTA free protease inhibitors cocktail (Roche Applied Sciences). *P. pastoris* spheroplasts were subsequently lysed with 10 strokes of Dounce homogenizer whilst on ice and the resulting cell lysate centrifuged at 10 000 *g* for 10 min. The supernatant was further centrifuged at 100 000 *g* for 1 h at 4ºC, with the resulting pellet resuspended in ice-cold lysis buffer, snap frozen in liquid nitrogen, and stored at -80ºC until required.

Western blot analysis: The expression of His_6 tagged CST was detected by Western blotting following transfer to a PVDF membrane using a mouse monoclonal anti-His_6 (1:10 000 dilution, Cell Signaling Technologies) and a goat anti-mouse horseradish peroxidase conjugated (1:10 000 dilution, Bio-Rad Laboratories) as the primary and secondary antibodies, respectively. Visualisation was performed using SuperSignal® West Pico Chemioluminescent Substrate (Pierce Biotechnology).

NMR experiments: All NMR experiments were performed on a Bruker 600 Ultrashield™ at 285 K, equipped with standard triple resonance CryoProbe, in deuterated 20 mM Tris buffer, supplemented with 2 mM $MgCl_2$, (D_2O) pH 7.5. 300 µL of sample in Shigemi tubes (Shigemi Co.) were used for all the acquisitions. STD spectra were acquired using 200 µg equivalent of Golgi-enriched fractions and a protein/ligand ratio of 1:100. A saturation time of 3 s and a total of 1500 scans were used. The protein signals were saturated using a Gaussian pulse train cascade for 3 s at an on-resonance frequency of -1 ppm and off-resonance of 300 ppm. A WATERGATE sequence was used to suppress residual HDO signal. The on- and off- resonance spectra FIDs were stored and processed separately and the subtraction of the on-resonance and the off-resonance spectrum resulted in the STD NMR spectrum. The STDD NMR spectrum was obtained by superimposing two indi-vidual STD NMR spectra followed by a subtraction. The STD NMR spectrum of the GeF-I:CMP-Neu5Ac complex was used to calculate the relative STD NMR effects for CMP-Neu5Ac. A STDD spectrum (GeF-I:CMP – GeF-wt:CMP) was used to calculate the relative STD NMR effects for CMP. All STD NMR effects were calculated accord-ing to the formula $A_{STD} = (I_0 \times I_{sat})/I_0 = I_{STD}/I_0$ using the H1' (Rib) proton to 100%.