

1 ***O*-fucosylation of thrombospondin-like repeats is required for processing of MIC2 and for**
2 **efficient host cell invasion by *Toxoplasma gondii* tachyzoites**

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9 **Supporting Information**

10 Supporting Experimental Procedures S-2

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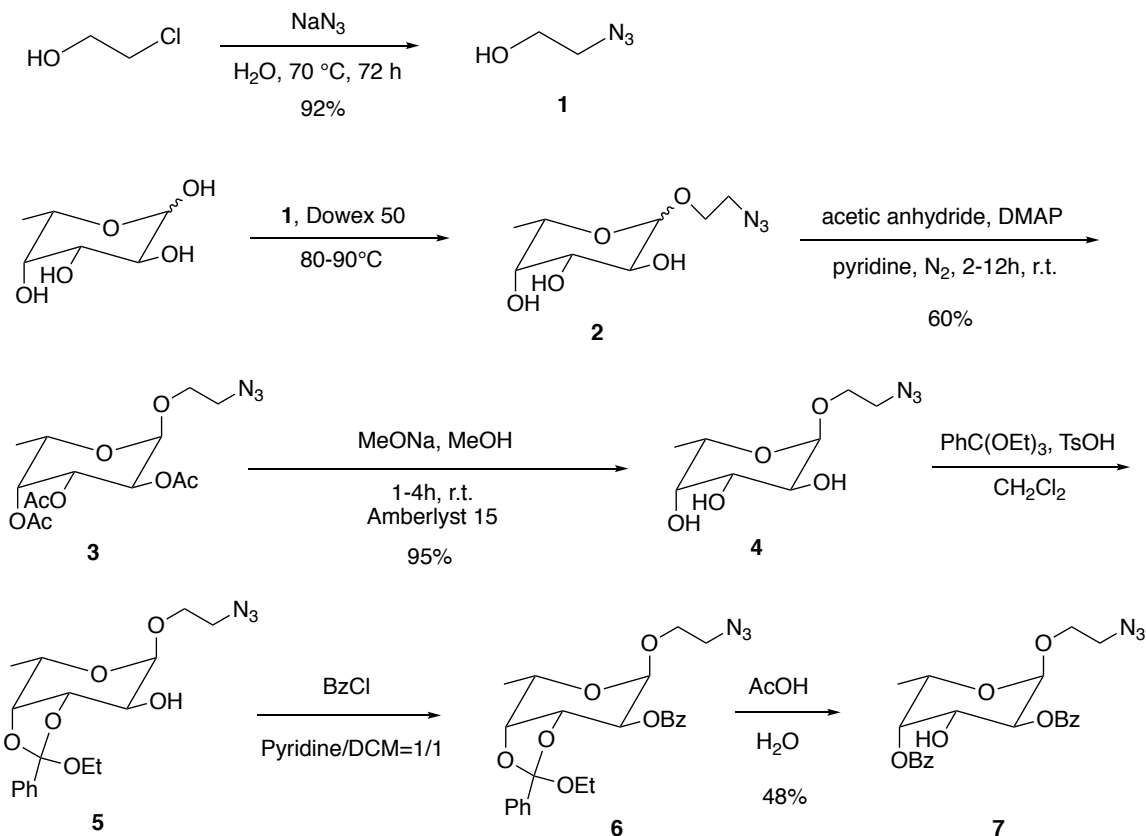
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15 **Supporting Information**

16

17 **Supporting Experimental Procedures**

18 **Synthesis of Glc- β -1,3-Fuc- α -KLH (1-4).**



28 **α/β -Azidoethyl fucoside (2).** α/β L-fucose mixture, Dowex-50 (120 mg/mmol) and 4 Å
29 molecular sieves were taken together in a two-neck round bottom flask and followed by addition
30 of (1) (4 ml/mmol). The reaction mixture was stirred at 80-90 °C for 2 h. After completion of the
31 reaction, the reaction mixture was filtered through cotton funnel and resin was washed thoroughly
32 with distilled MeOH. The filtrate was concentrated under reduced pressure to get the crude
33 residue. The mixture contained both α/β -azidoethyl fucoside (2) and residue 2-azidoethanol. It
34 was not further purified and used in the next step for peracetylation.

35 **Peracetylated α -azidoethyl fucoside (3).** DMAP (catalytic amount) and acetic anhydride (12
36 eq.) were added dropwise under N₂ to (2). The reaction was continued with stirring for 12 h at
37 RT. After completion of the reaction (TLC: hexane/ethyl acetate = 2/1), two to three cubes of ice
38 were added and stirred. The reaction mixture was diluted with ethyl acetate and washed with 1 M
39 HCl, saturated NaHCO₃, and brine. The combined organic layers were dried over anhydrous
40 Na₂SO₄, filtered and the filtrate was concentrated to dryness under vacuum. Column
41 chromatography (230-400 mesh) was carried out using solvent hexane/ethyl acetate = 4/1 to
42 separate the α - and β - isomers, yield for α -isomer (3) in the two steps 60%. For α -isomer, ¹H
43 NMR (400 MHz, CDCl₃) δ ppm: 1.16 (d, 3H, H-6, *J* = 6.4 Hz), 2.00 (s, 3H, -COCH₃), 2.08 (s,
44 3H, -COCH₃), 2.17 (s, 3H, -COCH₃), 3.43 (m, 2H, -CH₂), 3.62 (m, 1H, -CH_a), 3.86 (m, 1H, -
45 CH_b), 4.18 (q, 1H, H-5, *J* = 6.4 Hz), 5.12 (d, 1H, H-4, *J* = 4.0 Hz), 5.14 (dd, 1H, H-3, *J* = 4.0,
46 10.0 Hz), 5.32 (d, 1H, H-1, *J* = 3.2 Hz), 5.38 (dd, 1H, H-2, *J* = 3.2, 10.0 Hz).

47 **α -Azidoethyl fucoside (4).** To a well-stirred solution of peracetylated α -azidoethyl fucoside in
48 MeOH (4 ml/mmol) was added catalytic amount of MeONa and stirring was continued for 2 h at
49 room temperature. After TLC (hexane/ethyl acetate = 3/2) showed no starting material, reaction
50 mixture was diluted with MeOH and neutralized with Amberlyst 15 until pH 7. Resin was filtered
51 off and the filtrate was concentrated to yield the compound quantitatively.

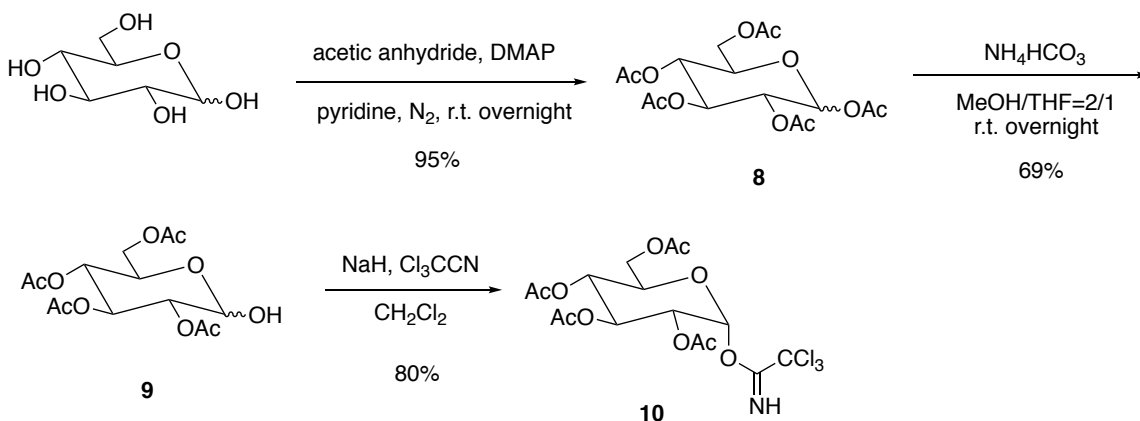
52 **3,4-protected α -azidoethyl fucoside (5).** Compound 4 was dissolved in dry dichloromethane (5

53 ml/mmol) with 4 Å molecular sieves and a speck of TsOH under Ar. Triethyl orthobenzoate (4
54 eq.) was slowly added to the mixture and stirred at RT overnight. After completion of the reaction
55 (TLC: hexane/ethyl acetate = 1/1), a small amount of NaOH was added and the mixture was
56 filtered. The filtrate was co-evaporate with toluene to yield brown liquid as the product.

57 **2,3,4-protected α -azidoethyl fucoside (6).** Compound **5** was dissolved in dry dichloromethane
58 (DCM) (5 ml/mmol) and was dropwise added to the pre-mixed benzoyl chloride (3 eq.) in dry
59 pyridine (equivalent amount to DCM) under Ar at 0 °C with ice bath. The mixture was stirred at
60 RT overnight until reaction was complete (TLC: hexane/ethyl acetate = 3/1). The product was
61 opaque brownish orange color.

62 **2,4-protected α -azidoethyl fucoside (7).** Acetic acid (1.7 ml/mmol) and water (1.1 ml/mmol)
63 were added dropwise to the above reaction mixture. The mixture was stirred at RT overnight to
64 achieve full conversion (TLC: hexane/ethyl acetate = 3/1), diluted with DCM, filtered, and
65 poured into sat. NaHCO₃. The aqueous phase was extracted with DCM and the combined organic
66 layer was washed with NaHCO₃ and brine, dried over Na₂SO₄, concentrated to yield orange
67 liquid. Column chromatography (230-400 mesh) was carried out using solvent hexane/ethyl
68 acetate = 4/1 to get compound **7** as yellow syrup. The yield for the three steps is 48%. ¹H NMR
69 (500 MHz, CDCl₃) δ ppm: 1.26 (d, 3H, H-6, J = 6.5 Hz), 3.40 (m, 2H, -CH₂), 3.66 (ddd, 1H, -
70 CH_a, J = 3.5, 7.0, 10.5 Hz), 3.93 (ddd, 1H, -CH_b, J = 3.5, 7.0, 10.5 Hz), 4.32 (q, 1H, H-5, J = 6.5
71 Hz), 4.54 (dd, 1H, H-3, J = 3.5, 10 Hz), 5.28 (d, 1H, H-1, J = 3.5 Hz), 5.38 (dd, 1H, H-2, J = 3.5,
72 10 Hz), 5.60 (d, 1H, H-4, J = 3.5 Hz), 7.45 (t, 2H, -Ar, J = 7.5 Hz), 7.49 (t, 2H, -Ar, J = 7.5 Hz),
73 7.58 (t, 1H, -Ar, J = 7.5 Hz), 7.60 (t, 1H, -Ar, J = 7.5 Hz), 8.10 (d, 2H, -Ar, J = 7.5 Hz), 8.15 (d,
74 2H, -Ar, J = 7.5 Hz), 8.62 (s, 1H, -OH).

75



76

77 **Scheme 2.** Preparation of glucoside donor (compound **10**)

78

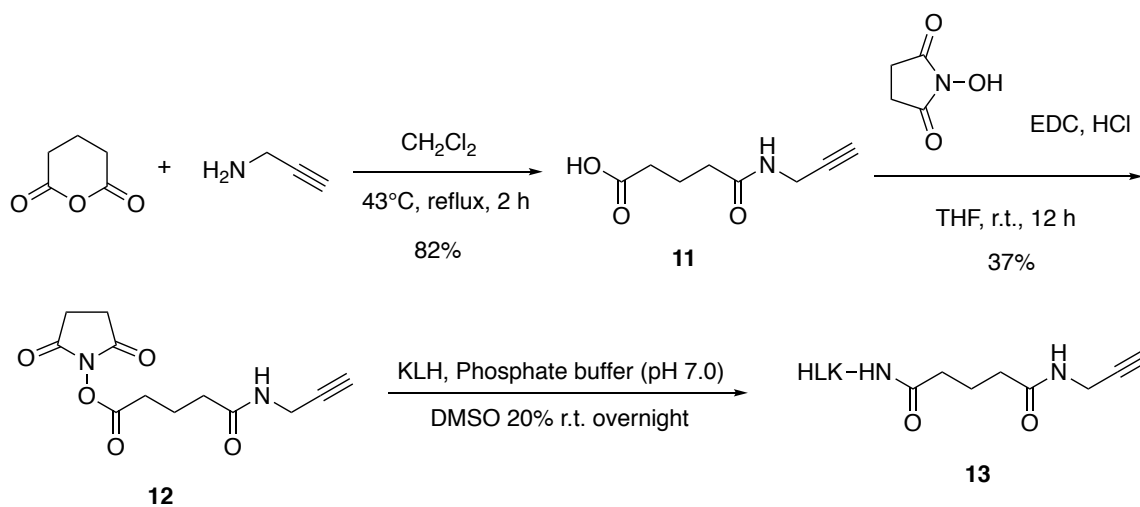
79 **Peracetylated- α/β -glucose (8).** D-glucose and DMAP (0.1 eq.) was dissolved in dry pyridine
 80 (1.5 ml/mmol) followed by dropwise addition of acetic anhydride (10 eq.) over 20 min at 0 °C
 81 under N₂. The reaction was stirred at RT overnight to completion. The mixture was diluted with
 82 ethyl acetate, the organic layer washed with 1 M HCl, sat. NaHCO₃ and brine, and dried over
 83 MgSO₄. After filtration, the solvent was evaporated under reduced pressure, affording the product
 84 in quantitative yield.

85 **2,3,4,6-Tetra-acetyl- α/β -glucose (9).** Compound **8** and ammonium acetate (4 eq.) in
 86 MeOH/THF (2/1, 4 mL/mmol) were stirred at RT with 4-Å molecular sieves overnight under N₂.
 87 Upon completion (TLC: hexane/ethyl acetate = 1/1), the molecular sieves were filtered off and
 88 the solvent was evaporated *in vacuo*. The residue was re-dissolved in ethyl acetate, washed with 1
 89 M HCl, sat. NaHCO₃ and brine, dried over MgSO₄, concentrated and purified by silica gel
 90 column chromatography (hexane/ethyl acetate = 1/1) to give product **9** as a white solid, yield
 91 69%.

92

93 **2,3,4,6-Tetra-acetyl- α -glucosyl trichloroacetimidate (10).** Compound **9** was dissolved in dry
 94 DCM (5 ml/mmol) under N₂ and trichloroacetonitrile (4.5 eq.) was added. The solution was put
 95 in an ice bath and sodium hydride (1.5 eq.) added in three portions. The reaction was stirred at RT

96 for 12 h and achieved full conversion (TLC: hexane/ethyl acetate = 3/2). The mixture was filtered
 97 over a celite bed and concentrated under vacuo. Column chromatography (230-400 mesh) was
 98 carried out using solvent hexane/ethyl acetate = 3/1 to get compound **10** as colorless oil, yield
 99 80%. ¹H NMR (500 MHz, CDCl₃) δ ppm: 2.02 (s, 3H, -CH₃), 2.04 (s, 3H, -CH₃), 2.05 (s, 3H, -
 100 CH₃), 2.08 (s, 3H, -CH₃), 4.14 (dd, 1H, H-6, *J* = 2.2, 12.0 Hz), 4.22 (m, 1H, H-5, *J* = 2.2, 4.0 Hz),
 101 4.28 (dd, 1H, H-6', *J* = 4.0, 12.0 Hz), 5.14 (dd, 1H, H-2, *J* = 3.5, 10 Hz), 5.18 (t, 1H, H-3, *J* =
 102 10.0 Hz), 5.57 (t, 1H, H-4, *J* = 10.0 Hz), 6.57 (d, 1H, H-1, *J* = 3.5 Hz), 8.70 (s, 1H, NH).
 103



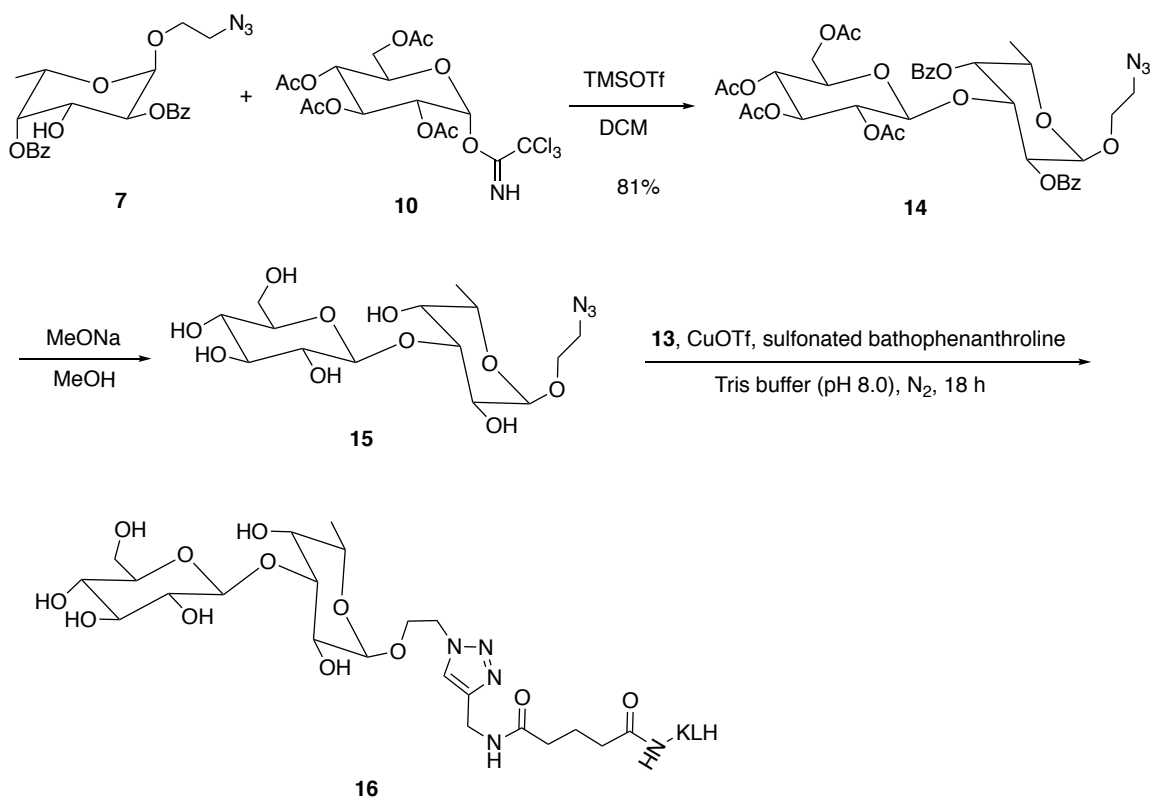
105 **Scheme 3.** Preparation of NHS-linker (compound **13**)

106

107 **5-oxo-5-(prop-2-yn-1-ylamino)pentanoic acid (11).** Glutaric anhydride was dissolved in dry
 108 DCM. Propargylamine (1 eq.) was added and the solution was refluxed at 43 °C for 2 h until full
 109 conversion (TLC: dichloromethane/MeOH = 9/1). The solvent was evaporated and the mixture
 110 was purified by column chromatography (dichloromethane/MeOH = 9/1) to get compound **11** as
 111 yellow liquid, yield 82%. ¹H NMR (500 MHz, MeOD) δ ppm: 1.92 (p, 2H, -CH₂, *J* = 7.5 Hz),
 112 2.29 (t, 2H, -CH₂, *J* = 7.5 Hz), 2.37 (p, 2H, -CH₂, *J* = 7.5 Hz), 2.59 (t, 1H, NH, *J* = 2.5 Hz), 3.38
 113 (s, 1H, alkyne-H), 3.97(d, 2H, -CH₂, *J* = 2.5 Hz), 5.00 (br, 1H, COOH).

114 **2,5-dioxopyrrolidin-1-yl 5-oxo-5-(prop-2-yn-1-ylamino)pentanoate (12).** Compound **11**, *N*-

115 hydroxy succinimide (1.4 eq.) and EDC·HCl (1 eq.) was dissolved in dry THF (10 ml/mmol).
116 The mixture was degassed with argon and stirred at RT overnight until completion (TLC:
117 dichloromethane/MeOH = 19/1). The solvent was evaporated and the mixture was re-dissolved in
118 DCM, washed with H₂O, NaHCO₃ and brine. The organic layer was collected, dried over Na₂SO₄
119 and concentrated *in vacuo*. Column chromatography (230-400 mesh) was carried out using
120 solvent DCM/MeOH = 98/2 to get compound **12** as clear liquid, yield 37%. ¹H NMR (500 MHz,
121 CDCl₃) δ ppm: 1.93 (p, 2H, -CH₂, *J* = 7.5 Hz), 2.20 (m, 2H, -CH₂, *J* = 7.5 Hz), 2.56 (t, 2H, -CH₂,
122 *J* = 7.5 Hz), 2.68 (s, 1H, alkyne-H), 2.72 (s, 2H, NHS-H), 3.88(d, 2H, -CH₂, *J* = 2.5 Hz), 6.75 (t,
123 1H, NH, *J* = 2.5 Hz).
124 **NHS linker-KLH (13)**. KLH was dissolved in PBS (pH 7.4) to make a 4 mg/ml stock solution
125 and the NHS linker (compound **12**) was dissolved in DMSO. NHS linker (340 equivalent) was
126 added to KLH solution to make a final 20% DMSO in water (for 8 mg of KLH, 1.85 mg of NHS
127 linker was used). The solution was agitated under room temperature overnight and dialyzed in 2 L
128 of distilled water for 24 h twice.
129



130

131 **Scheme 4.** Synthesis of Glc-β-1,3-Fuc-α-KLH antigen (compound **16**)

132

133 **Peracetylated glucosyl-β-1,3-(2,4-benzoyl)α-azidoethyl fucoside (14).** Compound **7** (1 eq) and
 134 **10** (2.5 eq) was dissolved in dry DCM (5 ml/mmol) with 4 Å molecular sieves under argon. After
 135 stirring at RT for 30 min, pre-diluted TMSOTf (0.35 eq) in DCM (0.03 M) was added dropwise
 136 to the solution. The reaction was stirred at RT overnight until completion (TLC: hexane/ethyl
 137 acetate = 1/1). The mixture was diluted with DCM and solid NaHCO₃ was added and stirred for
 138 10 min. The solution was filtered and the filtrate was concentrated and purified by column
 139 chromatography (hexane/ethyl acetate = 2/1) to get compound **14** as yellow oil, yield 81%. ¹H
 140 NMR (400 MHz, CDCl₃) δ ppm: 1.22 (d, 3H, H-6, *J* = 6.4 Hz), 1.53 (s, 3H, -COCH₃), 1.88 (s,
 141 3H, -COCH₃), 1.98 (s, 3H, -COCH₃), 2.01 (s, 3H, -COCH₃), 3.32 (ddd, 1H, -CH_a, *J* = 3.5, 7.0,
 142 10.5 Hz), 3.45 (ddd, 1H, -CH_b, *J* = 3.5, 7.0, 10.5 Hz), 3.66 (m, 2H, -CH₂), 3.88 (m, 1H, H-5'),
 143 3.93 (m, 1H, H-6'), 4.22 (m, 1H, H-6''), 4.25 (m, 1H, H-2'), 4.28 (m, 1H, H-5), 4.63 (dd, 1H, H-

144 3, $J = 3.2, 10.4$ Hz), 4.78 (d, 1H, H-1', $J = 8.8$ Hz), 4.98 (t, 1H, H-4', $J = 9.6$ Hz), 5.13 (t, 1H, H-
145 3', $J = 9.6$ Hz), 5.31 (d, 1H, H-1, $J = 3.6$ Hz), 5.44 (dd, 1H, H-2, $J = 3.6, 10.4$ Hz), 5.64 (d, 1H, H-
146 4, $J = 3.2$ Hz), 7.43 (t, 2H, -Ar, $J = 7.6$ Hz), 7.47 (t, 2H, -Ar, $J = 7.6$ Hz), 7.59 (t, 1H, -Ar, $J = 7.6$
147 Hz), 7.60 (t, 1H, -Ar, $J = 7.6$ Hz), 8.08 (d, 2H, -Ar, $J = 7.6$ Hz), 8.11 (d, 2H, -Ar, $J = 7.6$ Hz).

148 **Glucosyl- β -1,3- α -azidoethyl fucoside (15)**. Compound **14** was dissolved in dry MeOH and
149 sodium methoxide (2 eq) was added. The reaction was stirred at RT overnight until completion
150 (TLC: ethyl acetate/MeOH = 5/1). The solution was neutralized with Amberlyst 15 until pH 7,
151 filtered and concentrated *vacuo*. Prep-TLC was performed (ethyl acetate/MeOH = 5/2) to get
152 compound **15** as colorless syrup with quantitative yield. ^1H NMR (600 MHz, MeOD) δ ppm: 1.28
153 (d, 3H, H-6, $J = 6.6$ Hz), 3.30 (m, 1H, H-2'), 3.37 (m, 2H, H-6'), 3.43 (m, 1H, -CH_a), 3.55 (m,
154 1H, H-5'), 3.57 (m, 1H, -CH_b), 3.66 (m, 2H, -CH₂), 3.69 (m, 3H, -CH₂, H-3'), 3.86 (m, 1H, H-4'),
155 3.89 (m, 1H, H-4), 3.92 (m, 1H, H-2), 4.01 (dd, 1H, H-3, $J = 3.0, 10.2$ Hz), 4.05 (q, 1H, H-5, $J =$
156 6.6 Hz), 4.45 (d, 1H, H-1', $J = 7.8$ Hz), 4.87 (s, 1H, H-1).

157 **Glc- β -1,3-Fuc- α -KLH antigen (16)**. All the reagents were dissolved in argon degassed double
158 distilled water and the reaction was carried out under argon. In the NHS linker-KLH solution,
159 Tris buffer (pH 8.0), compound **15**, and the ligand bathophenanthroline sulfonated sodium salt,
160 CuOTf were added sequentially to make a final solution containing 2 mg/ml NHS-KLH, 0.1 M
161 Tris, 0.3 mM disaccharide, 2 mM ligand, and 1 mM CuOTf. The mixture was degassed with
162 argon for 1 min and then agitated at RT overnight followed by dialyzing in 2 L of distilled water
163 for 24 h twice. After dialysis, the solution was snap-frozen in liquid N₂ and lyophilized to yield
164 blue powder.

165

166 **Disaccharide agarose beads preparation**. A slurry of agarose-alkyne beads in DMF was treated
167 with compound **15** (4 eq.), 2,6-lutidine (8 eq.), 2,2'-bipyridine (8 eq.), cuprous bromide (4 eq.),
168 and sodium ascorbate (8 eq.). The solution was degassed with argon for 1 min and agitated at RT
169 overnight. The resulting mixture was washed sequentially with DMF, H₂O, MeOH, 0.1 M

170 aqueous EDTA, H₂O, and DMF.

171

172 **References**

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183

184 **Supporting Tables**

185 **Table S1. *In silico* identification putative *T. gondii* POFut2 protein acceptors.**

| Gene ID | Protein Name | POFut2 consensus sequence(s) |
|--------------|---|---|
| TGGT1_201780 | microneme protein MIC2 (MIC2) | see Fig. 1A |
| TGGT1_209060 | thrombospondin type 1 domain-containing protein | [460]WSPCTASCEGGE[473] [586]WSACSATCGEGW[599] |
| TGGT1_218310 | microneme protein MIC14 (MIC14) | [639]WSECSRTCRRSGG[652] |
| TGGT1_223480 | sushi domain (scr repeat) domain-containing protein | [538]WSDCSTTCGEGQ[551] [593]WGPCGASC GGGE[606] [756]WSVCTASCGFGT[769] [872]WGPCSATCGGGQ[885] [934]FRECDATCGVGR[947] [999]WSSCSVTCGGGQ[1012] [1113]WTGCSQSCGLGG[1126] [1282]WTPCSVTCGVGK[1295] [1338]WSTCSASC GGGV[1351] |

186

187

TableS2. Primers and homologous recombination sequences (ROs) used in this study

| Primer | Sequence (5'-3') |
|--------|--|
| P20 | AGCGCTTCTGTGXGCTGTCGTTATTTCCAGCAGTTAACGCGGGTTCGAAGGCTGCT AGTACTG |
| P21 | GAGCGGATCGCGACCAAACGTTACAGTGTCGAACTGGGGTGCTTAACACCATTG CATTCC |
| P22 | AGGCCTGGAGATTCTGGCCGCCGCTTTTCCCCCAGATCACCTCGAAGGCTGCTAG TACTG |
| P23 | TACGTAAACGGTATCTCCCTTTTGAAGTGGGTTTCGGCTTAACACCATTGCATTCC |
| P24 | AAGTTGAGTGCATGCAGGCAGCACCGG |
| P25 | AAAACCGGTGCTGCCTGCATGCACTCA |
| P26 | AAGTTGCAGTGTCGAACTGGGGTCCAG |
| P27 | AAAACCTGGACCCCAGTTCGACACTGCA |
| P28 | AAGTTGCAGGTATCGACCTACCAGGG |
| P29 | AAAACCCTGGTAGGTCGATACCTGCA |
| P30 | AAGTTGTGTCTGGCAAAGTTGCAGATG |
| P31 | AAAACATCTGCAACTTTGCCAGACACA |
| P32 | GAGTTTACACTGGACGCAG |
| P33 | CAAACAGACCGGCAGGTGCT |
| P34 | GCTTAATTAATACTTTTGTATAGTTCATCCATG |
| P35 | GTATACGCTCGCGACTCTC |
| P36 | CATGCATTGTCAACTAGG |
| P37 | CTTCCGTTTCCTCGTGTAC |
| P38 | CATAAGGAGACTCCTGACC |
| P39 | GTAGTAACAGTGTCTTACACG |
| P40 | CACCGTTCAAGTCTTCCTCGG |
| P41 | CCATGGATGAGGCGCCACGTCTCAGTT |
| P42 | CAGATCTATGCATTGTCAACTAGGAGG |
| P43 | CCTAGGCAGTGTCGAACTGGGGT |
| RO1 | TGG CCA GAA GGA CGC AGT CGA AGT GAG CTC AGG AGG AAA TAC TGG CCT TCa TTG GAC CCC AGT TCG ACA CTG GAA CAA AAG TTG ATT TCT GAA GAA GAT TTG AAC GGT GAA CAA AAG CTA ATC TCC GAG GAA GAC TTG AAC GGT GCT AGG GCC GAG GAG CAG AAG CTG ATC TCC GAG GAG GAC CTG TAA CGT TTG GTC GCG ATC CGA CTC CCC ATT TTC GTT TCG GGC ATC TTG GAG ACG TCA CGC TGC TGC CAG CTG |

191 **Table S3. Statistical analysis of the attachment/ invasion assay comparing parental strain**
 192 **and mutants.**

| Comparison | <i>p</i> value |
|---|----------------|
| Δ ku80 vs. <i>pofut2KO</i> | 0.0004 |
| Δ ku80 vs. <i>nst2KO</i> | 0.03 |
| Δ ku80 vs. <i>mic2KO</i> | 0.0002 |
| <i>pofut2KO</i> vs. <i>nst2KO</i> | n.s. (0.28) |
| <i>pofut2KO</i> vs. <i>mic2KO</i> | n.s. (0.43) |
| <i>nst2KO</i> vs. <i>mic2KO</i> | n.s. (0.1) |
| Δ ku80 vs. <i>pofut2KO</i> | 0.0005 |
| <i>pofut2KO</i> vs. <i>pofut2KO</i> + POFUT2 | 1.8xE-06 |
| Δ ku80 vs. <i>pofut2KO</i> + POFUT2 | n.s. (0.87) |

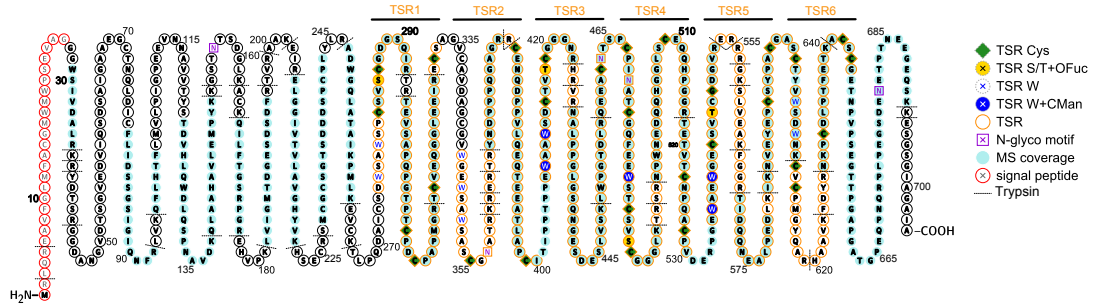
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194 n.s.: not significant

195

196 Supporting Figures

197 Figure S1.

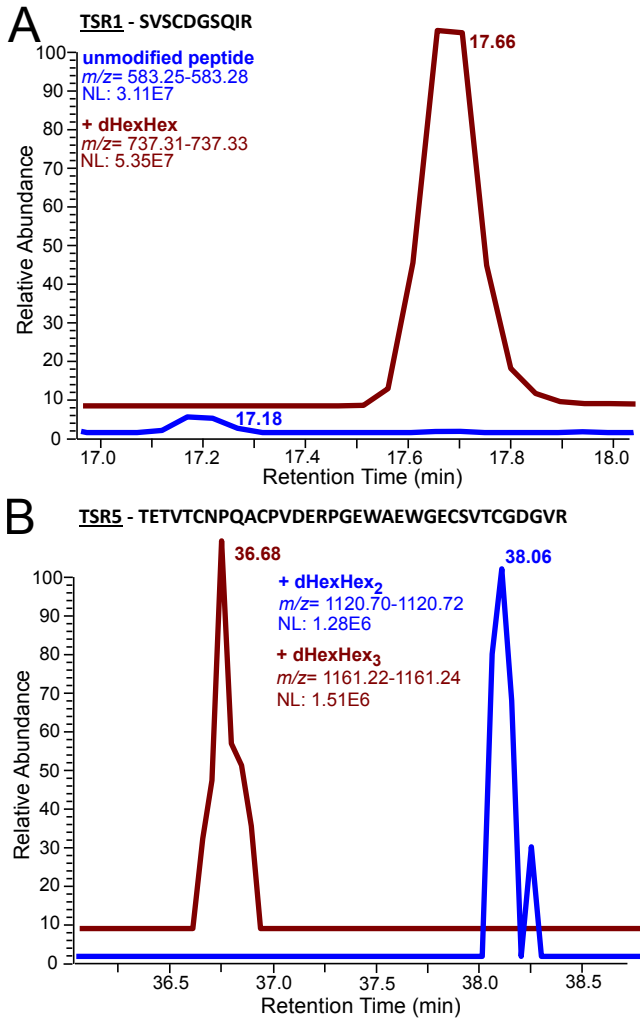


198

199 **Figure S1. Mass spectrometry analysis of MIC2 performed in this study.** Protter was used to
200 build a graphic representation of the MIC2 peptides detected in this study. The sequence of
201 secreted MIC2 (plus signal peptide) was used. A legend is shown above.

202

203 **Figure S2.**



204

205 **Figure S2. Extracted Ion Chromatograms (XIC) comparing the relative abundances of**

206 **MIC2 TSR1 and TSR5 observed glycoforms.** *A.* The semi-tryptic peptide

207 [282]SVSCDGSQIR[294] from TSR1 was observed as unglycosylated (m/z 583.2670) and

208 modified by dHexHex on S284 (m/z 737.3221), however the unglycosylated species is a very

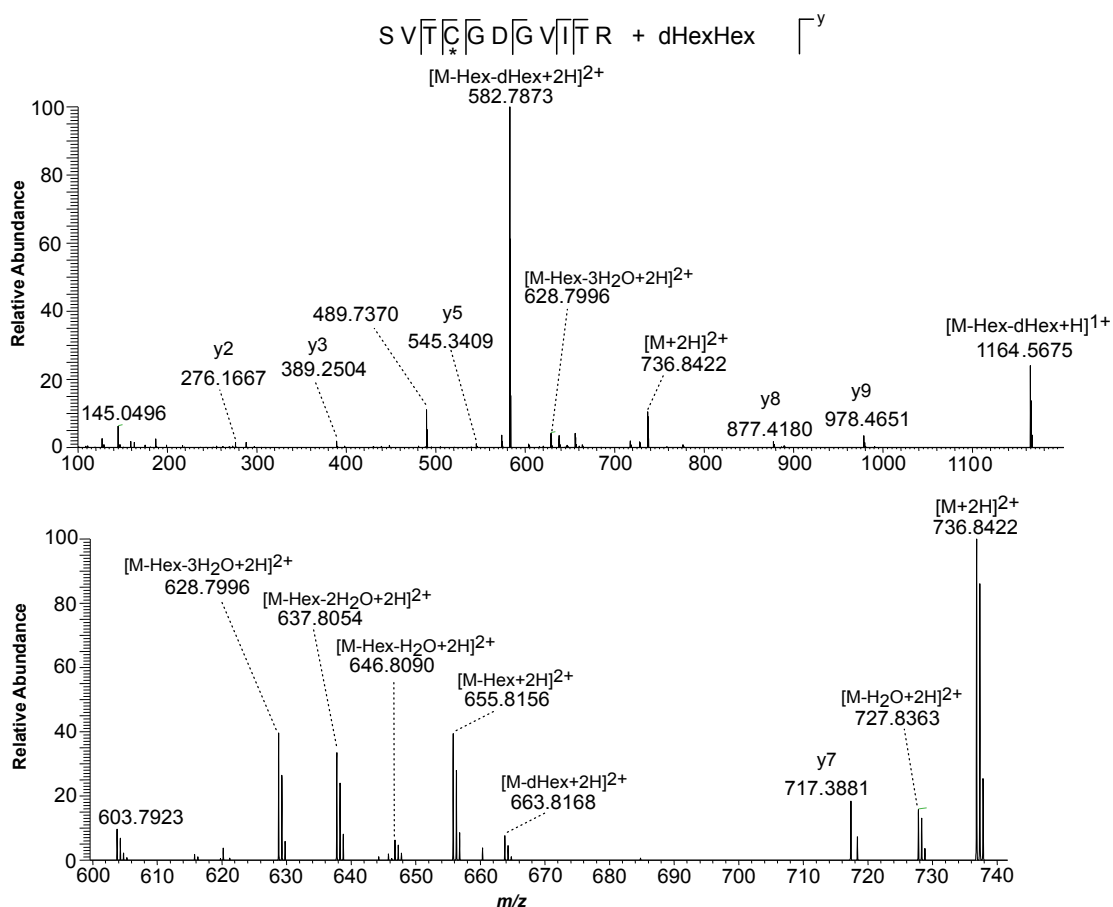
209 minor isoform. *B.* Two glycoforms were consistently observed for peptides from TSR5

210 differentiated by the presence of a dHex or dHexHex on the POFUT2 site. The tryptic peptide

211 (one missed cleavage) [517]TETVTCNPQACPVDERPGEWAEWGECVTCGDGVR[553] was

212 used to assess the relative abundance of these two glycoforms. Comparison of the XIC for m/z

213 1120.7179 (dHexHex₂) and 1161.2313 (dHexHex₃) suggests they have similar abundances.

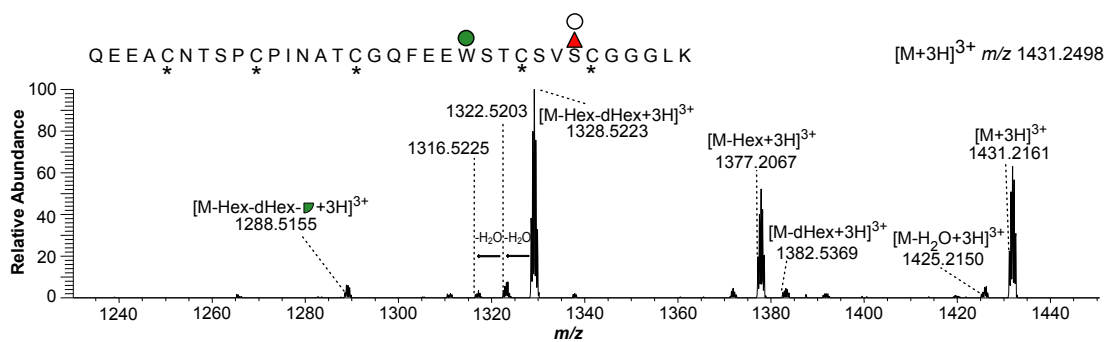


215

216 **Figure S3. Low energy HCD of a semi-tryptic glycopeptide from the TSR2 of**
 217 **thrombospondin 1.** A collision energy of 15 eV was used: the *top panel* shows the full MS/MS
 218 spectrum and an enlargement of the *m/z* range 600-740 is shown at the *bottom*. Together with
 219 product ions corresponding to the precursor minus a Hex and a dHexHex, with or without the
 220 additional loss of 1-3 H₂O molecules, we observed a low abundance product ion corresponding to
 221 the precursor minus dHex ([M+2H]²⁺ *m/z* 663.8168). The detection of this product ions
 222 originating from a TSP1 glycopeptide suggest that, both in this case and for the MIC2
 223 glycopeptides, a very minor but nevertheless detectable glycan rearrangement took place during
 224 the mass spectrometry analysis.

225

226 **Figure S4.**



227

228 **Figure S4. Low collision energy HCD MS/MS of an *O*-fucosylated and *C*-mannosylated**
229 **glycopeptide from the TSR4 of MIC2.** A collision energy of 10 eV was used. The MS/MS
230 spectrum shows product ions corresponding to the precursor minus a Hex, a dHexHex, or minus
231 dHexHex and 120.04 Da (broken green circle). As observed for the TSR3, a minor product ion
232 arising from the precursor minus a dHex is also observed and it is likely due to rearrangement of
233 the glycan. Red triangle: fucose; Green circle: mannose; White circle: Hex; Asterisk: cysteine
234 carbamidomethylation.

235

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TgPOFUT2      1 MHCQLGGQARAL I FLML ESCRN SWHVSPWSSV PFL P S PCL F F S F S A L P F P H P L S Q F I A C R G S S A T W L L P V ; 70
PiPOFUT2
SmPOFUT2
MmPOFUT2
DmPOFUT2
HsPOFUT2

TgPOFUT2      71 S S G T L V L R Y A L A A S P P R G T M R P S T N P R I A E G G R P W L I H A T Q P G P O T A S V L S L F P A V N A G F F S A G R R R R R G G 140
PiPOFUT2
SmPOFUT2
MmPOFUT2
DmPOFUT2
HsPOFUT2

TgPOFUT2      141 R P P C L L N H R R L L L G L V S V L T V F L S C L P F T T A T V S P A A L Q D V C Y A F G N I S R K L S S F L V P S R V T C P R G A T L L 210
PiPOFUT2      1 - - - - - M K F I T V L L L F F F F K - - - - - V I D R V I C V T P Q K L I C 29
SmPOFUT2      1 - - - - - M N L S C I I L I I L A - - - - - C M S R V C I Y S - - - - - 21
MmPOFUT2      1 - - - - - M A A L S V V C L L L A - - - - - A A S W R P V S A S G E E F 26
DmPOFUT2      1 - - - M R G S W P R L G F P A L L L L L H L L T G S D A A - - - - - V R N G T A K R E I G D S R G S S G T C V K G F L Q E I 54
HsPOFUT2      1 - - - - - M A T L S F V F L L L G - - - - - A V S W P P A S A S G Q E F 26

TgPOFUT2      211 S G V E A Q D S L E H P E I P D F R F L V Y D V K N G E G F H L Q K E V I Y R V A L V I S L L N A R A A Q Q G R M T D V H T A E K A R E D R 280
PiPOFUT2      30 L K E D V Y L G D F F F L K R K K Y I M Y D V N I G E G F N L O K E I F Y R L S L V I Y N L N - - - - - V I D R V I C V T P Q K L I C 77
SmPOFUT2      22 - - - - - K R Y L F Y D V F Y G E G F N L R R D V Y I R V A N T V R L L R D P S Q I P D - - - - - 60
MmPOFUT2      27 W P G Q S A A D I L S G A A S R R R Y L L Y D V N P P E G F N L R R D V Y I R V A S L L K T L - - - - - 73
DmPOFUT2      55 L P L P A T C P P E V L G M R G A V Y I L Y D V N I S E G F N L R R D V Y I R M A V F V R R L D - - - - - 102
HsPOFUT2      27 W P G Q S A A D I L S G A A S R R R Y L L Y D V N P P E G F N L R R D V Y I R I A S L L K T L - - - - - 73

TgPOFUT2      281 G H P Q A S H M L C A S S S F S H A C S A R S T F P F P M W V L V L P P W C R L A H W I F S E E T I T A M A E N - - S W L K H V R W G T F 348
PiPOFUT2      78 - - - - - K K D K I N I Y Y L V L P P W C Y V T H W I I R K G N N L R - - - - - W E F F 111
SmPOFUT2      61 - - - - - F A L H N I N V K N L T G D D W I L V L P P W G P L P H W F N D R S Y E R Y T N H S Y F N N W S G I P W S M F 115
MmPOFUT2      74 - - - - - L K T E E W V L V L P P W G R L Y H W S P D I H Q V R - - - - - I P W S E F 107
DmPOFUT2      103 - - - - - R R R R F R H V R L V L P P W P R L Y H W S Q G L Q Q S G - - - - - L P W S H F 138
HsPOFUT2      74 - - - - - L K T E E W V L V L P P W G R L Y H W S P D I H Q V R - - - - - I P W S E F 107

TgPOFUT2      349 F D F Q D L G E R L P V M E Y E D F L T Y Q L M R P D P W G E R R Q R K E Q T T P V E L D V V L S V R F S S T P S S R S L P F C A C L S S 418
PiPOFUT2      112 F N T D I M K K V I P I I E Y E E Y E K L Y G N Y S D I M I N - S K Y I L D N Y K E K S F L I L P F E - - E C N I N V N R F K Q F C K K C 178
SmPOFUT2      116 F D L N S L S L F I P V M D L I E F D R - S N V L S Q Q S L L N R S S N H L L T V D L A L Q L V R G - - D F N R Q L E Q Y S N D N - - - 178
MmPOFUT2      108 F D L P S L N K N I P V I E Y E O F I A E S G G P F I D Q V Y V L Q G Y A E G W K E G T W E E K V D A - - R P C I D P L L Y S Q D K - - - 171
DmPOFUT2      139 F D L A S L R R Y A P V L D Y E E F L A E Q R L F G N G A P L V H V G - H A F R L Q H Y E V M L E Q - - G I F R D K F E R V T D K P C S E 205
HsPOFUT2      108 F D L P S L N K N I P V I E Y E O F I A E S G G P F I D Q V Y V L Q S Y A E G W K E G T W E E K V D E - - R P C I D Q L L Y S Q D K - - - 171

TgPOFUT2      419 R A S E I A D E Q A Q T D D V C C N V N D L P G C P Q I H A A L T P E E R Q R A P A Q R G G D P R E E I D E Q T G E F N E G H N P S G D G E 488
PiPOFUT2      179 H K Y N V L Y S G Y C T T I N T K Q S E G Y S Y N M I S N Y F I T S I L E N - - - - - Y Q S N L E D I L L F N N K L L S Y G N N Y I S N I L K T N - - 216
SmPOFUT2      179 D R K V D F C P F E L R K S Y R L N D S T L S S S S S S F S R P M E L F E G N S F N L O N G I L P M T A S A Y D C I T G D L E P I H L A P 248
MmPOFUT2      172 H E Y Y R G W F W G Y E E T R G L N V S C L S V Q G S A S I V A P V L L K N - - - - - E Y W T V R R S I T I A N H L R D I G D N Y R E T Y L H S N D I 314
DmPOFUT2      206 G S L S G G P L L Q Q A E L R V G R F H C V R F O G S A G L L E K L L R E A I D E D T A G - - - - - E Y W D T R R S M V F A K H L R A V G D E F R S O H L N S T D A 263
HsPOFUT2      172 H E Y Y R G W F W G Y E E T R G L N V S C L S V Q G S A S I V A P L L R N - - - - - H F W Q A R R S M R F A R R L E Q V A A D F R R Q A L D T T D A 308

TgPOFUT2      489 R E K R K P G R R S D T S R S R K E I Q E E A K V S D T W S G V S L W L A G F C E T V R A L E M W C A S L Y I A D A P R I A D L L W R S V A 558
PiPOFUT2      217 - - - - - L F L Y N I T S V L I K Q S T N I L V P F V N E L - - - - - Y Q S N L E D I L L F N N K L L S Y G N N Y I S N I L K T N - - 271
SmPOFUT2      249 F L I Q L I Q N S K K P I T T L Y L G S A Q S I I H G H W S E W S Q - - - - - E Y W T V R R S I T I A N H L R D I G D N Y R E T Y L H S N D I 314
MmPOFUT2      210 - - - - - T S A R S V M L D R A E N L L H D H Y G G R - - - - - E Y W D T R R S M V F A K H L R A V G D E F R S O H L N S T D A 263
DmPOFUT2      251 - - - - - P E D V D M R T Y A L S A E T V L H D H W G D E - - - - - H F W Q A R R S M R F A R R L E Q V A A D F R R Q A L D T T D A 308
HsPOFUT2      210 - - - - - T S A R S V M L D R A E N L L H D H Y G G K - - - - - E Y W D T R R S M V F A R H L R E V G D E F R S R H L N S T D D 263

TgPOFUT2      559 E R P P G A I Q T V W L K F G E N L L V P W P D V L L D A H L L D M L H V H P K L R Q I G D L F I N K F L S N R D K T E A G K G E R A S T E 628
PiPOFUT2
SmPOFUT2      315 S D R T V S P S I V E H L G P G S N W L R S Q W - - - - - 338
MmPOFUT2      264 A D K - - - - - M A P E E D W T K M K V - - - - - 278
DmPOFUT2      309 S A G - - - - - V Q R P A M W E L E R - - - - - 322
HsPOFUT2      264 A D R - - - - - I P F Q E D W M K M K V - - - - - 278

Motif I
TgPOFUT2      629 G G T E R D E N L A K H G Y I A A H L R R T D F Y L K R - S V P L Q R A A A Y L V S R M K E H G V F K - - - - - 679
PiPOFUT2      272 - - - - - H Y I S S H L R Y T D F K Y I S R Y N V P P I H I A L L K L L Y - - - - - I M F I N N C R 311
SmPOFUT2      339 - - - - - P L S P A L G G P Y V A V H W R R G D F V T T S T A T T T T T V T T S R S P N S V L A A Q Q I L N A V K I F N Q Y E D H Y I D 404
MmPOFUT2      279 - - - - - K L G S A L G G P Y L G V H L R R K D F W G H R E D V P S L E G A V K K I R S - - - - - L M K T H Q L D 326
DmPOFUT2      323 - - - - - P K R N A K G G D Y L C A H L R R G D F V R S R D A T T P T L K A A A Q Q V K Q - - - - - L L R G F N M T 370
HsPOFUT2      279 - - - - - K L G S A L G G P Y L G V H L R R K D F W G H R Q D V P S L E G A V R K I R S - - - - - L M K T H R L D 326

Motif II
TgPOFUT2      680 - A F I C T D G S E D E K R E L R D A V R R V G D A A S S P Y T V V F F D L P T V R R L M I K T L E A S S H V S D D G S F H D L K A V E T 748
PiPOFUT2      312 I I F I A S D E K V E I Q K V I N K D F H Q Y K K H F Y F Y - - - - - N N Q N N L H E G E - - - - - 351
SmPOFUT2      405 T I Y L A T D A D K K E L E N L K S L L Y P L Q I F H F E P - - - - - T E F E W I S Y G P G G - - - - - 446
MmPOFUT2      327 K V F V A T D A I R K E Q E E L R K L L P - - E M V R F E P - - - - - T W E E L E Y K D G G - - - - - 366
DmPOFUT2      371 T V F L A T D A T P Y E L M E L K E L F Y R F R L V H F A P E - - - - - S N V Q R R E L K D G G - - - - - 413
HsPOFUT2      327 K V F V A T D A V R K E Y E E L K K L L P - - E M V R F E P - - - - - T W E E L E Y K D G G - - - - - 366

Motif III
TgPOFUT2      749 P G Y S G P N H I S L L L H P G I T A L I E V W I A A R A A Y F I G T K D S R F S Q A I R W E R H L M G H P L E S S L E V F C V D S S P D Q 818
PiPOFUT2      352 - - - - - F S I I E Q W I C T R S Y I F I G N I F S R F T M N I N W E R H L I N K G O I N Q N I D L C S Y H I N D D 404
SmPOFUT2      447 - - - - - S A I I D Q W I C A H A R Y F I G T S S S T F T F R I I E E R S I M G F L S N T T L N N L C P N G P I H L 499
MmPOFUT2      367 - - - - - V A I I D Q W I C A H A R F F I G T S V S T F S F R I H E E R E I L G L D P K T T Y N R F C G D - - - - - 414
DmPOFUT2      414 - - - - - V A V V D Q L V C A Y A R Y F V G T Y E S T F T Y R I Y E E R E I L G F T Q A S T F N T F C K A L G G S C 466
HsPOFUT2      367 - - - - - V A I I D Q W I C A H A R F F I G T S V S T F S F R I H E E R E I L G L D P K T T Y N R F C G D - - - - - 414

TgPOFUT2      819 T G G Q A Q G K C F A T K S H D P P E G R S R S E L R R K Y W P S L D P S S T L - - - - - 858
PiPOFUT2      405 N D Q D I K N S Y K K I V H I F N H K A L Q K I K N I Y D N Y S D R D K K Y I N T I C Y N F L S H F P N N R S I Y R K E Y I T N T 469
SmPOFUT2      500 Y Q P Y H Y Y T D S S T C Q S L T E W P V I Y E K O Y T I S S S S P M T N D K L H L N K Y M K D E L - - - - - 550
MmPOFUT2      415 - - - - - Q E K A C E Q P T H W K I A Y - - - - - 429
DmPOFUT2      467 S R N A V W P I V W A D G D S D S E E S D P Y - - - - - 490
HsPOFUT2      415 - - - - - Q E K A C E Q P T H W K I T Y - - - - - 429
    
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238 **Figure S5. Sequence alignment of *T. gondii* POFUT2 with other eukaryotic POFUT2**
239 **highlights conserved motifs.** Conserved sequences are boxed in black (FTs superfamily
240 conserved motifs I-III) or gray (POFUT2-specific) (35, 37). The catalytic Glu residue is marked
241 by a black dot. Gray dashed boxes mark transmembrane helices. *Pf: Plasmodium falciparum*;
242 *Dm: Drosophila melanogaster*; *Hs: Homo sapiens*; *Mm: Mus musculus*; *Sm: Schistosoma*
243 *mansoni*.
244

245 **Figure S6.**

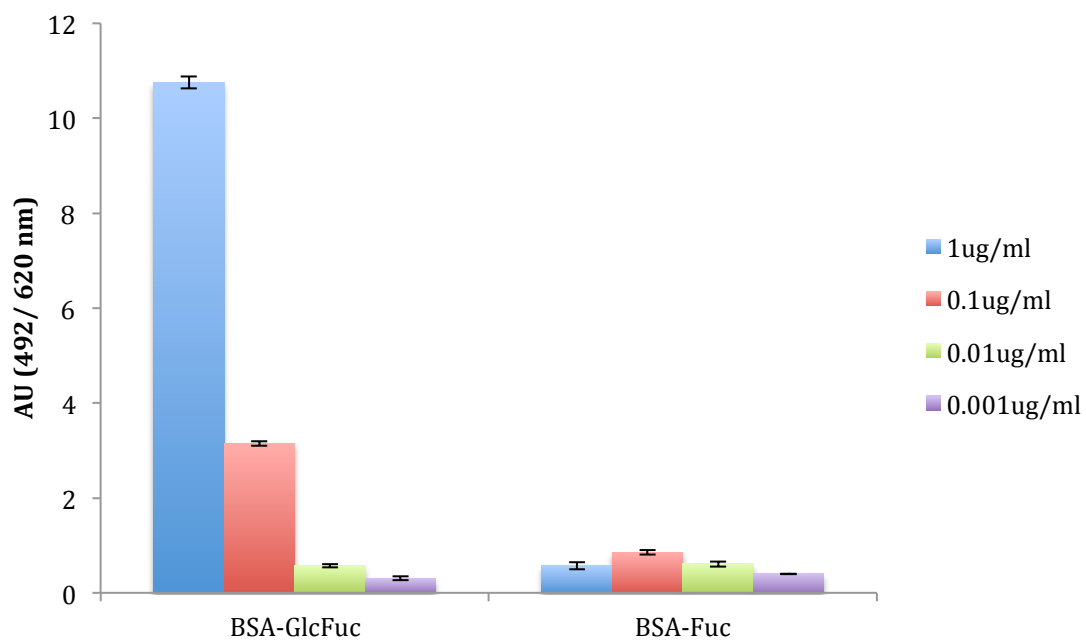


246

247 **Figure S6. Sequence alignment of *T. gondii* NST2 with other GDP-Fuc transporters.** The
 248 predicted topology and conserved motifs for the *HsFUCT1* (42) was used to annotate the
 249 alignment. TM1-10 mark the transmembrane domains. The two dashed boxes highlight the motif
 250 involved in guanidine and sugar binding, as recently described for the GDP-Mannose transporter
 251 (42). *Dd*: *Dictyostelium discoideum*; *Hs*: *Homo sapiens*; *At*: *Arabidopsis thaliana*.

252

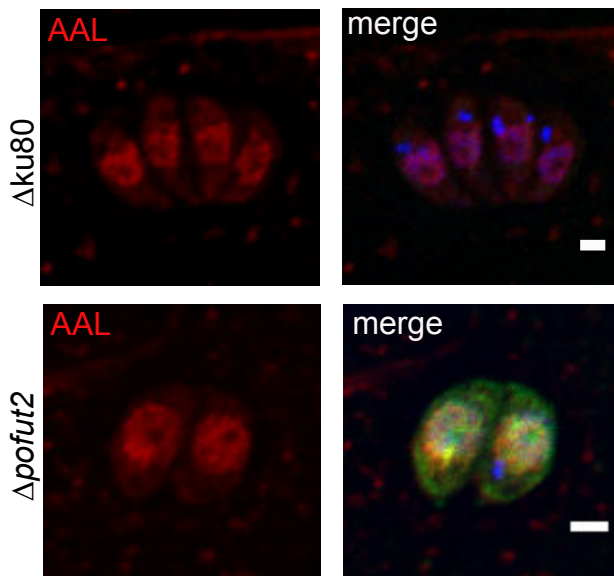
253 **Figure S7.**



254

255 **Figure S7. Anti-GlcFuc Antibody Titration.** Different concentrations of anti-GlcFuc polyclonal
256 IgY were tested by ELISA to identify the concentration that provided the best signal against
257 BSA-GlcFuc.

258 **Figure S8.**

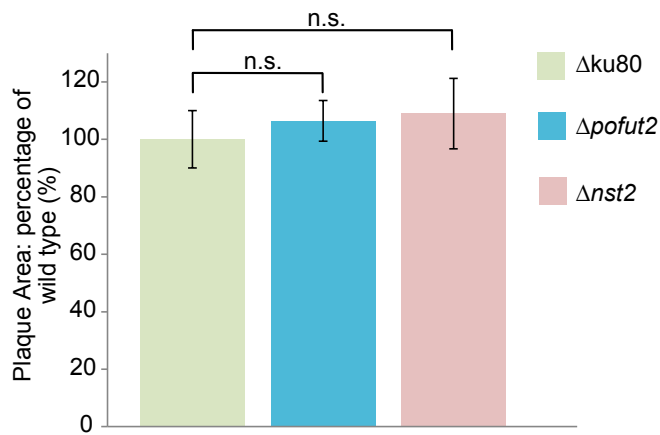


259

260 **Figure S8. Knockout of *pofut2* does not affect nuclear O-fucosylation.** IFA staining with
261 *Aleuria aurantia* lectin (AAL) of wild type and $\Delta pofut2$ tachyzoites shows that nuclear O-
262 fucosylated proteins are still present in the KO.

263

264 **Figure S9.**



265

266 **Figure S9. No difference in plaque area is observed between wild type and *pofut2* and *nst2***
267 **deficient parasites.** The lack of statistical difference in the size of plaques, suggest the *O*-
268 fucosylation deficient parasites are replicating as efficiently as the parental strain. Plaque areas
269 were determined as described in material and methods. The average of two biological repeats is
270 shown. n.s.: not significant.

271