Cell-specific Bioorthogonal Tagging of Glycoproteins in Co-culture

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SUMMARY

This work describes the development of a strategy to profile the glycoproteome with bioorthogonal tags in a cell-specific manner. We use a synthetic biology strategy in which an artificial biosynthetic AND gate leads to the cell-specific biosynthesis of a tagged UDP-sugar that, in turn, is used by the cellular glycosylation machinery. Comprehensive characterisation in mono- and co-culture experiments reveal a strong foundation for cell-specific glycoproteome analysis.

ABSTRACT

Interactions between cells fundamentally impact biological processes. In cancer development, such interactions define key stages of disease that cannot be adequately recapitulated in cell monoculture. Complex co-culture studies have been key to unraveling the complexity of these processes, usually by sorting cells and transcriptome or bulk proteome analyses. However, these methods invariably lead to sample loss and do not capture aberrant glycosylation as an important corollary of cancer formation. Here, we report the development of Bio-Orthogonal Cell line-specific Tagging of Glycoproteins (BOCTAG). Cells are equipped with a biosynthetic AND gate that uses bioorthogonally tagged sugars to generate glycosylation precursors. The cellular glycosylation machinery then introduces bioorthogonal tags into glycoproteins exclusively in cell lines expressing the enzymes of the biosynthetic AND gate. Modification with clickable reporter moieties allows for

imaging or enrichment with mass spectrometry-proteomics in a cell-specific fashion. Making use of glycans as a property of most cell surface proteins, we use BOCTAG as an efficient means for cell-specific protein tracing.

INTRODUCTION

Interactions between cells fundamentally shape physiological processes. In cancer, these interactions are aberrant, with tumour cells impacting non-cancerous bystander cells and vice versa. The complexity of such interplay is difficult to emulate in cell monoculture, requiring more elaborated co-culture systems.[1–3] Recent years have seen a massive increase in methods to probe the transcriptomes of different interacting cell populations, providing some insight into their state within a multicellular conglomerate.[4] However, the relationship between transcriptome and proteome is still less clear.[5] In addition, posttranslational modifications (PTMs) heavily impact the plasticity of the proteome. Glycosylation is the most complex and most abundant PTM, but difficult to probe due to the non-templated nature of glycan biosynthesis.[6] Glycans are generated by the combinatorial interplay of >250 glycosyltransferases (GTs) and glycosidases, mostly in the secretory pathway.[7] Certain cancer-derived glycoproteins are approved as diagnostic markers, but their discovery in complex model systems is a particular challenge.[8,9] This is especially true when *in vivo* or *in vitro* model systems comprise cell populations from the same organism that do not allow bioinformatic distinction of proteomes.[10,11] Methods to study the glycoproteome of a cell type in co-culture experiments are therefore a largely unmet need.

Metabolic oligosaccharide engineering (MOE) has revolutionized the field of glycoproteomics.[12] MOE reagents are membrane permeable monosaccharide precursors modified with chemical reactive tags amenable for bioorthogonal chemistry.[13] Many MOE reagents are based on chemically tagged analogues of *N*-acetylgalactosamine (GalNAc) that are activated by the biosynthetic GalNAc salvage pathway to derivatives of the nucleotide-sugar UDP-GalNAc.[14–17] Epimerisation at C4 position by the epimerase GALE yields UDP-*N*-acetylglucosamine (UDP-GlcNAc) analogues that can be used by different glycosyltransferases to chemically tag glycan structures in the secretory pathway and in the cytosol.[16–18] In turn, UDP-GalNAc analogues are used by members of the cancer-relevant *N*-acetylgalactosaminyltransferase (GalNAc-T) family consisting of 20 members (T1…T20).[14,19,20] We have recently used engineered double mutants (DM) of GalNAc-Ts to enable recognition of bulky analogues of UDP-GalNAc that are not used by WT-GalNAc-Ts.[21–23] Following incorporation, chemical tags are traced with enrichment handles or fluorophores by methods of bioorthogonal chemistry, including Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC).[24] Despite recent advances in the development of new MOE reagents, no chemical probes are yet available for the cell-specific tagging of glycoproteins.

Here, we developed Bio-Orthogonal Cell-specific Tagging of Glcyoproteins (BOCTAG) as an MOE strategy that allows for cell-specific investigation of the (glyco-)proteome in co-culture model systems. The strategy uses a biosynthetic AND-gate to generate an alkyne-tagged UDP-GalNAc analogue from a stable, readily available precursor. Only in cells carrying the pathway, wild-type GTs and engineered GalNAc-Ts use the nucleotide-sugar to chemically tag the glycoproteome. We demonstrate that BOCTAG allows for programmable glycoprotein tagging in living cells and in co-culture models from a simple, readily available precursor. We show that the chemical modification enters a range of glycan types, supporting the promiscuity of BOCTAG to

tag a large number of glycoproteins. The expression of engineered GalNAc-Ts preferentially accommodating the nucleotide-sugar further enhances labeling, rendering BOCTAG a promising tool for cell-specific glycoproteomics.

RESULTS

Metabolic engineering of a biosynthetic AND gate for a chemically tagged UDP-sugar

Design of a metabolic AND gate requires a linear 2-step biosynthetic pathway with the ability to supply the precursor and monitor conversions of both steps. We chose the GalNAc salvage pathway that consists of the kinase GALK2 and the pyrophosphorylase AGX1 to convert GalNAc first into GalNAc-1-phosphate and subsequently into UDP-GalNAc, respectively (Fig. 1A). The GalNAc salvage pathway is impervious to large modifications of the *N*-acyl group of GalNAc, e.g. a 6-carbon hex-5-ynoate chain in the substrate herein called GalN6yne (Fig. 1A).[21,25,26] In contrast, a shorter 5-carbon pent-4-ynoate chain in the substrate GalNAlk can be used as a substrate in low efficiency.[17] This is corroborated by the crystal structures of both GALK2 and AGX1 (Fig. S1). We thus designed BOCTAG to contain an artificial salvage pathway for GalN6yne which was hypothesized to not be used by the native GalNAc salvage pathway. We reasoned that interconversion by GALE to the C4 epimer, UDP-GlcN6yne, would affect chemical glycoprotein labelling in the living cell (Fig. 1A). We and others have mutated AGX1 at residue Phe383 to smaller amino acids to accommodate chemical modifications at the *N*-acyl position of sugar-1-phosphate substrates.[21,27] AGX1^{F383A}, herein called mut-AGX1, converted synthetic GalN6yne-1-phosphate to UDP-GalN6yne in an *in vitro* liquid chromatography-mass spectrometry (LC-MS) assay, while WT-AGX1 did not accept GalN6yne-1-phosphate at both 2 h and 16 h reaction times (Fig. 1B, Fig. S2A). In contrast to AGX1, the human kinase GALK2 was deemed unsuitable for engineering because the amino acids in close proximity to the *N*-acyl group are part of a hydrogen bond network that appear to stabilise positioning of the Mg^{2+} ion in the active site (Fig. S1). We instead turned to bypassing the GALK2 step with a more promiscuous kinase. Bacterial *N*-acetylhexosaminyl kinases (NahK) are widely used to phosphorylate GalNAc derivatives in chemoenzymatic syntheses.[28] Recombinant NahK from various species converted GalN6yne to GalN6yne-1-phosphate almost quantitatively in an *in vitro* assay, in contrast to GALK2 and the human galactose-specific kinase GALK1 (Fig. 1B). We chose to incorporate the most widely-used NahK from *Bifidobacterium longum* as a codon-optimised version for human expression in our BOCTAG strategy. Stable bicistronic expression of both *B. longum* NahK and mut-AGX1 in K-562 cells biosynthesized UDP-GalN6yne from a membrane-permeable per-acetylated precursor Ac_4GalN6yne (Fig. 1C). Expression of either enzyme alone or WT-AGX1 led to inefficient biosynthesis at best compared to levels of native UDP-sugars (Fig. S3). We further confirmed that by-passing of both enzymatic steps is needed for UDP-GalN6yne biosynthesis by feeding cells a protected precursor of GalN6yne-1-phosphate. Following non-specific uncaging in the living cell, UDP-GalN6yne was biosynthesised only in cells expressing mut-AGX1. Equally, a GalNAc analogue with a shorter side chain, GalNAlk, was efficiently converted to UDP-GalNAlk by cells only expressing mut-AGX1. In all cases, alkyne-tagged UDP-GalNAc analogues were interconverted to the corresponding UDP-GlcNAc analogues by GALE, which was corroborated for UDP-GalN6yne in an *in vitro* epimerization assay (Fig. 1C, fig. S2C, fig. S3). Thus, installing a biosynthetic AND gate for salvaging GalN6yne led to the biosynthesis of both UDP-GalN6yne and UDP-GlcN6yne, enabling chemical tagging of a wide range of different glycan subtypes by BOCTAG.

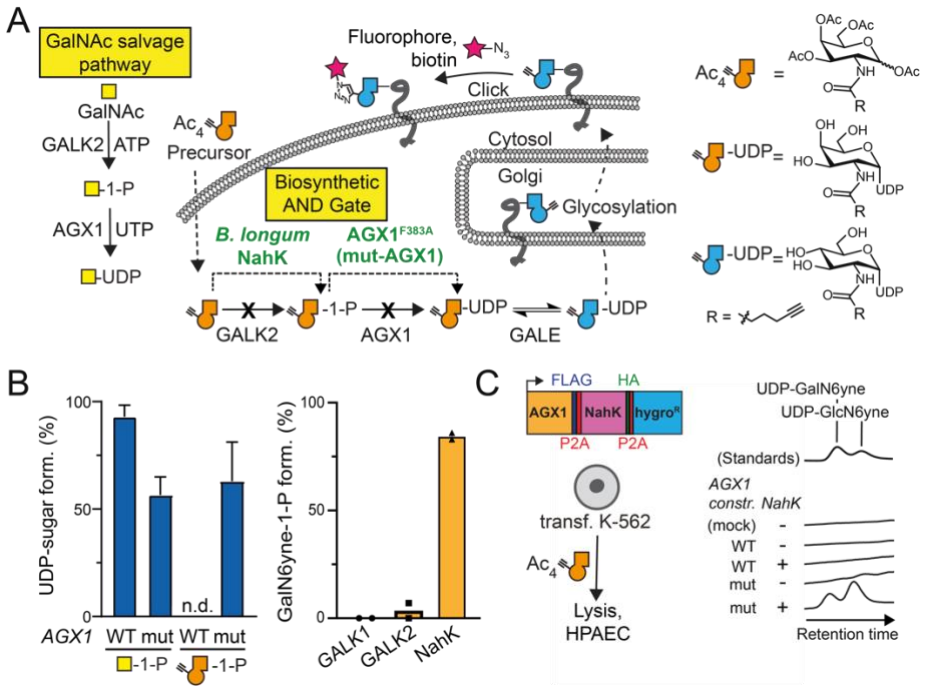

Fig. 1: Development of a metabolic AND gate for biosynthesis of UDP-GalN6yne 1. *A*, strategy of BOCTAG. The native GalNAc salvage pathway accepts GalNAc to synthesise UDP-GalNAc, but does not accept a GalNAc analogue with an *N*-hexynoyl side chain. *B. longum* NahK and mut-AGX1 biosynthesise UDP-GalN6yne and, by epimerization, UDP-GlcN6yne. Incorporation into GlcNAc-containing glycoconjugates can be traced by CuAAC. *B, in vitro* evaluation of UDP-GalN6yne biosynthesis by WT- or mut-AGX1 (left) and evaluation of GalN6yne-1-phosphate biosynthesis by human GALK1, GALK2 and *B. longum* NahK (right). Data are from LC-MS assays and integrated ion counts. Data are from three independent experiments and depicted as means ± SD (left) or two independent experiments and depicted as individual data points and means (right). *C*, biosynthesis of UDP-GalN6yne and UDP-GlcN6yne in cells stably expressing both NahK and mut-AGX1, as assessed by high performance anion exchange chromatography (HPAEC). Data are one representative out of two independent experiments.

To prove the validity of the MOE approach based on Ac_4GalN6yne, we confirmed chemical tagging of the cell surface glycoproteome. Alkyne tags were readily visualized by in-gel fluorescence in glycoproteomes of cells stably transfected with NahK and mut-AGX1 that were fed with Ac_4GalN6yne and treated with the clickable fluorophore CF-680 by CuAAC (Fig. 2A). Cells only expressing either NahK or mut-AGX1 exhibited no or sixfold reduced fluorescent labelling intensity, respectively. The promiscuous MOE reagent Ac_4ManAlk that enters the sialic acid pool gave fluorescent labelling regardless of the enzyme combination expressed. Fluorescent labelling was selective for NahK and mut-AGX1 expression over three orders of magnitude, underlining the robustness of programmable glycoprotein tagging (Fig. 2B).

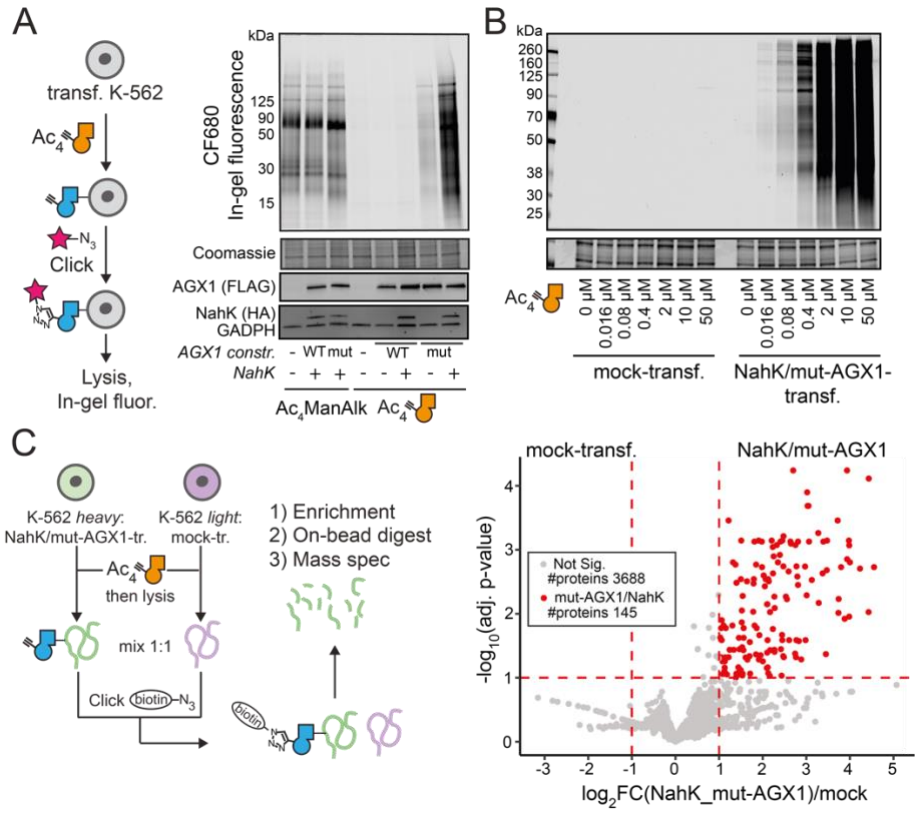

Fig. 2: A metabolic AND gate enables programmable chemical tagging of the glycoproteome. *A,* evaluation of cell surface glycoproteome tagging after treating K-562 cells transfected with NahK/AGX1 combinations 50 µM Ac_4GalN6yne or 10 µM Ac_4ManNAlk. *B,* dose-response experiment of cell surface tagging. Data in *A* and *B* are from one representative out of two independent experiments each. *C,* quantitative measurement of glycoprotein labelling by SILAC. Data were analysed from three independent experiments with forward (heavy mock, light NahK/mut-AGX1) and reverse analyses incorporated, as a total of six replicates.

A metabolic AND gate allows for programmable enrichment of the proteome.

We used Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) to quantify chemical tagging. BOCTAG- or mock-transfected K-562 cells were individually grown in heavy or light media in the presence of Ac_4GalN6yne. Lysates of these cells were mixed to contain equal amounts of heavy and light protein, and clickable biotin-picolyl azide was installed by CuAAC. Enrichment on neutravidin beads allowed to exclusively isolate biotin-tagged proteins that, after double digestion with LysC and trypsin, were analyzed by quantitative MS. Statistical analyses contained three independent experiments in which both combinations of heavy and light SILAC labelling each were used to give a total of six replicates (Fig. 2C). We found peptides from 145 proteins to be significantly enriched in BOCTAG-transfected cells. NahK/mut-AGX1-transfected cells fed with DMSO as a vehicle control gave similar labelling to mock-transfected cells fed with Ac_4GalN6yne. More than 99% of the proteins selectively enriched from NahK/mut-AGX1-transfected cells have been previously annotated as either Asn(N)- or Ser/Thr(O)-glycosylated, including the nucleoporins Nup62 and Nup153 and the cell surface glycoproteins CD47 and NOTCH1, confirming the stringency of the approach for glycoproteins.

Assessing and manipulating glycan promiscuity of GalN6yne

We next sought to address the glycan targets of our MOE approach. We were prompted by our recent findings that GalNAc analogues with bulky *N*-acyl chains such as GalN6yne are exclusively incorporated into mucin-type O-GalNAc glycans by DM-GalNAc-Ts (Fig. 3A).[21–23] We stably co-expressed WT- or DM-versions of GalNAc-T1 or T2 with NahK and mut-AGX1 from the same plasmid through a bidirectional promoter in K-562 cells. Expression of DM-GalNAc-Ts increased the intensity of in-gel fluorescence by more than sevenfold over expression of WT-GalNAc-Ts (Fig. 3B). WT-AGX1 expressing cells lacked UDP-GalN6yne/GlcN6yne biosynthesis and did not show any discernible fluorescent labelling over vehicle control DMSO. We assessed the glycan targets of chemical tagging by digestion with the hydrolytic enzymes PNGase F (reduces N-glycosylation), StcE (digests mucin-type glycoproteins) and OpeRATOR (digests O-GalNAc glycoproteins in the presence of the sialidase SialEXO) prior to in-gel fluorescence.[29] In cells expressing NahK, mut-AGX1 and WT-GalNAc-Ts, fluorescent labelling was slightly sensitive to PNGase F treatment, indicating that the major target structures of BOCTAG alone are N-glycoproteins (Fig. 3C). Co-expression of DM-GalNAc-Ts with NahK/mut-AGX1 led to additional fluorescent labelling of a small number of presumably mucin-type O-glycoproteins with high intensity and sensitivity to both StcE and OpeRATOR/SialEXO (Fig. 3C). Thus, DM-GalNAc-Ts broaden the target scope of BOCTAG to include mucin-type O-glycoproteins with high incorporation efficiency. Concomitant with this finding, we performed quantitative MS-proteome analysis by SILAC of cell lines expressing BOCTAG+DM-T2. In contrast to cells expressing only BOCTAG, we observed an increase of 37% to 61% of enriched annotated O-GalNAc glycoproteins (Table S1, table S2).

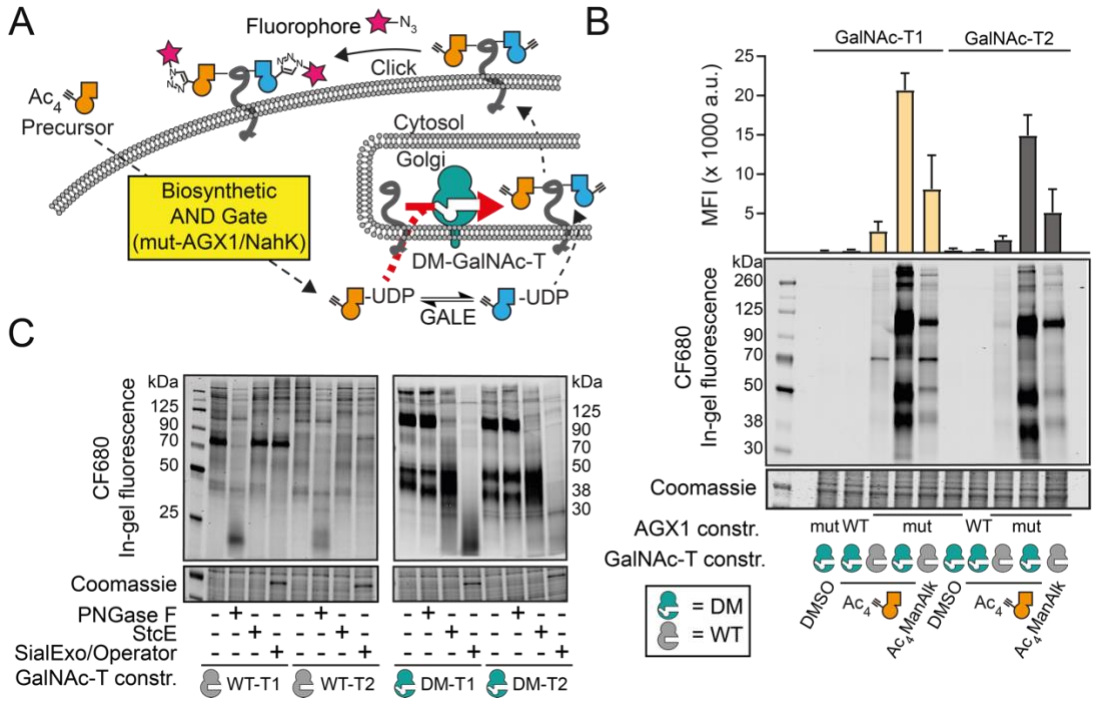

Fig. 3: Enhancement of programmable glycoprotein tagging by expression of DM-GalNAc-Ts. *A*, strategy of labelling O-GalNAc glycans in addition to GlcNAc-containing glycans. Expression of DM-GalNAc-Ts selectively engineered to accommodate bulky chemical tags mediates preferential O-GalNAc tagging in cells expressing NahK/mut-AGX1. *B*, evaluation of labelling efficiency by feeding transfected K-562 cells with

DMSO, 1 μM Ac_4GalN6yne or 2 μM Ac_4ManNAlk, analysis of labelling by in-gel fluorescence and quantification as means ± SD from three independent experiments. All cell lines used here expressed NahK. *C*, assessment of labeled glycans by treatment with hydrolytic enzymes of the samples analysed in *B*. Data are from one representative out of a total of four replicate labelling experiments performed on two different days.

Cell type-specific glycoporeome tagging in co-culture

We assessed the suitability of the synthetic salvage pathway as a metabolic AND gate as a BOCTAG cell type-specific (glyco)proteome labelling technique by fluorescence microscopy. Colonies of NahK/mut-AGX1-transfected, GFP-expressing 4T1 breast cancer cells were established on a monolayer of non-transfected MLg fibroblast cells by co-culturing for 72 h before media supplementation with Ac_4GalN6yne (Fig. 4A). Clickable biotin-picolyl azide was installed by CuAAC followed by Streptavidin-AF647 staining to visualize chemical tagging and counter-stained with phalloidin. Streptavidin-AF647 signal was strongly and reproducibly restricted to GFP-expressing cells only when Ac_4GalN6yne was fed (Fig. 4B, C), indicating a localised BOCTAG signal. In contrast, the promiscuous MOE reagent Ac_4ManNAlk was non-specifically incorporated throughout the entire co-culture (Fig. 4B, C). Taken together, BOCTAG enables cell-specific tagging of cell surface glycoproteins in co-culture.

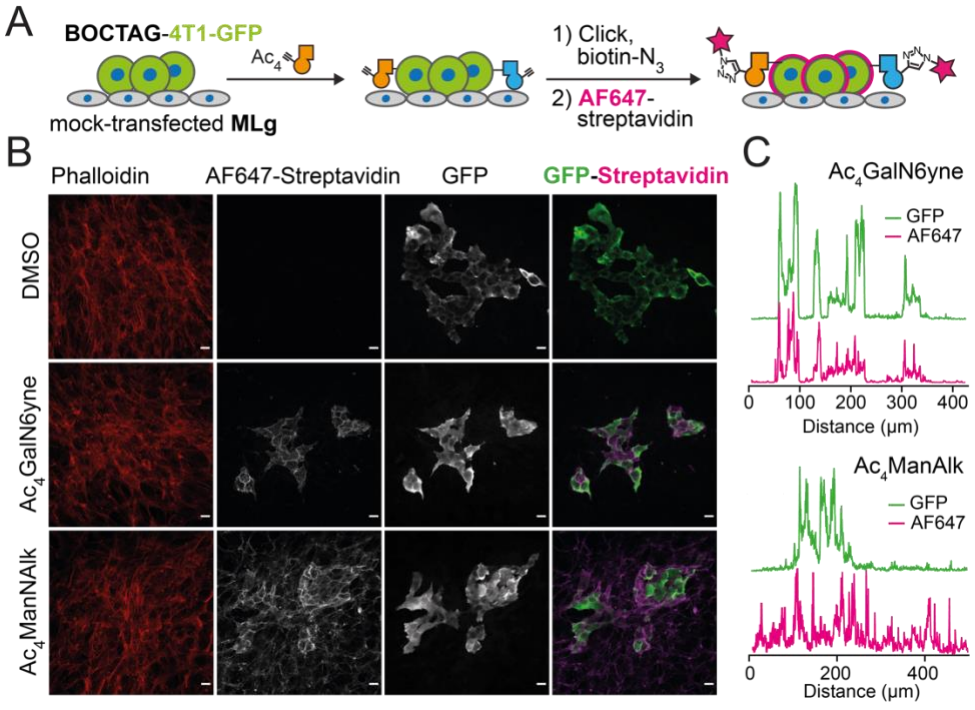

Fig. 4: Bioorthogonal cell-specific glycoprotein tagging in co-colture. *A*, schematic of the 4T1-MLg co-culture experiment. GFP-tagged 4T1 cells transfected with NahK/mut-AGX1 should be selectively positive for AF647-labelling in BOCTAG. *B*, fluorescence microscopy, using co-cultures fed with 50 μM Ac_4GalN6yne or 50 μM Ac_4ManNAlk as well as phalloidin as a counterstain. *C*, intensity profile of fluorescent signal between GFP and AF647 in Ac_4GalN6yne- (top) or Ac4ManNAlk-fed (bottom) co-cultures.

DISCUSSION

Our incentives to develop BOCTAG were twofold. First, there is still an unmet need for chemical tagging of proteins produced by a particular cell type. While glycans are a means to an end in this respect, the large signal-to-noise ratio in our fluorescent labelling experiments paired with the absence of toxicity in MOE indicates that our BOCTAG is complementary to other techniques, including the use of unnatural amino acids and proximity biotinylation.[30,31] Second, directly incorporating glycans in the analysis will give insight into cell-type-specific glycosylation sites and glycan structures to add another dimension to cell-type-specific glycoproteome profiling. A metabolic AND gate was necessary to ensure minimal background labelling while being able to supply the tagged monosaccharide as an easy-to-synthesise MOE reagent, which is in marked difference to highly unstable caged sugar-1-phosphates used previously.[19,21] To enable BOCTAG, cells require transfection with at least two transgenes. However, the design of a bicistronic, transposase-responsive plasmid ensures that transfection efforts are straightforward. BOCTAG will be used to profile the cell-specific glycoproteome in complex co-culture model systems.

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