1 **Expanding the repertoire of GalNAc analogues for cell-specific bioorthogonal**

2 **tagging of glycoproteins**

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19

20 **Abstract**

21 Glycosylation is a ubiquitous modification of proteins, necessitating approaches for

22 visualization and characterization. Bioorthogonally tagged monosaccharides have

23 been instrumental to this end, offering a chemical view into the cell biology of

24 glycans. Understanding the use of such monosaccharides by cellular biosynthetic

25 pathways has expanded their applicability in cell biology, for instance through the

26 strategy Bioorthogonal Cell-specific Tagging of Glycoproteins (BOCTAG). Here, we

27 show that the cellular use of two azide-tagged analogues of the monosaccharide *N*-

28 acetylgalactosamine can be promoted through expression of two biosynthetic

29 enzymes. Cellular expression of the kinase NahK and the engineered

30 pyrophosphorylase AGX1^{F383A} led to biosynthesis of the corresponding activated

31 nucleotide-sugars and subsequent bioorthogonal tagging of the cellular

32 glycoproteome. We explore the use of both sugars for BOCTAG, demonstrating the

33 visualization of cell surface glycosylation in a specific cell line in a co-culture system.

34 Our work adds to the toolbox of glycoprotein analysis in biomedicine.

Introduction

The proteome is substantially expanded through posttranslational modifications (PTMs). Glycosylation is the most abundant and complex PTM, and tools to understand the glycoproteome are thus essential to contribute to understanding in biology. Bioorthogonal chemistry was first developed to study glycosylation due to the lack of genetic methods to directly manipulate glycans. Glycobiology has since played a key role in enhancing bioorthogonal chemistry.

A number of monosaccharides have been furnished with chemical tags to allow for bioorthogonal incorporation of reporter compounds such as fluorophores or enrichment reagents. Azide groups were among the first such tags, as they are inert to cellular metabolic processes and are amenable to copper-catalysed (CuAAC) or strain-promoted azide-alkyne (SPAAC) cycloaddition reactions to append reporter groups such as fluorophores.[1–4] Other tags have included alkynes, alkenes, and many others that are suitable for distinct bioorthogonal reactions.[4–13] The successful use of tagged monosaccharides relies on their acceptance by biosynthetic enzymes to generate UDP-sugars, and on glycosyltransferases using these UDP-sugars as substrates. Appending an azido group to the side chain of *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc) to render GalNAz or GlcNAz, respectively, allows acceptance by both types of enzymes and incorporation into the glycoproteome.[14–16] However, even slightly bigger modifications can render monosaccharides refractory to incorporation (Fig. 1A).[2,17–20]

We and others have found that acylamide moieties in GalNAc and GlcNAc with sterically more demanding modifications are often not converted to the corresponding UDP-sugar analogues. In parallel with Chen and colleagues, we have shown that UDP-sugar biosynthesis can be engineered into a cell line for cell-selective incorporation in co-culture systems alongside more advanced *in vivo* systems.[17,20,21] This strategy, termed Bio-Orthogonal Cell-specific TAgging of Glycoproteins (BOCTAG), employs the bacterial sugar-1-kinase NahK that is more promiscuous towards chemical modifications than the human kinases GALK1 and GALK2.[17,22] An engineered human pyrophosphorylase AGX1[F383A] then converts GalNAc-1-phosphates to UDP-GalNAc analogues.[2,18,21,23,24] Chen and colleagues used the similarly engineered (F383G) pyrophosphorylase AGX2, an isoenzyme to AGX1, along with a bioorthogonal GlcNAc analogue to mediate UDP-sugar biosynthesis.[20] Both studies employed linear alkynoate side chains as bioorthogonal

69   tags, either a pentynoate side chain e.g. in the sugar GalNAlk, or a hexynoate side

70   chain in the sugar GalN6yne. The corresponding UDP-GalNAc analogues are

71   interconverted into the corresponding UDP-GlcNAc analogues and vice versa by the

72   activity of the epimerase GALE, leading to labelling of various sub-types of the

73   glycome. However, only a limited set of sugars have been used to this end. A set of

74   bioorthogonal sugars with alternative chemical tags and potential incorporation into

75   different sub-sections of the glycome would be useful to expand our repertoire of

76   tools to study the glycoproteome.

77   We have previously identified the bioorthogonal azide-containing GalNAc analogue

78   GalNAzMe to be selectively incorporated into O-GalNAc glycans (Fig. 1A).[2]

79   Selectivity was conferred by the branched nature of the acylamide modification,

80   rendering the corresponding nucleotide-sugar UDP-GalNAzMe resistant to cellular

81   epimerisation by GALE to the respective analogue termed UDP-GlcNAzMe.[2] In

82   contrast, the nucleotide-sugar UDP-GalNPrAz, an isomer of UDP-GalNAzMe with a

83   linear acylamide, was epimerised to UDP-GlcNPrAz *in vitro*. While no cellular

84   labelling studies with GalNPrAz were attempted at the time, we reasoned that

85   GalNPrAz may be a more promiscuous metabolic chemical reporter of glycosylation.

86   Testing these hypotheses was challenging at the time since biosynthesis of UDP-

87   GalNAzMe in cells was only possible from a synthetically complex caged sugar-1-

88   phosphate in cells expressing AGX1^{F383A}.[2,21] Our later findings suggesting that the

89   kinase NahK can prime the biosynthesis of chemically tagged UDP-sugars led us to

90   evaluate the metabolic fate of GalNAzMe and GalNPrAz (Fig. 1A). Here, we

91   demonstrate that the BOCTAG principle can be expanded to allow biosynthesis and

92   use of UDP-GalNAzMe and UDP-GalNPrAz from synthetically accessible precursors

93   to increase the variability of metabolic reporters for cell-selective glycoproteome

94   evaluation.

95

96   **Results and Discussion**

97

98   ***In vitro* enzymatic synthesis of chemically modified UDP-GalNAc analogues**

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100   To anticipate cellular biosynthesis, we investigated how GalNPrAz and GalNAzMe

101   can be enzymatically converted to their respective UDP-GalNAc analogues. We first

102 subjected each monosaccharide alongside GalNAc to recombinant human kinases

103 GALK1, GALK2 or the bacterial kinase NahK and measured turnover to sugar-1-

104 phosphates by ultra-performance liquid chromatography with mass spectrometry

105 detection (UPLC-MS) after either 3 h or 16 h reactions (Fig. 1B, Supporting Fig. 1).

106 GALK2 accepted both GalNAc and GalNPrAz as substrates to afford > 60% and

107 near-complete turnover in 3 h and 16 h reactions, respectively. In contrast,

108 conversion of GalNAzMe by GALK2 proceeded slowly and with approximately 3-

109 times lower turnover after a 16 h reaction. The more promiscuous kinase NahK

110 accepted all three substrates, with > 80% conversion of the synthetic compounds

111 GalNAzMe and GalNPrAz after 16 h (Fig. 1B). The galactose-specific kinase GALK1

112 did not accept any of the three monosaccharides, in line with our previous findings

113 on GalNAc analogues.[17] We next assessed conversion of sugar-1-phosphates to

114 UDP-sugars by AGX1^{F383A} (Fig. 1C, Supporting Fig. 2). Monosaccharides were

115 converted to sugar-1-phosphates by NahK *in situ* and treated in a one-pot

116 multienzyme (OPME) reaction with 125 nM WT-AGX1 or AGX1^{F383A}. Measuring

117 UDP-sugar biosynthesis by UPLC with UV detection, we found that WT-AGX1 used

118 chemically modified GalNAc analogues with lower turnover (< 20%) compared to

119 GalNAc (91%). In contrast, AGX1^{F383A} yielded a 67% turnover for UDP-GalNPrAz

120 and 27% turnover for UDP-GalNAzMe in a 16h reaction (Fig. 1C). The *in vitro*

121 turnover reactions by AGX1 variants matched our previous cellular biosynthesis

122 data.[2] Reactions stopped after 3 h showed generally low turnover, with the same

123 trends as observed for 16 h reactions (Supporting Fig. 2A). We tested a higher

124 concentration of 500 nM recombinant AGX1 to corroborate these results (Supporting

125 Fig. 2B). Including alkyne-tagged GalNAc analogues GalNAlk and GalN6yne in

126 OPME reactions (Supporting Fig. 2C), we similarly found that NahK together with

127 AGX1^{F383A} produced the corresponding UDP-sugars in near-quantitative turnover in

128 3 h and 16 h reactions.[17,21,24] Taken together, the combination of the enzymes

129 NahK/AGX1^{F383A} that featured in our BOCTAG approach successfully synthesised a

130 range of chemically modified UDP-GalNAc analogues from free monosaccharides.

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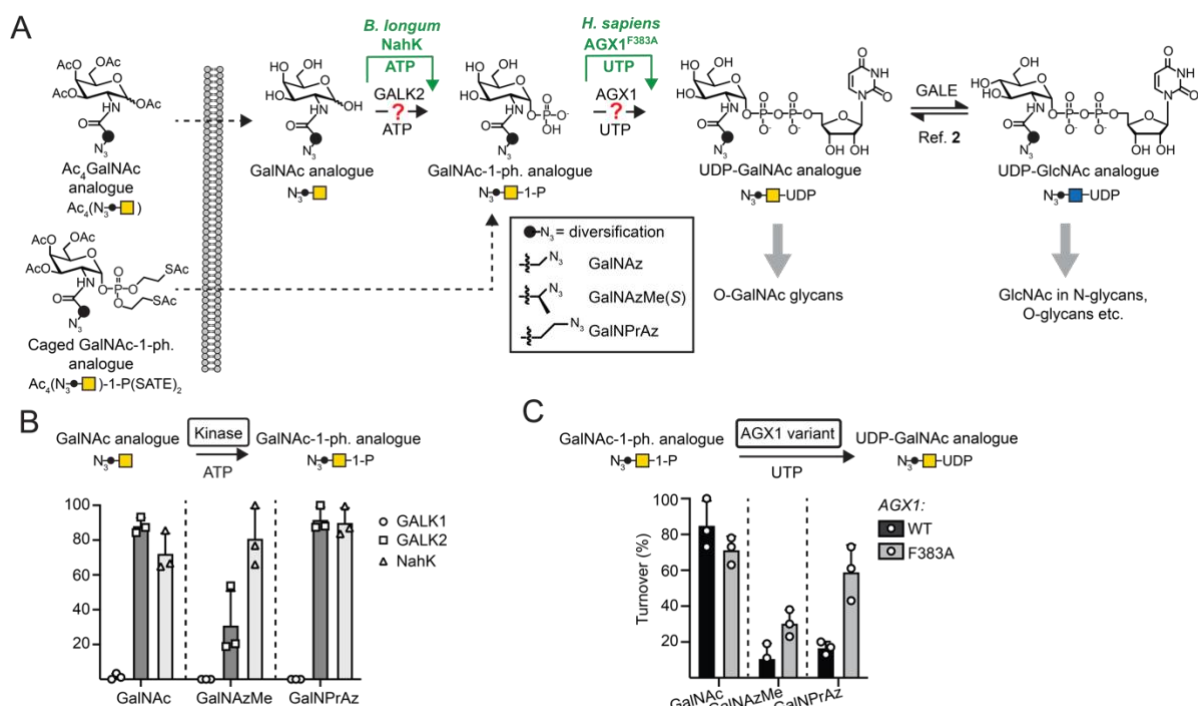
Fig. 1: Engineered biosynthesis for UDP-GalNAc analogues. *A*, biosynthetic pathways. UDP-GalNAc is biosynthesized by GALK2 and AGX1 activities, while modifications often require the enzymes NahK and AGX1[F383A]. *B*, acceptance of GalNAc as well as azide-tagged analogues by sugar-1-kinases as measured by UPLC-MS in 16 h reactions. Data are means + SD from three independent replicates. *C*, acceptance of GalNAc-1-phosphate as well as azide-tagged analogues by AGX1 constructs as assessed by UPLC. GalNAc-1-phosphate analogues were generated *in situ* by reaction of the corresponding monosaccharides with NahK as shown in *B*. Data are means + SD from three independent replicates.
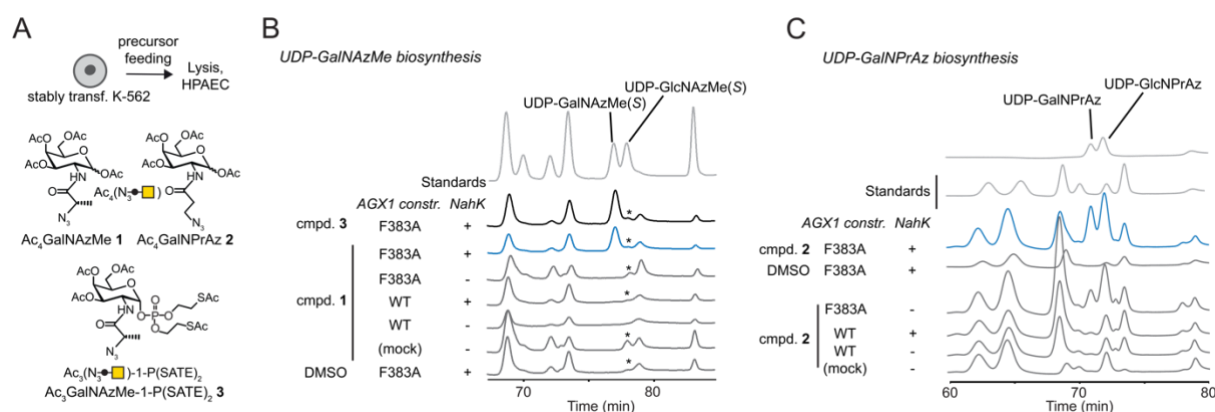

Cellular biosynthesis of UDP-GalNAzMe and UDP-GalNPrAz

We assessed biosynthesis in K-562 cells expressing combinations of NahK and AGX1 constructs. Per-acetylated sugars Ac4GalNAzMe **1** and Ac4GalNPrAz **2** were used as synthetically convenient monosaccharide precursors (Fig. 2A). A caged GalNAzMe-1-phosphate **3** previously used by us was employed as a control for biosynthesis.[2] Mindful that high concentrations of per-acetylated compounds can lead to non-enzymatic background labelling even without UDP-sugar biosynthesis,[25,26] we included a control cell line transfected with an empty plasmid not encoding either of the biosynthetic enzymes. Cells were fed with sugar precursors, and UDP-sugar biosynthesis was assessed by high performance anion exchange chromatography (HPAEC) (Fig. 2A, B).[2,18] Comparison with synthetic standards indicated that cells expressing both NahK and AGX1[F383A] biosynthesized

155   UDP-GalNAzMe from Ac$_4$GalNAzMe **1** alongside GalNAzMe-1-phosphate **3** (Fig.

156   2B). Both NahK and AGX1[F383A] were needed for UDP-GalNAzMe biosynthesis from

157   Ac$_4$GalNAzMe, as cell lines expressing only one of the components or

158   overexpressing WT-AGX1 did not yield a peak at the corresponding retention time

159   (Fig. 2B). Our previous work on the biosynthetic fate of tagged monosaccharides

160   indicated that branched acylamide side chains such as in UDP-GalNAzMe suppress

161   epimerization between UDP-GalNAc analogues and UDP-GlcNAc analogues.[2] We

162   therefore included the synthetic compound UDP-GlcNAzMe as a standard in our

163   HPAEC experiments.[2] We could not conclusively rule out epimerisation of UDP-

164   GalNAzMe in these experiments since a peak with variable intensity from cell lysates

165   eluted at the retention time of UDP-GlcNAzMe (Fig. 2B, asterisk). Feeding cells with

166   increasing concentrations of Ac$_4$GalNAzMe **1** did not correlate with increasing

167   intensity of this peak (Supporting Fig. 3A). We therefore concluded that

168   epimerization of UDP-GalNAzMe was not detectable in our assay.[2] UDP-GalNPrAz

169   was successfully biosynthesized in cells expressing NahK and AGX1[F383A], although

170   small amounts were produced in the absence of NahK (Fig. 2C, Supporting Fig. 3B).

171   Including the corresponding UDP-GlcNAc analogue which we termed UDP-

172   GlcNPrAz as a standard, we observed clear epimerization of UDP-GlcNPrAz in cells,

173   although the corresponding peak likewise overlapped with a background peak (Fig.

174   2C, Supporting Fig. 3B). Taken together, these data suggested that the presence of

175   the BOCTAG enzymes is an efficient way to biosynthesize both UDP-GalNAzMe and

176   UDP-GalNPrAz.

177

178   **Fig. 2:** Engineered cellular biosynthesis of UDP-GalNAzMe and UDP-GalNPrAz. *A*, experimental

179   layout and synthetic compounds used. *B*, biosynthesis of UDP-GalNAzMe in stably transfected cells

180   as measured by HPAEC. Cells were fed with 50 µM synthetic compounds or DMSO. Data are from

181   one representative out of at least two replicates. Asterisk denotes a peak likely due to an artifact of

182   chromatography conditions. *C*, biosynthesis of UDP-GalNPrAz in stably transfected cells as

183   measured by HPAEC. Cells were fed with 50 µM compound **2** or DMSO. Data are from one

184   representative out of at least two replicates.

185

186   **Incorporation of GalNAzMe and GalNPrAz into cell-surface glycoproteins**

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188   We assessed incorporation of GalNAc analogues into cellular glycans by on-cell

189   CuAAC with a near-infrared fluorophore. This experimental setup ensured that only

190   cell surface glycosylation was detected by ensuing in-gel fluorescence of

191   lysates.[2,18,21,27] In cells fed with per-acetylated GalNAc analogues Ac$_4$GalNAzMe **1**

192   and Ac$_4$GalNPrAz **2**, both sugar precursors led to a dose-dependent increase in

193   fluorescence signal in cells expressing AGX1[F383A] and NahK (Fig. 3A). The minimal

194   concentration required for visible signal after Ac$_4$GalNAzMe **1** feeding was 25 µM.

195   We observed substantial background fluorescence signal in cell lines fed with

196   Ac$_4$GalNAzMe **1**, particularly at concentrations above 50 µM. We attributed this

197   signal to non-enzymatic S-glyco-modification as a side product seen with per-

198   acetylated bioorthogonal sugars described by Chen and colleagues.[25,26] However,

199   glycosylation in cells expressing NahK/AGX1[F383A] led to a clear increase of

200   fluorescence intensity that was specific to individual glycoprotein bands, for instance

201   those at approximately 100 kDa. We attributed this signal to the mucin-like

202   glycoprotein CD43 that is heavily expressed in K-562 cells and chemically tagged by

203   GalNAzMe.[2,28] Cells expressing NahK and AGX1[F383A] showed visible fluorescent

204   signal when fed more than 2.5 µM Ac$_4$GalNPrAz **2**. In the absence of NahK, feeding

205   with Ac$_4$GalNPrAz **2** also led to a dose-dependent fluorescence signal above a

206   minimum concentration of 12.5 µM Ac$_4$GalNPrAz **2**. Incorporation of GalNPrAz was

207   observed in a much larger range of glycoproteins than GalNAzMe, commensurate

208   with UDP-GalNPrAz being epimerized into UDP-GlcNPrAz that could be used by

209   GlcNAc transferases.[2] In experiments comparing the labelling intensity of optimal

210   Ac$_4$GalNAzMe **1**/Ac$_4$GalNPrAz **2** concentrations, we further found that

211   overexpression of WT-AGX1 alone or together with NahK does not lead to

212   discernible fluorescence signal (Fig. 3B). Ac$_4$GalNAzMe **1** feeding required both

213   NahK and AGX1[F383A] for bioorthogonal tagging whereas Ac$_4$GalNPrAz **2** requires at

214   least AGX1[F383A]. These data were corroborated in two other cell lines, the murine

215   green fluorescent protein (GFP)-expressing 4T1 murine cancer cell line (4T1-GFP)

and the murine MLg fibroblast cell line (Supporting Fig. 4). Both cell lines incorporated GalNPrAz and GalNAzMe into their glycoproteomes when expressing both NahK and AGX1^{F383A}, but not when left untransfected, further confirming that both biosynthetic enzymes lead to reproducible bioorthogonal tagging of the glycoproteome.

The nature of glycoproteins into which GalNPrAz and GalNAzMe had been incorporated was subsequently investigated. Lysates of cells expressing both NahK and AGX1^{F383A} fed with per-acetylated GalNAc analogues were incubated with a panel of glycosidases and glycoproteases (Fig. 3C). The well-characterized, promiscuous GalNAc analogue Ac$_4$GalNAz served as a control to compare labelling band patterns by in-gel fluorescence.[15] The mucinase SmE was recently demonstrated to cleave the protein backbone of mucin-domain glycoproteins with broad tolerance for dense glycosylation and high glycan complexity.[29] Using SmE, the 100 kDa fluorescent glycoprotein band disappeared across samples fed either Ac$_4$GalNAzMe **1**, Ac$_4$GalNPrAz **2** or Ac$_4$GalNAz. A new band appeared at approximately 90 kDa which we attributed to be a digestion product of CD43. A commercial endo-α-*N*-acetylgalactosaminidase was next used to remove short core 1 O-glycans in the presence of a neuraminidase.[30] This treatment also led to disappearance of the 100 kDa band across samples fed with all sugars, with the emergence of a new band at approx. 70 kDa that likely lacks untagged O-glycans. PNGaseF removed N-linked glycans from glycoproteins. The band corresponding to CD43 at approximately 100 kDa remained in all samples, though appeared to be shifted to a slightly lower molecular weight (Ac$_4$GalNPrAz **2** treated) or slightly higher molecular weight (Ac$_4$GalNAzMe **1** treated). CD43 is known to carry a single N-glycan,[28] the removal of which may impact the charge state of CD43 and thus its migration by electrophoresis. Bands observed at approx. 60, 160 and 260 kDa in cells treated with either Ac$_4$GalNPrAz **2** or Ac$_4$GalNAz disappeared upon PNGase F treatment, suggesting cleavage of fluorescently labelled N-glycans. Taken together, these results suggest GalNPrAz and GalNAzMe are indeed incorporated into mucin-type O-glycans, and that Ac$_4$GalNPrAz **2** recapitulates the labelling of N- and O-glycoproteins of Ac$_4$GalNAz.
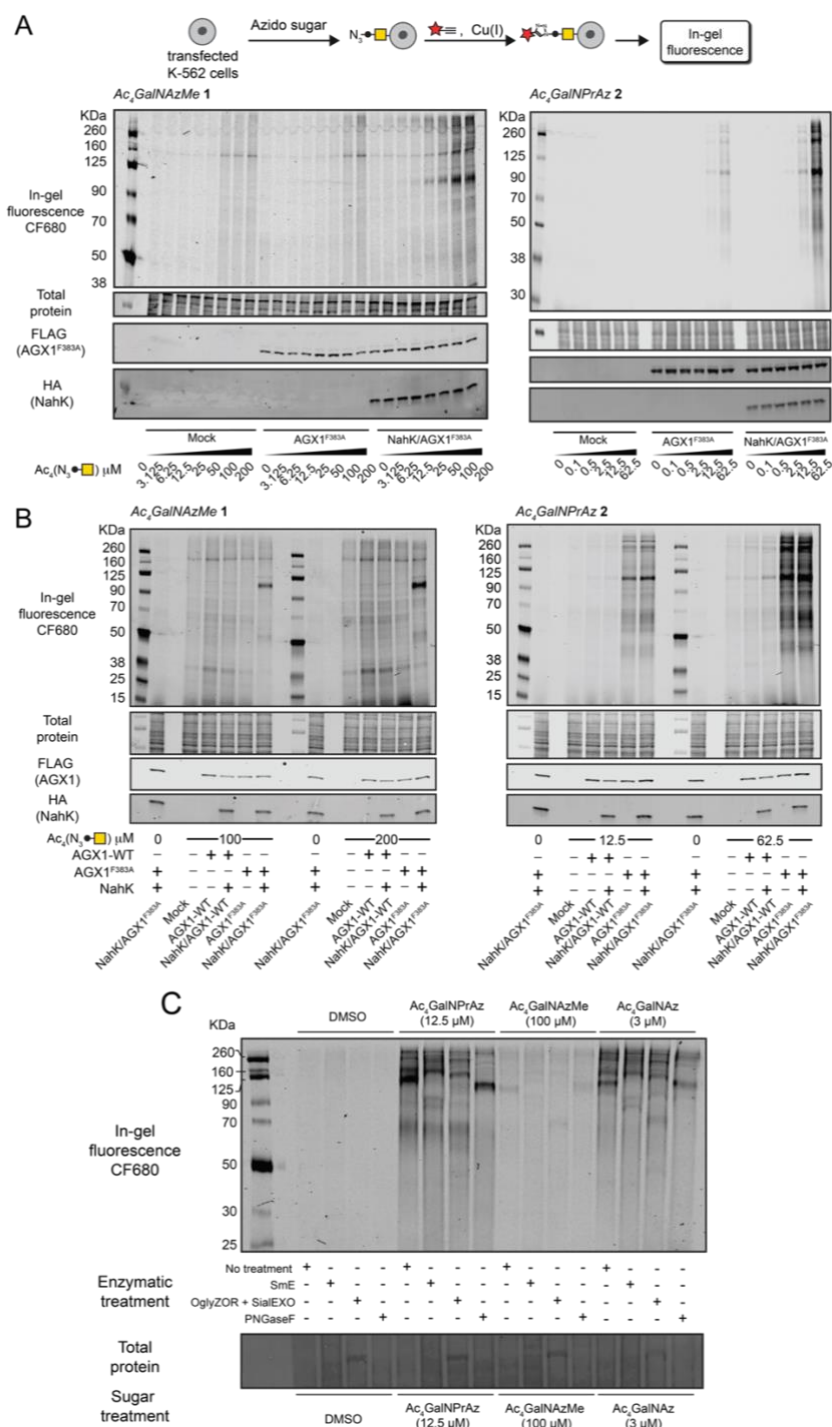
Fig. 3: Cell surface incorporation and cell-specific bioorthogonal tagging of azidosugars. *A*, dose-dependent incorporation of GalNAzMe (left) and GalNPrAz (right) into the cell surface glycoproteome of stably transfected K562 cells as assessed by in-gel fluorescence. Cells were fed with the indicated concentrations of compounds **1** or **2**, subjected to cell-surface CuAAC with CF680-alkyne and glycosylation detected by fluorescence scanning. Data are one representative out of two independent replicates. *B*, comparison of stably transfected K562 cell lines for incorporation of GalNAzMe or GalNPrAz based on optimised feeding concentrations. Data are one representative out of two independent replicates. *C*, comparison of different glycoprotease or glycosidase digestions on lysates

256 of cells expressing both NahK and AGX1F383A fed with per-acetylated GalNAc analogues assessed

257 by in-gel fluorescence.

258

Expanding the monosaccharide repertoire for cell-specific bioorthogonal tagging of glycoproteins

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262 We next tested whether GalNPrAz and GalNAzMe are suitable substrates for

263 BOCTAG, employing a co-culture system between 4T1-GFP and MLg cells.[17] 4T1-

264 GFP cells were transfected with NahK and AGX1^{F383A} or with an empty plasmid, and

265 grown in a co-culture system with non-transfected MLg cells. The co-culture was fed

266 with either Ac$_4$GalNAzMe **1**, Ac$_4$GalNPrAz **2**, or the more promiscuous reagent

267 Ac$_4$ManNAz that enters the pool of *N*-acetylneuraminic acid.[31] Incorporation of a

268 clickable fluorophore allowed visualization of cell-surface glycosylation (Fig. 4A). We

269 found that only 4T1-GFP cells expressing the BOCTAG enzymes NahK/AGX1^{F383A}

270 efficiently incorporated GalNPrAz, leading to cell-specific bioorthogonal tagging of

271 glycoproteins (Fig. 4B).
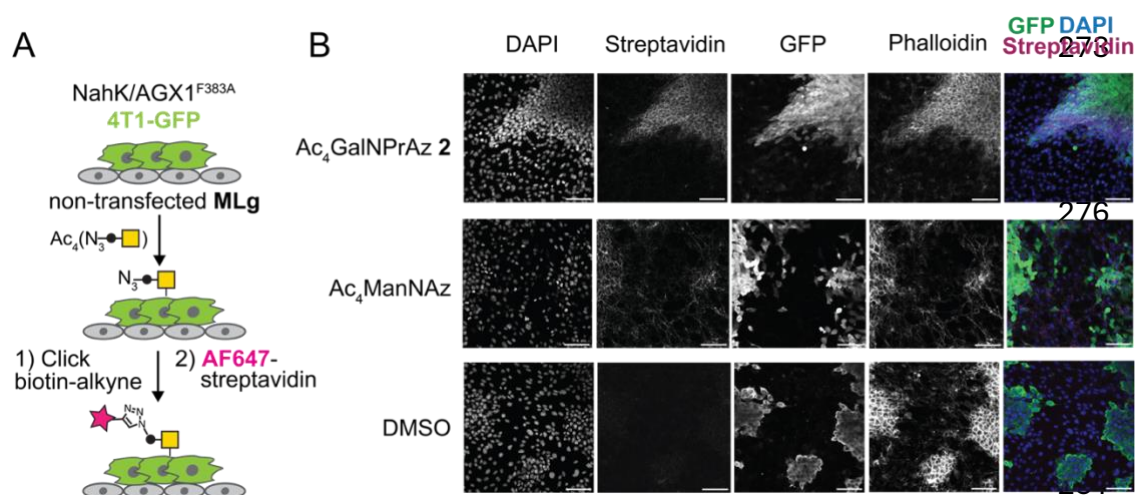
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283 **Fig. 4:** Cell-specific bioorthogonal tagging of glycoproteins with GalNPrAz. *A,* schematic describing

284 set-up of co-culture system between stably transfected 4T1-GFP cancer cells and MLg murine

285 fibroblasts. Cells were fed with compound **2** (62.5 μM), Ac$_4$ManNAz (10 μM) or an equivalent volume

286 of DMSO, subjected to on-cell CuAAC with biotin-alkyne, and imaged using fluorescently labelled

287 streptavidin. *B,* Microscopy data from one experiment. Scale bar set to 100 μm.

288

289 In contrast, Ac$_4$ManNAz feeding led to ubiquitous cell surface fluorescence on all cell

290 lines. In contrast, we observed no discernible cell-specific fluorescent labelling with

291   Ac$_4$GalNAzMe, indicating that labelling intensity was too weak for fluorescence

292   microscopy (Supporting Fig. 5). These data are consistent with our previous findings

293   suggesting that GalNAzMe requires further boosting of signal through engineered

294   glycosyltransferases.[2] We concluded that Ac$_4$GalNPrAz **2** allows for cell-specific

295   bioorthogonal tagging of glycoproteins in the presence of the BOCTAG enzymes

296   NahK/AGX1^{F383A}.

297

298   **Conclusions**

299   Bioorthogonal labelling techniques have rapidly evolved over the last decades,

300   fuelled by the need to probe the glycome. In recent years, the field has started to

301   map the metabolic fates of bioorthogonal monosaccharides, including their

302   compatibilities with biosynthetic enzymes as well as incorporation into different parts

303   of the glycome. Such metabolic precision is essential to allow the use of metabolic

304   tools in biomedical research. For instance, we have previously shown that

305   GalNAzMe allows investigation of the O-GalNAc glycoproteome in a genome-wide

306   genetic knockout screen.[2] In contrast, GalNPrAz is poised to enter O-GalNAc

307   glycans as well as other cellular glycans, presumably N-glycans. This finding renders

308   GalNPrAz a more promiscuous tool with higher incorporation efficiency, similar to the

309   alkynoate-containing sugar GalNAlk.[21]

310   Various strategies have been employed to allow for cell-specific chemical tagging of

311   proteins, including the use of biotin ligases and unnatural amino acids that directly

312   modify the peptide sequence.[32–34] BOCTAG is complementary to these techniques,

313   targeting glycans as a ubiquitous PTM. Application of the BOCTAG tactic to

314   monosaccharides that enter different glycan subtypes further expands the toolbox for

315   cell-specific labelling of glycoproteins, employing accessible per-acetylated

316   monosaccharide precursors for convenient use in biomedical research.

317

329 **Competing Interests**

330 S.A.M. is a consultant for InterVenn Biosciences and Arkuda Therapeutics, and a

331 coinventor on a Stanford nonprovisional utility patent application that has been filed

332 and is pending in the US (number US20220003777) related to the use of mucinases

333 for mass spectrometry analysis of mucin-domain glycoproteins.

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