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Supporting Information

Visualization of Protein-Specific Glycosylation inside Living Cells

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Supporting Information

Supporting Figures S1 – S11 and Table S1
Experimental section

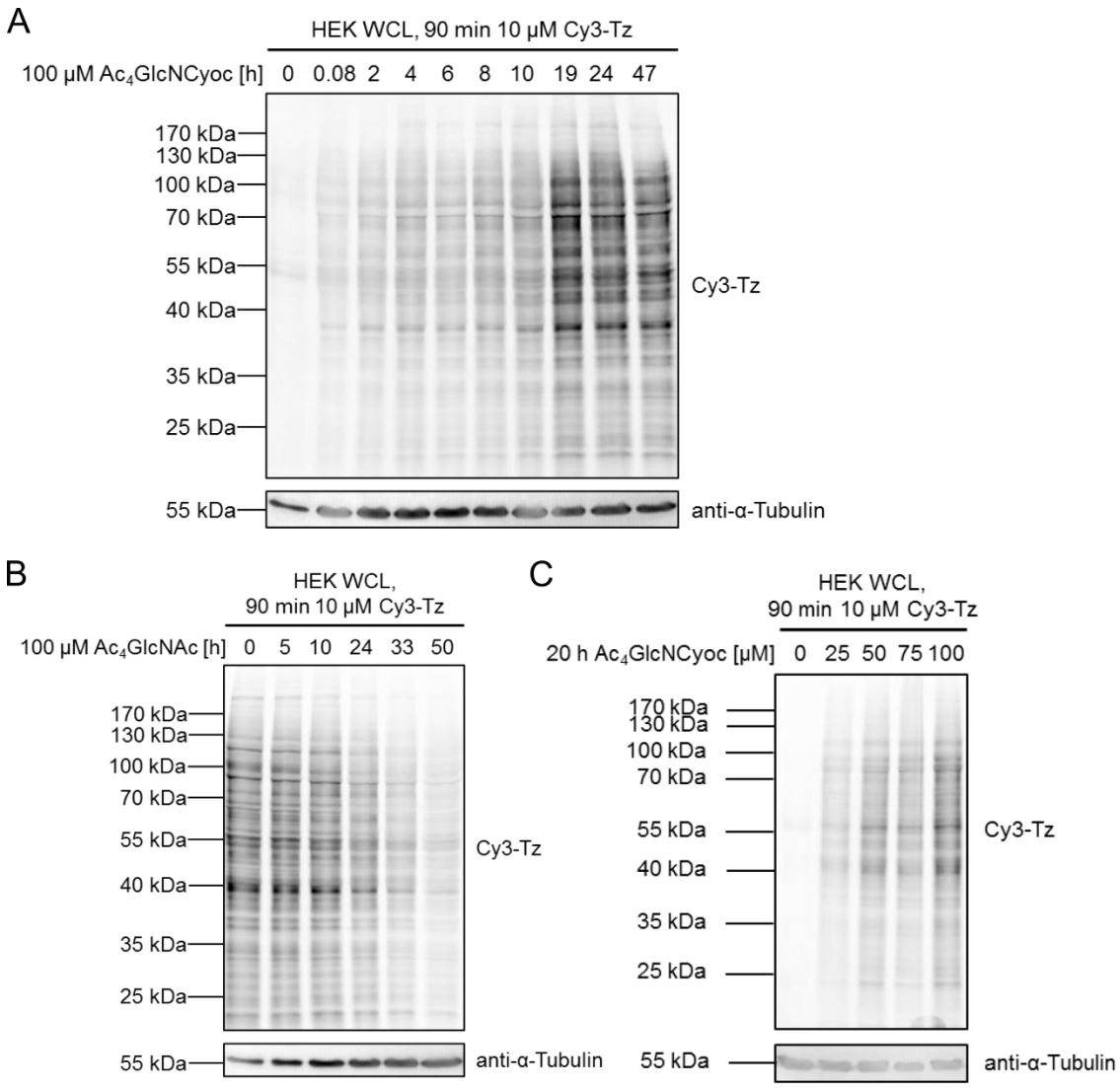


Figure S1. Incorporation of Ac_4GlcNCyoc into the glycome of HEK293T cells is maximal at 100 µM for 20 h. (A) Incorporation kinetics were investigated by treating HEK293T cells with 100 µM Ac_4GlcNCyoc for different periods of times. (B) To investigate degradation, cells were treated with 100 µM Ac_4GlcNCyoc for 20 h, washed and subsequently treated with 100 µM Ac_4GlcNAc for different periods of times. (C) Optimal concentration was assessed by treating cells with different concentrations of Ac_4GlcNCyoc ranging from 0 to 100 µM for 20 h. Afterwards, cells were lysed and the DAinv reaction was performed with 10 µM Cy3-Tz for 90 min at room temperature. Fluorescence of Cy3 was recorded from Western blots. Equal loading was verified by blotting against α-Tubulin. WCL= whole cell lysate.

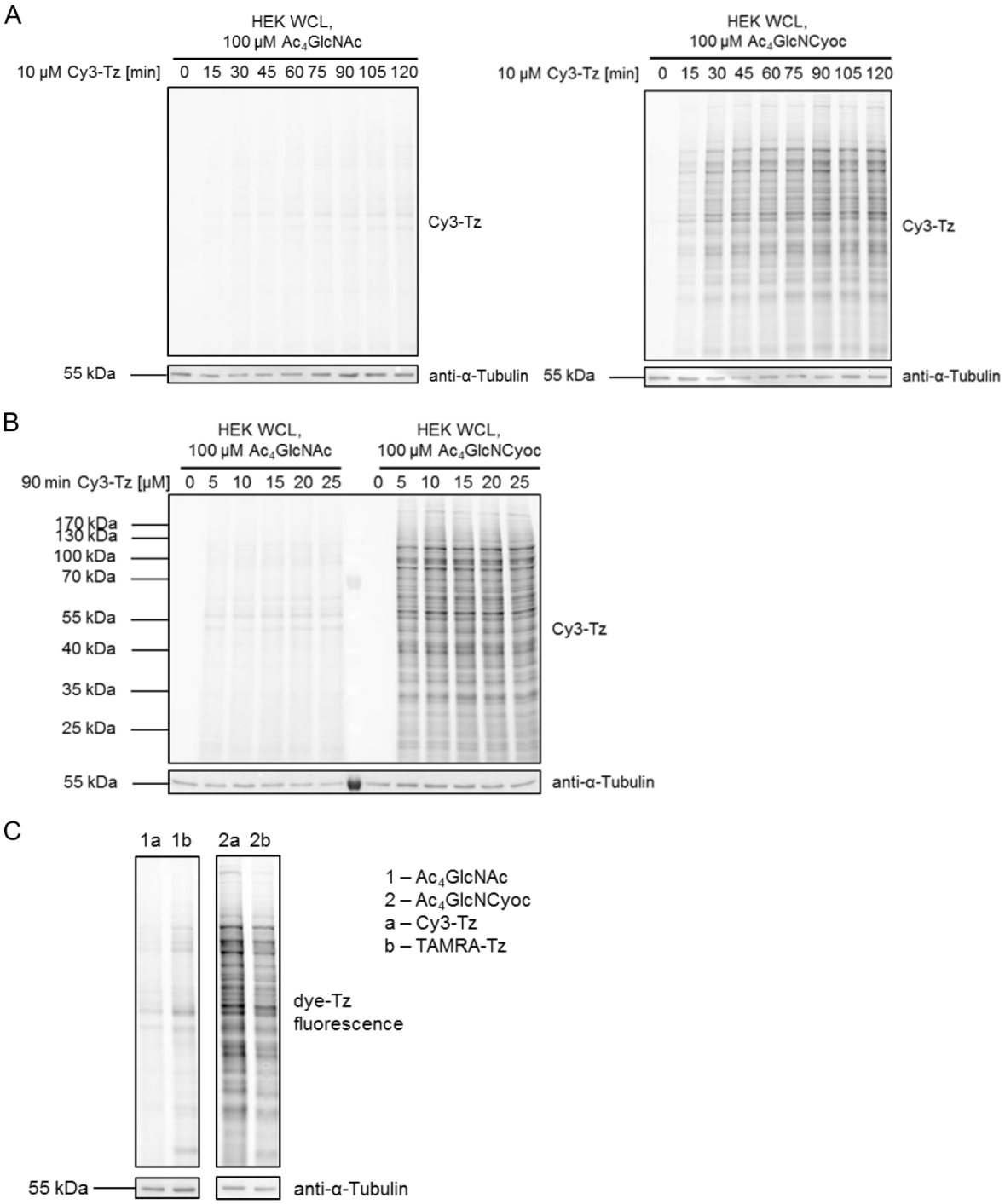


Figure S2. Labeling of Ac_4GlcNCyoc with dye-tetrazine conjugates in lysates of HEK293T cells is optimal at 10 µM for 90 min. Cells were treated with 100 µM Ac_4GlcNAc or 100 µM Ac_4GlcNCyoc for 20 h and lysed. (A) Labeling kinetics were followed by incubating cell lysates with 10 µM Cy3-Tz for the indicated times or (B) for 90 min with Cy3-Tz concentrations varying from 0 to 25 µM. (C) Cy3-Tz (a) and TAMRA-Tz (b) were compared by labeling lysates with 10 µM each for 90 min. TAMRA-Tz exhibits a slightly higher background signal compared to Cy3-Tz. Equal loading was verified by blotting against α-Tubulin.

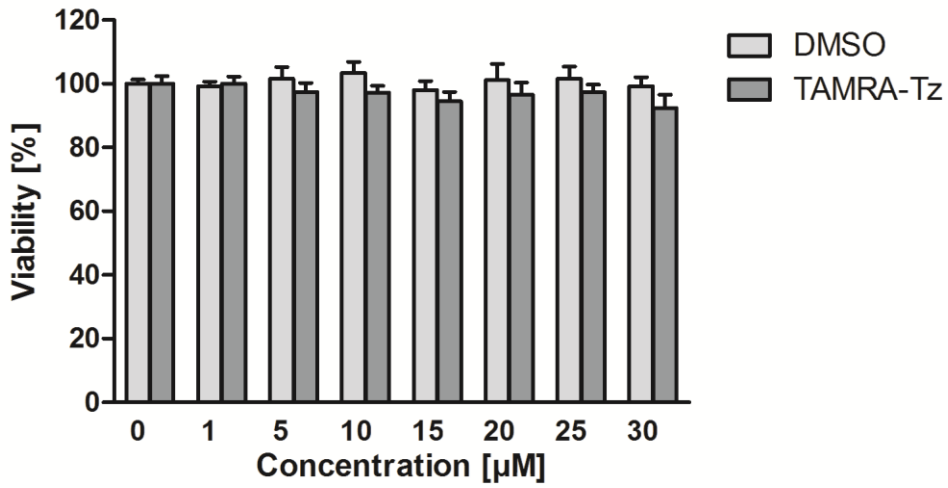


Figure S3. Impact of TAMRA-Tz on cellular viability. HEK293T cells were treated with 0 to 30 µM TAMRA-Tz or 0 to 0.3 V-% DMSO only for 1 h. Cell viability was assessed using an ATP assay. Columns show means of three independent experiments with four replicates each. Error bars represent standard errors of the means (SEM). TAMRA-Tz and DMSO have no influence on cellular viability in the tested concentration range.

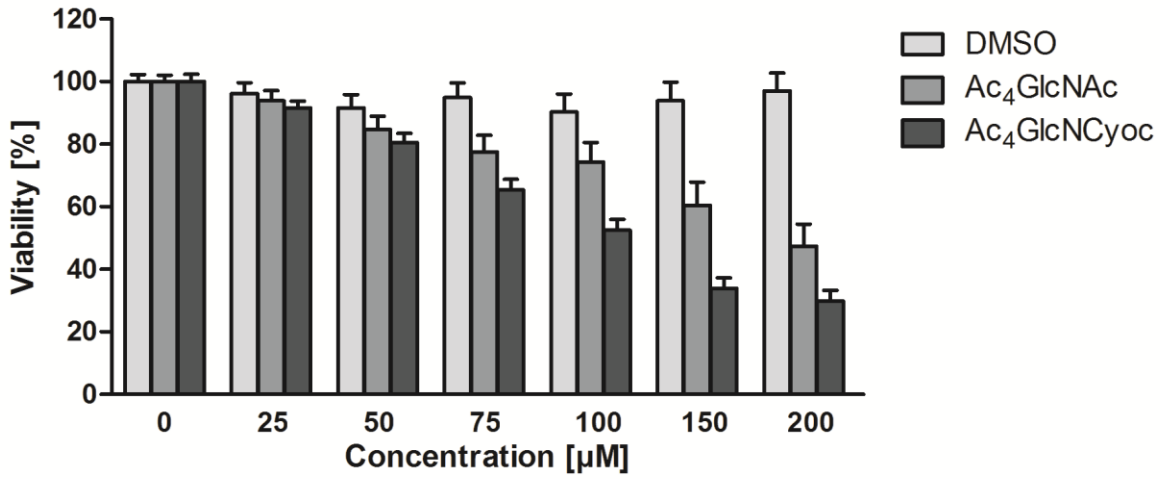


Figure S4. Impact of Ac_4GlcNCyoc and Ac_4GlcNAc on cellular viability. HEK293T cells were treated with 0 to 200 µM Ac_4GlcNCyoc, Ac_4GlcNAc or 0 to 0.2 V-% DMSO for 20 h. Viability was assessed using an AlamarBlue assay. Columns show means of three independent experiments with four replicates each. Error bars represent standard errors of the means (SEM). The EC50 values of Ac_4GlcNCyoc and Ac_4GlcNAc are 106 µM and 190 µM, respectively.

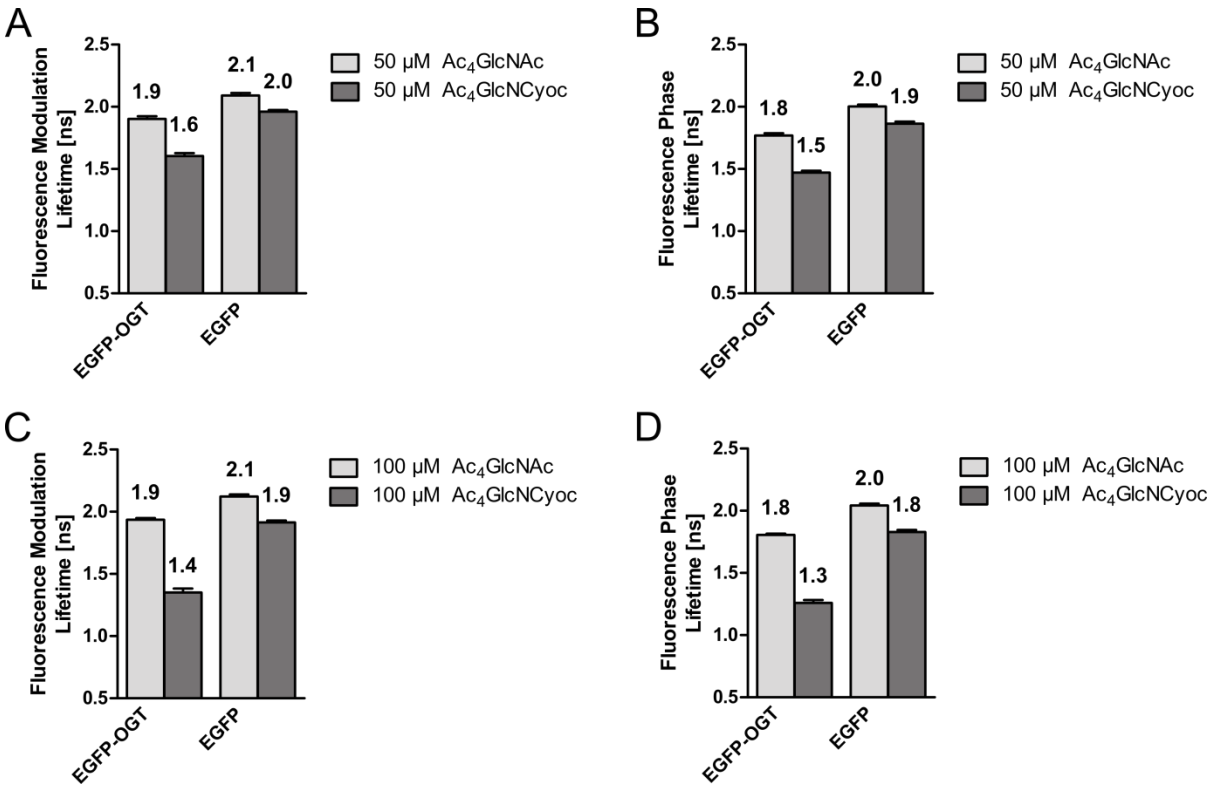


Figure S5. Fluorescence lifetimes of EGFP-OGT and EGFP measured in living cells. HEK293T cells were transfected with expression vectors for EGFP-OGT or EGFP, treated with Ac_4GlcNAc or Ac_4GlcNCyoc for 20 h, and incubated with 25 µM TAMRA-Tz for 60 min. Fluorescence lifetimes were calculated from three independent experiments with five cells each being imaged per experiment. Modulation and phase lifetimes obtained from cells treated with 50 µM sugar are presented in A and B, respectively. Modulation and phase lifetimes obtained from cells treated with 100 µM sugar are presented in C and D, respectively. Columns represent means (values in ns) and error bars standard errors of the means (SEM).

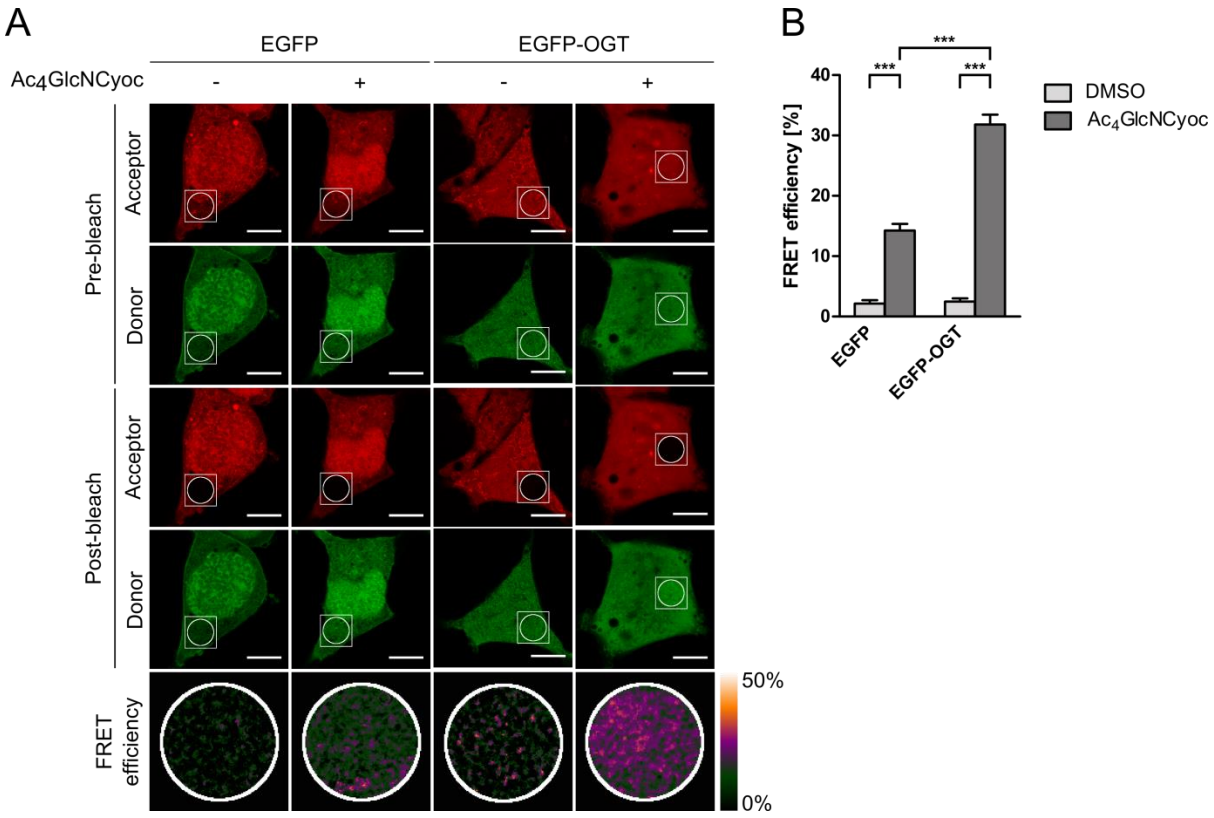


Figure S6. FRET in fixed cells assessed by acceptor photobleaching. HEK293T cells were transfected with expression vectors for EGFP or EGFP-OGT, treated with 0.1 V-% DMSO (-) or 100 µM Ac_4GlcNCyoc (+) for 18-20 h, fixed, permeabilized, and incubated with 10 µM Cy3-Tz for 60 min. Acceptor photobleaching was carried out and FRET efficiencies were calculated as described in the Experimental Section. (A) Representative confocal images show EGFP/EGFP-OGT (donor) and Cy3 (acceptor) fluorescence signals before and after photobleaching the acceptor. FRET efficiencies are depicted in the lowest panel. Scale bars: 10 µm. A high FRET efficiency was only observed in EGFP-OGT-transfected cells treated with Ac_4GlcNCyoc. (B) Three independent experiments with five cells each were performed. For quantitative analysis, FRET efficiencies were calculated in images from these measurements. Columns represent means and error bars standard errors of the means (SEM). Statistical significance was calculated with a One-Way ANOVA and the Tukey-Kramer posttest. The degree of significance is *** $p < 0.001$.

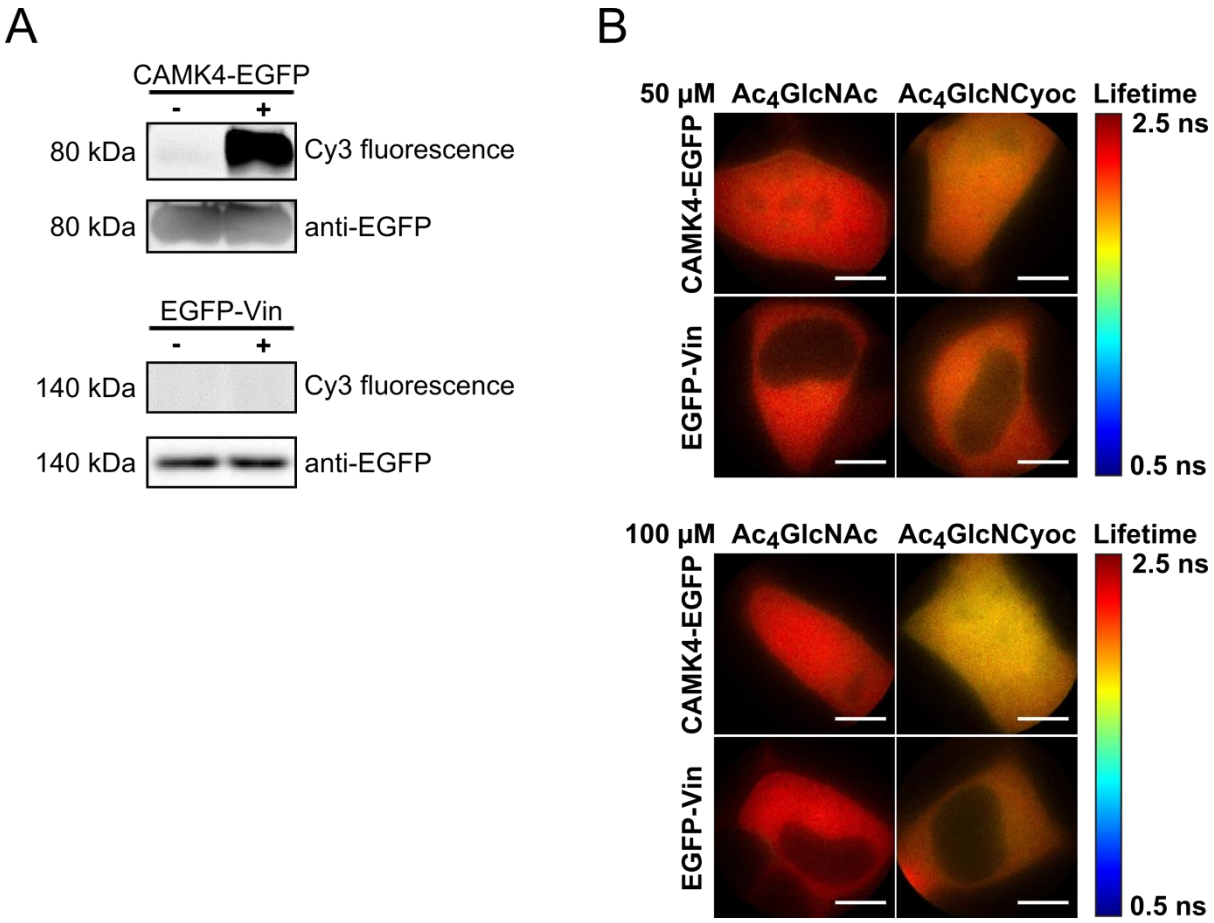
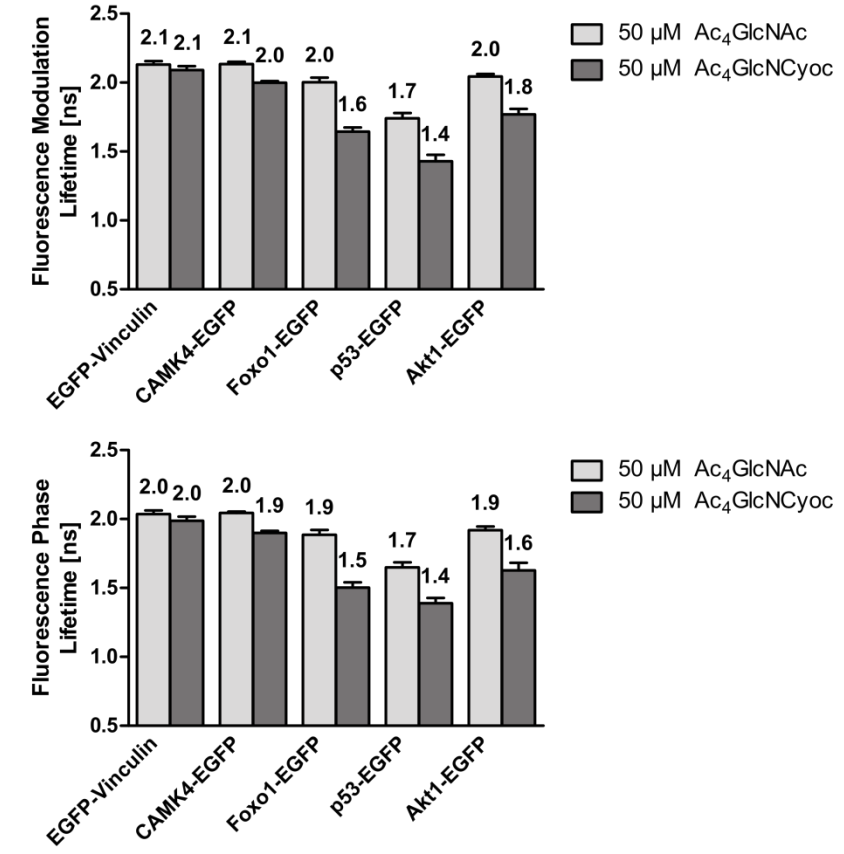


Figure S7. EGFP-Vinculin and CAMK4-EGFP show only slight reductions in fluorescence lifetimes. (A) HEK293T cells were transfected with vectors for EGFP-Vinculin or CAMK4-EGFP. 30 h later, cells were treated with 100 µM Ac_4GlcNAc (-) or Ac_4GlcNCyoc (+) for 20 h. They were lysed and immunoprecipitates against GFP were performed. Cy3 fluorescence was only detected for CAMK4-EGFP treated with Ac_4GlcNCyoc. Equal loading was verified by blotting against GFP. (B) After transfection and treatment with 50 µM or 100 µM Ac_4GlcNAc or Ac_4GlcNCyoc, cells were treated with 25 µM TAMRA-Tz for 1 h and live-cell FLIM-FRET microscopy was assessed. Modulation lifetime images are shown. Scale bars: 10 µm.

A


Fluorescence Modulation Lifetime [ns]
2.5
2.0
1.5
1.0
0.5
2.1 2.1
2.1 2.0
2.0 1.6
1.7 1.4
2.0 1.8
EGFP-Vinculin
CAMK4-EGFP
Foxo1-EGFP
p53-EGFP
Akt1-EGFP
50 µM Ac4GlcNAc
50 µM Ac4GlcNCyoc
Fluorescence Phase Lifetime [ns]
2.0 2.0
2.0 1.9
1.9 1.5
1.7 1.4
1.9 1.6
EGFP-Vinculin
CAMK4-EGFP
Foxo1-EGFP
p53-EGFP
Akt1-EGFP
50 µM Ac4GlcNAc
50 µM Ac4GlcNCyoc

B

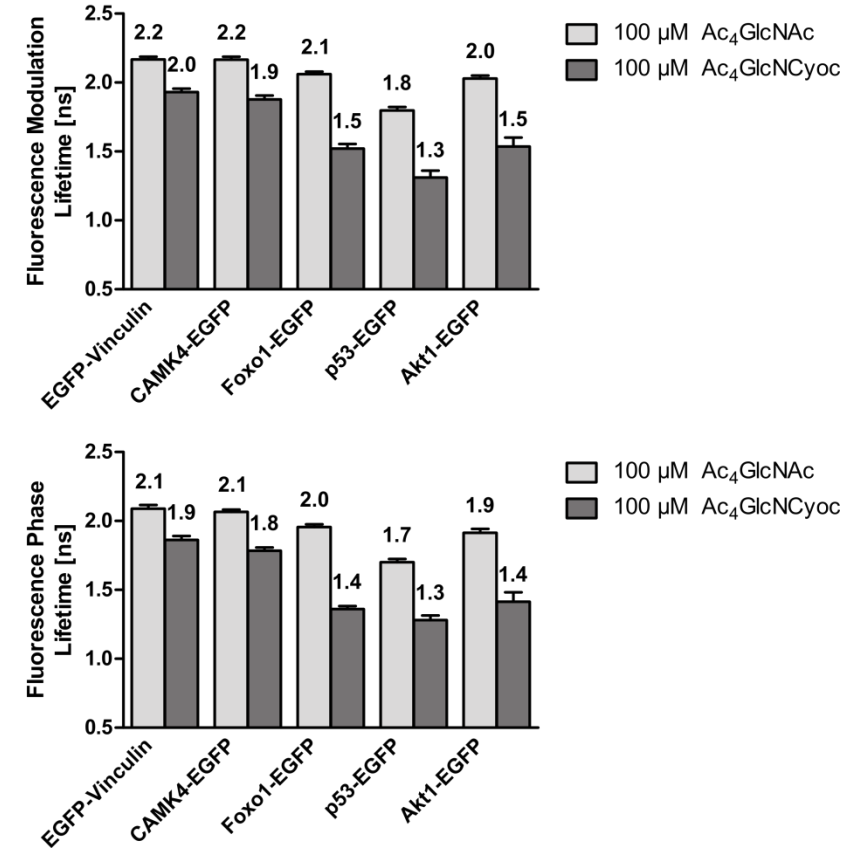

Fluorescence Modulation Lifetime [ns]
2.5
2.0
1.5
1.0
0.5
2.2 2.0
2.2 1.9
2.1 1.5
1.8 1.3
2.0 1.5
EGFP-Vinculin
CAMK4-EGFP
Foxo1-EGFP
p53-EGFP
Akt1-EGFP
100 µM Ac4GlcNAc
100 µM Ac4GlcNCyoc
Fluorescence Phase Lifetime [ns]
2.1 1.9
2.1 1.8
2.0 1.4
1.7 1.3
1.9 1.4
EGFP-Vinculin
CAMK4-EGFP
Foxo1-EGFP
p53-EGFP
Akt1-EGFP
100 µM Ac4GlcNAc
100 µM Ac4GlcNCyoc

Figure S8. Fluorescence lifetimes of EGFP-Vinculin, CAMK4-EGFP, Foxo1-EGFP, p53-EGFP, and Akt1-EGFP measured in living cells. HEK293T cells were transfected with constructs for EGFP-Vinculin, CAMK4-EGFP, Foxo1-EGFP, p53-EGFP or Akt1-EGFP, treated with Ac_4GlcNAc or Ac_4GlcNCyoc for 20 h, and incubated with 25 µM TAMRA-Tz for 60 min. Fluorescence lifetimes were calculated from three independent experiments with five cells being imaged per experiments. Modulation and phase lifetimes obtained from cells treated with 50 µM and 100 µM sugar are presented in A and B, respectively. Columns represent means (values in ns) and error bars standard errors of the means (SEM).

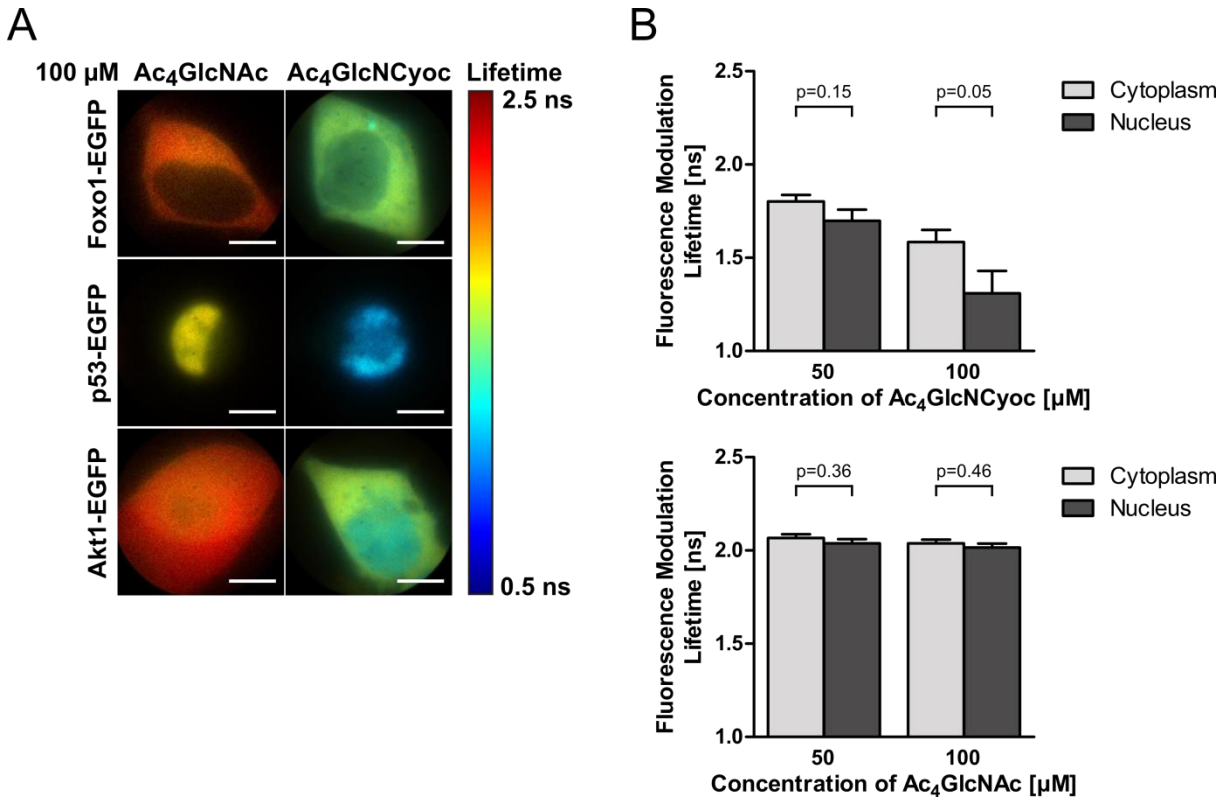


Figure S9. For Foxo1-EGFP, p53-EGFP- and Akt1-EGFP significant reductions in fluorescence lifetimes were observed. (A) HEK293T cells were transfected with vectors for Foxo1-EGFP, p53-EGFP and Akt1-EGFP. 30 h later, cells were treated with 100 µM Ac_4GlcNAc or Ac_4GlcNCyoc. After 20 h, 25 µM TAMRA-Tz was added for 1 h and live-cell FLIM-FRET microscopy was performed. Modulation lifetime images are shown. Scale bars: 10 µm. (B) For Akt1-EGFP, fluorescence lifetimes were measured in the cytoplasm and the nucleus for all Ac_4GlcNCyoc- and Ac_4GlcNAc-treated cells. The location of the nucleus was determined based on the fluorescence intensity and fluorescence lifetime of Akt1-EGFP. Columns represent means ± standard errors of the means (SEM). P-values were determined using two-tailed t-tests for unpaired observations.

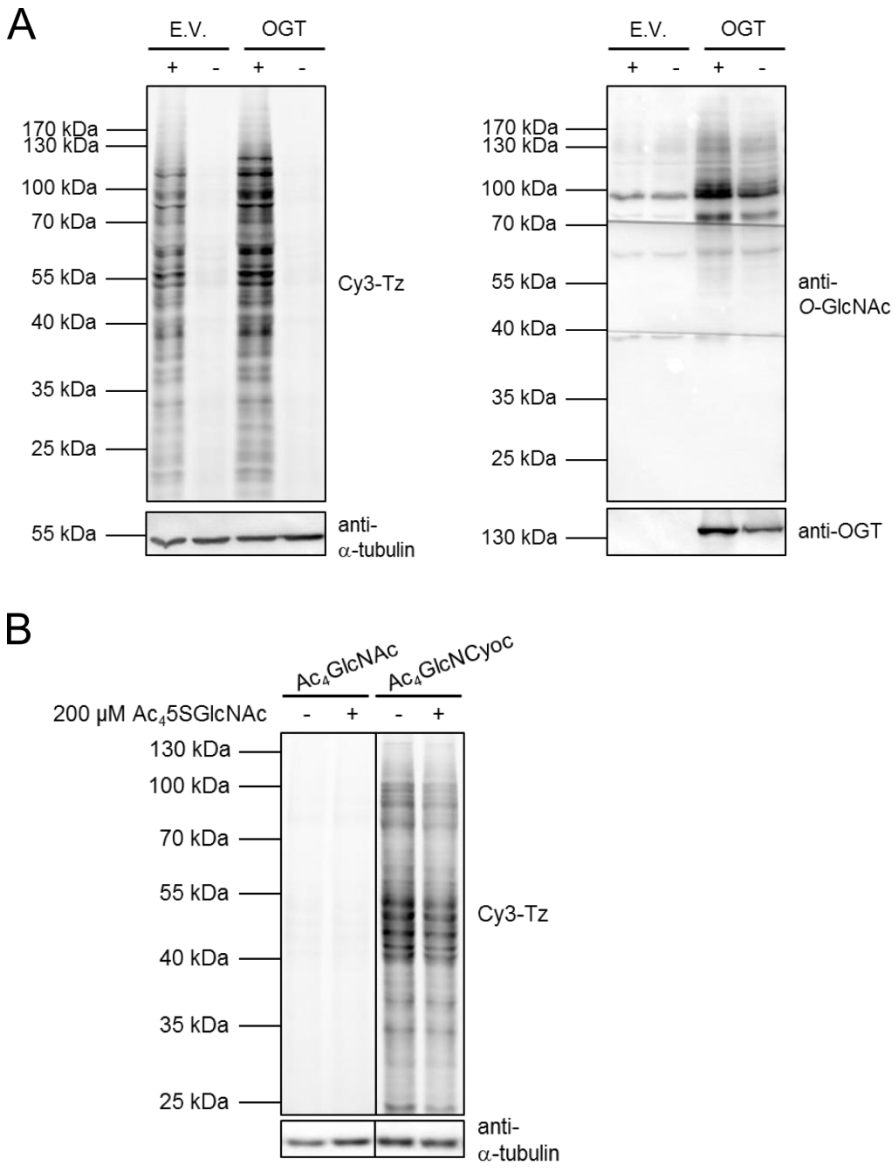


Figure S10. Incorporation of Ac_4GlcNCyoc depends on OGT activity. (A) HEK293T cells were transfected with an expression vector for an empty vector (E.V.) or EGFP-OGT (OGT). 30 h later, cells were treated with 100 µM Ac_4GlcNCyoc (+) or 0.1 V-% DMSO (-) for 20 h. Subsequently, cells were lysed. Lysates were incubated with 10 µM Cy3-Tz for 90 min. Overexpression of EGFP-OGT was confirmed by immunoblotting against OGT. OGT overexpression increased cellular *O*-GlcNAc levels and also Cy3-fluorescence. Equal loading was verified by blotting against α-tubulin. (B) HEK293T cell lysates were treated with 100 µM Ac_4GlcNAc or Ac_4GlcNCyoc with (+) or without (-) the addition of 200 µM $Ac_4$5SGlcNAc for 20 h at 37 °C. Subsequently, DAinv reaction with 10 µM Cy3-Tz was performed for 90 min at room temperature. Fluorescence of Cy3 was recorded from Western blots. $Ac_4$5SGlcNAc reduces incorporation of Ac_4GlcNCyoc. Equal loading was verified by blotting against α-tubulin.

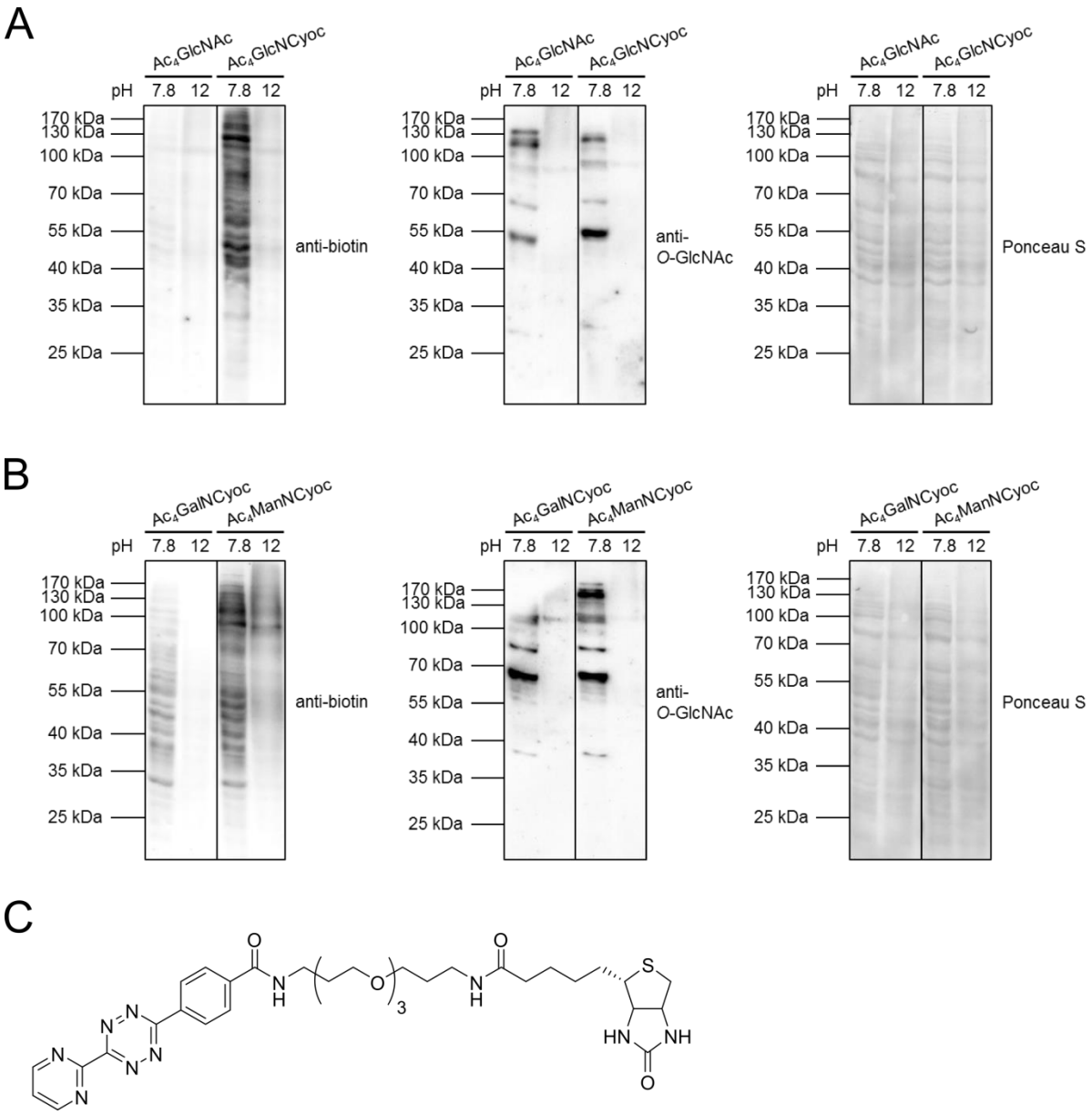


Figure S11. Ac_4GlcNCyoc is attached to serine and threonine residues. (A) HEK293T cells were treated with 100 µM Ac_4GlcNAc or Ac_4GlcNCyoc for 20 h and subsequently lysed. Lysates were incubated with 150 µM biotin-Tz for 90 min. Then pH was changed from 7.8 (lysis buffer) to 12 by addition of NaOH to a final concentration of 55 mM. *O*-GlcNAc as well as biotin signal are removed at pH 12. Equal loading of samples was verified by protein staining with Ponceau S. (B) Control experiments were performed with 100 µM Ac_4GalNCyoc[1] and Ac_4ManNCyoc[2]. Base-induced β-elimination only occurs with *O*-linked glycans whereas *N*-linked glycans are not cleaved under these conditions.[3] Consistent with recent finding suggesting that Ac_4GalNCyoc is incorporated in *O*-glycans,[1,4] the biotin signal of Ac_4GalNCyoc-treated cells vanishes at pH 12. For Ac_4ManNCyoc, the biotin signal is only partially reduced at pH 12. This is in line with previous findings showing that mannosamine derivatives are incorporated into sialoglycans that can be either *O*-linked to serine/threonine or *N*-linked to asparagine.[5] (C) Chemical structure of biotin-Tz.

Protein	Localization	Number of amino acids	*O*-GlcNAc-site(s)	EGFP-Tag	Function
OGT	N+C[6]	1046	(S3, S4)[7]	N-terminal	O-linked N-acetylglucosamine (GlcNAc) transferase[8]
Forkhead box protein O1 (Foxo1)	(N+)C[9]	655	T317[10], S333[11], S550[10], T646[12], T648[10], S654[10]	C-terminal	Transcription factor and main target of insulin signaling, regulates metabolic homeostasis in response to oxidative stress[13]
Akt1	C+N+M[14]	480	(S126, S129, T305, S312)[15], S473[16]	C-terminal	Serine/threonine kinase and regulator of glucose metabolism, cell proliferation, apoptosis, transcription and cell migration[17]
p53	N[18]	393	S149[19]	C-terminal	Tumor suppressor and regulator of cell cycle and apoptosis[20]
Calcium/ calmodulin-dependent protein kinase type IV (CAMK4)	C+N[21]	473	(T57/S58, S137, S189, S344/S345, S356)[22]	C-terminal	Member of the CaMKK-CaMK4 signaling cascade and regulator of several transcription activators in immune response, inflammation, and memory consolidation[23]
Vinculin	C [24]	1134	sites unknown [25]	N-terminal	Cytoplasmic actin-binding protein[26]

Table S1. Information on localization, size, function, and *O*-GlcNAc-sites of proteins examined within this study. N=nucleus, C=cytoplasm, M=membrane.

Experimental section

Cell culture and transfection

The human embryonic kidney cell line 293T (HEK293T cells) was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin at 37 °C and 5% CO_2. The cell line was authenticated by STR profiling at the Leibniz-Institut DSMZ, Braunschweig, Germany. Mycoplasma contamination was negatively tested using the Venor®GeM Classic Kit (Minerva Biolabs). The cells were transfected with expression vectors by standard calcium phosphate co-precipitation using a total amount of 2-8 µg plasmid DNA per 10 cm culture dish as described previously.[27]

Plasmids

The mammalian expression vector pcDNA3.1 (empty vector) was purchased from Invitrogen-Life technologies (Thermo Fisher Scientific), pEGFP-C1-loxP (EGFP) from Clontech. Human *O*-GlcNAc transferase DNA was amplified by polymerase chain reaction (PCR) from pOTB7-hOGT (obtained from RZPD, Berlin, Germany) with the following primers: 5'-GAAGTTATCAGTCGACGCGTCTTCCGTGGGCAACGTG-3' and 5'-ATGGTCTAGAAAGCTTGTTTATGCTGACTCAGTGACTTCAACAGGC-3'. The coding sequence was inserted into pDNR-dual MCS via SalI/HindIII to yield pDNR-dual hOGT. The cDNA was subsequently transferred into pEGFP-C1-loxP by Cre-mediated recombination to yield pEGFP-hOGT. The pEGFP-N1-Foxo1 plasmid was a gift from Domenico Accili (Addgene plasmid # 17551).[9] pLPS-3'EGFP Akt1 (HsCD00026041) and pLPS-3'EGFP CAMK4 (HsCD00026148) were obtained from the PlasmID Repository at Harvard Medical School.[28] Human p53 DNA was amplified by polymerase chain reaction (PCR) from p53 plasmid (kindly provided by Martin Scheffner, University of Konstanz).[29] The following primers were used: 5'-TAAGCAGTCGACATGGAGGAGCCGCAGTCA-3' (forward primer) and 5'-TGCTTAGGATCCAAGTCTGAGTCAGGCCCTTCTGT-3' (reverse primer). The coding sequence was inserted into pEGFP-N1 (Clontech) via BamHI/SalI restriction sites to yield pEGFP-N1-p53. EGFP-Vinculin plasmid was kindly provided by Kris DeMali (Carver College of Medicine, University of Iowa).[24]
Complete plasmid sequences are available upon request.

Reagents and antibodies

Rhodamine 6G was from Radiant Dyes Laser & Accessories GmbH, coumarin 6 from Sigma Aldrich, TAMRA-Tz from Jena Bioscience GmbH and Ac_4GlcNAc from Sigma Aldrich. Ac_4GlcNCyoc, Ac_4GalNCyoc, Ac_4ManNCyoc, and Cy3-Tz were synthesized as previously described.[1-2] Stock solutions for Ac_4GlcNAc, Ac_4GlcNCyoc, Ac_4GalNCyoc, and Ac_4ManNCyoc were 100 mM in DMSO, for Cy3-Tz 5 mM in PBS, and for TAMRA-Tz 10 mM in DMSO. PUGNAc was purchased from Sigma Aldrich, DNAse I from Thermo Scientific, protease inhibitors cOmplete™ EDTA-free from Roche, Protein A/G PLUS-Agarose from Santa Cruz Biotechnology and GFP-Trap®_A from chromotek. $Ac_5$5SGlcNAc was a gift from David Vocadlo.[30] Biotin-Tz was synthesized as described.[31] AlamarBlue Cell Viability Reagent was obtained from Thermo scientific, CellTiter-Glo® Luminescent Cell Viability Assay from Promoega. Anti-α-tubulin antibody AA4.3 was prepared from hybridoma supernatant.[1] Monoclonal mouse antibody against GFP (clone JL-8) was from Clontech. Anti-OGT antibody (HPA030751) was purchased from Sigma. Anti-*O*-GlcNAc antibody RL2 was purchased from life technologies. Anti-biotin antibody was from Abnova. The secondary horseradish-peroxidase-conjugated anti-mouse antibody was purchased from Dianova (goat anti-mouse igG (H+L)-HRO), the secondary horseradish-peroxidase-conjugated anti-rabbit antibody was purchased from Sigma Aldrich (goat anti-rabbit).

Viability tests

AlamarBlue Assay.
15000/well HEK293T cells were seeded in 96-well plates. 6 h later, cells were treated in quadruplicates with 0 - 200 µM Ac_4GlcNAc, Ac_4GlcNCyoc or 0 - 0.2 V-% DMSO as control. After 20 h, AlamarBlue Cell Viability Reagent was added. Plates were incubated for 1 h at 37 °C and 5 % CO_2. Fluorescence was read out using the Synergy HT from Biotek with a 530/25 bandpass excitation filter and a 590/30 bandpass emission filter. Experiments were performed three times. EC50 values were determined by linear regression.

ATP Assay.
The AlamarBlue Viability Assay is an inexpensive, standard viability test and was therefore used for viability tests with Ac_4GlcNCyoc. However, it is not suitable in combination with TAMRA as it is based on the readout of resorufin fluorescence which spectrally overlaps with the TAMRA fluorescence. Hence, viability tests for TAMRA-Tz were performed with the CellTiter-Glo® Luminescent Cell Viability Assay according to the manufacture's protocol. 15000/well HEK293T cells were seeded in 96-well plates. 26 h later, cells were treated in quadruplicates with 0 - 30 µM TAMRA-Tz

or 0 - 0.3 V-% DMSO as control for 1 h. Luminescence was read out using the Synergy HT from Biotek. No emission filter was used. Signals were corrected for potentially occurring bioluminescent resonance energy transfer from Luciferin to TAMRA. Experiments were performed three times.

Immunoprecipitation and Western blotting

Cell lysis, immunoprecipitation and Western blotting were performed as described previously[1,32] and repeated at least once. 1 Million HEK293T cells were seeded in 10 cm dishes one day prior transfection. 30-32 h after transfection, cells were treated with 100 µM Ac_4GlcNCyoc or Ac_4GlcNAc for 20 h. Subsequently, cells were solubilized in 600 µl lysis buffer (0.5 V-% Triton X-100, 25 mM Tris pH 7.4, 300 mM NaCl, 5 mM EDTA, 20 mM beta-glycerophosphate, 20 mM NaF, 0.3 mM NaV, 10 U/ml DNAse I, 1x protease inhibitors, 100 µM *O*-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) *N*-phenylcarbamate (PUGNAc)). The lysates were cleared by centrifugation (30 min at 14550 g). 20 µl of the supernatant were incubated with 10 µM Cy3-Tz for 90 min at 25 °C under constant shaking. SDS sample buffer was added and samples were incubated for 10 min at 98 °C. They were stored at -25 °C until usage.
For immunoprecipitation, 500 µl of the lysate were incubated with 3 µg of anti-GFP antibody for 4 h at 4 °C with overhead rotation. Antibody complexes were recovered by addition of 25 µl Protein A/G PLUS-Agarose beads for 1 h at 4 °C. Subsequently, the beads were washed 3 times with Triton buffer (1 V-% Triton X-100, 50 mM Hepes pH 7.4, 150 mM NaCl, 10 V-% Glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM Na-pyrophosphate, 100 mM NaF, 1 mM Na-orthovanadate, 1x protease inhibitors) by centrifugation at 8600 g for 2 min. After final removal of the supernatant, the remaining 20 µl of the sample containing the Agarose beads were incubated with 10 µM Cy3-Tz for 90 min at 25 °C under constant shaking. SDS sample buffer was added and samples were incubated for 10 min at 98 °C. They were stored at -25 °C until usage.

Foxo1-EGFP and CAMK4-EGFP were immunoprecipitated using GFP-Trap®_A. First, 10 µl of GFP-Trap®_A bead slurry was diluted with 500 µl wash buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) and centrifuged for 2 min at 3000 g. The supernatant was discarded. This step was repeated twice. Lysates of HEK293T cells transfected with expression vectors for Foxo1-EGFP or CAMK4-EGFP and treated with either 100 µM Ac_4GlcNCyoc or Ac_4GlcNac were diluted with wash buffer to reduce Triton X-100 concentration to 0.2 V-% and added to the beads. The mixture was incubated for 60 min at 4 °C under constant shaking. Following, beads were sedimented by centrifugation (3000 g for 2 min). They were washed three times with 500 µl wash buffer. After final removal of the supernatant, the remaining 30 µl of the sample containing the GFP-Trap_A beads were incubated with 10 µM Cy3-Tz for 90 min at 25 °C under constant shaking. SDS sample buffer was added and samples were incubated for 10 min at 98 °C. They were stored at -25 °C until usage.

Proteins were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels and transferred to nitrocellulose membranes (BioRad). Transfer efficiency and equal loading was analyzed with Ponceau S staining. The Cy3 or TAMRA fluorescence was detected with the Typhoon FLA 9500 Fluorescence Imager (GE Healthcare, Life Sciences) using a 575 nm long pass filter at 532 nm excitation. Afterwards, the membranes were blocked in 5% milk in PBS with 0.5 V-% Tween 20 (PBS-T) for 1 h at room temperature followed by incubation with primary antibodies in 2% BSA in TBS containing 0.05% Tween 20 and 0.05% sodium azide (anti-GFP: 1:3000, anti-α-tubulin: 1:200, anti-*O*-GlcNAc: 1:1000, anti-OGT: 1:1000, anti-biotin 1:2000) overnight at 4 °C. The membranes were washed 3 times with PBS-T for 10 min each, incubated with secondary horseradish-peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:3000 in milk, 1 h, room temperature) and washed again 3 times with PBS-T for 10 min each. The blots were developed by an ECL detection system (clarity Western ECL substrate, BioRad) and visualized using the ChemiDoc™ Touch Imaging System (BioRad).

FLIM of mammalian cells

HEK293T cells were transfected with mammalian expression vectors for EGFP-fusion proteins and seeded in 8-well ibiTreat µ-Slides (ibidi) (50000 cells/cm²). 6h later, cells were treated with Ac_4GlcNAc or Ac_4GlcNCyoc for 20 h. Cells were washed with PBS and DAinv reaction in living cells was performed by addition of 25 µM TAMRA-Tz. After 60 min, cells were washed with PBS and phenol red-free DMEM containing 10 µM HEPES was added.
FLIM was performed using the wide-field frequency domain approach. For measurements in the frequency domain, an intensity modulated continuous wave laser is used to excite the fluorophore at a MHz frequency ω.[33] This results in a modulated emission signal with a different phase and modulation depth as compared to the excitation light. Both phase shift ($\varphi_{em} - \varphi_{ex}$) and relative demodulation $\frac{M_{ex}}{M_{em}}$ are used to calculate the fluorescence lifetime at each pixel of an image.
For mono-exponential decays, modulation and phase lifetimes are theoretically equal and can be calculated as

(1) $\tau_\varphi = \frac{\tan(\varphi_{em}-\varphi_{ex})}{\omega}$

(2) $\tau_M = \frac{1}{\omega}\sqrt{(\frac{M_{ex}}{M_{em}})^2 - 1}.$

Most samples of interest display more than one decay time. In this case the lifetimes calculated from the phase shift and relative demodulation measured at a particular frequency are only apparent values and are the result of a complex weighting of various components in the emission. This is also the case, when the fluorescence decay becomes biexponential due to FRET, and explains why phase and modulation lifetimes can differ.[34] The imaging system was based on a DMI 6000B inverted microscope (Leica) equipped with an oil immersion objective lense (100x, 1.4 NA, PL APO, Leica). The microscope was equipped with a heating stage connected to a temperature controller allowing for the maintenance of living biological samples at 37 °C (Tempcontrol 37-2 digital, Pecon). The PI-MAX4:1024i RF CCD camera coupled to a GenIII intensifier (Princeton Instruments) was used as detector. 12 phase images with an exposure time of 100 ms each were acquired in a random recording order to suppress photobleaching induced artifacts.[35] 10 exposures per phase image were averaged resulting in a total exposure time of 1000 ms per phase image. A LDM488.20.A350 diode laser (Omicron) was modulated at 70 MHz and used to excite the donor EGFP. A laser clean up filter ZET488/10 (Chroma) was used as excitation filter. For homogenous illumination of the sample, the laser light was coupled into the microscope via a shaken 0.25 NA multi-mode fiber (Thorlabs), and passed onto the sample with a QuadLine zt440/488/560/635rpc dichroic mirror (Chroma). The donor fluorescence was separated using a 525/50 BrightLine HC bandpass filter (Semrock) and a 515/30 ET bandpass filter (Chroma). No bleedthrough of Cy3-Tz/TAMRA-Tz was detected in the donor channel. The acquisition parameters were the following: Intensifier gain: 90x, bit depth: 16 bit, pixel size: 60 nm x 60 nm, image dimensions: 30.72 µm x 30.72 µm (512 x 512 pixel after 2 x 2 binning).

The performance of the setup was tested using a series of rhodamine 6G solutions in water quenched by KI as previously described.[36] Before the experiment, the reference was calibrated daily by averaging 3 lifetime measurement of a 100 µM solution of coumarin 6 in ethanol with a known lifetime of 2.5 ns.[34] The reference measurement was verified by measuring a 10 µM solution of rhodamine 6G in water with a known lifetime of 3.9 ns (http://www.iss.com/resources/reference/data_tables/LifetimeDataFluorophores.html).[37] The lifetimes of the reference solutions were confirmed by TCSPC measurements. All acquisition parameters were kept constant between reference and sample measurements. The raw images were background-corrected and sorted immediately after acquisition with the Lightfield acquisition software (Princeton Instruments). Lifetime data were analyzed using SimFCS 3.0 developed at the Laboratory for Fluorescence Dynamics (http://www.lfd.uci.edu/globals/, University of California, Irvine, USA).[38] Modulation lifetime images were calculated from the phase sequence using Matlab macros (MATLAB version R2013a, The MathWorks Inc.). These Matlab scripts were developed at the Department of Chemistry, Ludwig-Maximilians-Universität München, Munich, Germany. Correction of the irising effect was performed using a reference measurement of a homogenous fluorophore solution. For the representation of modulation lifetime images, a 2 x 2 moving average filter was applied. The jet colormap was used for false-color representation with the hue corresponding to the lifetime and the brightness to the intensity.

For calculation of the apparent FRET efficiencies E_{app}, fluorescence modulation lifetimes (τ) were averaged over single cells and the mean value was determined from 15 cells in total. Three independent experiments were performed with 5 cells being imaged per experiment. E_{app} was calculated from the averaged lifetime values as

(3) $E_{app} = \left(1 - \frac{\tau_{Ac_4GlcNCyoc}}{\tau_{Ac_4GlcNAc}}\right) \cdot 100\%.$

Confocal microscopy and acceptor photobleaching experiments of fixed mammalian cells

HEK293T cells (50000/cm²) were seeded in 8-well ibiTreat µ-Slides (ibidi) 22-24 h after transfection. The wells had been coated overnight at 4 °C with Poly-L-Lysine (33 µg/ml) and Fibronectin (2 µg/ml). 4 h later the cells were incubated for 18-20 h with 100 µM Ac_4GlcNCyoc containing medium. Medium without Ac_4GlcNCyoc was added as negative control. The cells were washed with PBS and fixed for 20 min with 4% paraformaldehyde in PBS. They were washed again with PBS and twice for 2 min with 50 mM ammoniumchloride in PBS. The cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min. Unspecific binding sites were blocked by washing twice with 3 wt-% BSA in PBS for 2 min before the cells were incubated with 10 µM Cy3-Tz for 60 min in PBS. Afterwards, the cells were washed once with PBS and twice with 3 wt-% BSA in PBS. PBS was added and the samples were stored at 4 °C in the dark. They were measured within 24 h at room temperature.

Fluorescence microscopy was performed on a TCS SP5 confocal laser scanning microscope (Leica) using an oil immersion objective lense (63 x, 1.4 NA, PLAPO, Leica) and the LAS AF (Leica) acquisition software. Acceptor photobleaching experiments were performed using the implemented FRET acceptor bleaching wizard of the Leica TCS SP5. Bleaching of the acceptor Cy3-Tz was performed to at least 20% of total Cy3 fluorescence with high laser intensity at 561 nm (two times with 25%). The bleaching area was kept constant with a diameter of 7 µm. Prebleach and postbleach images of the donor EGFP and the acceptor Cy3 were serially acquired with excitation at 488 nm (10%) and 561 nm (10%) using appropriate emission bands (500-550 nm, 570-630 nm). The acquisition parameters such as excitation intensity, line average, scan speed, and pixel dimensions were adapted to minimize acquisition bleaching (line average: 2, scanning speed: 200 lines/s, bit depth: 8 bit, pixel size: 68.7 nm x 68.7 nm, image dimensions: 70.3 µm x 70.3 µm (1024 x 1024 pixel)). The total fluorescence intensity within the bleached area was measured in the donor channel (IntD) before and after photobleaching the acceptor.[39] The apparent FRET efficiency was calculated as

$$(4)\quad \mathrm{E_{app}} = \left(1 - \frac{\mathrm{Int\ D_{prebleach}}}{\mathrm{Int\ D_{postbleach}}}\right) \cdot 100\% = (E_{FRET} \cdot \alpha).$$

The apparent FRET efficiency E_{app} is a product of the FRET efficiency E_{FRET} itself and the fraction α of molecules exhibiting FRET. The FRET efficiency E_{FRET} depends on the Förster Radius R_o of the respective FRET pair (donor-acceptor distance for which the FRET efficiency amounts to 50%) and on the distance r between the two fluorophores: $E_{FRET} = R_o^6/(R_o^6+r^6)$. The Förster-Radius for EGFP/TAMRA is R_0 = 5.8 nm and R_0 = 6.1 nm for EGFP/Cy3.
Donor and acceptor pre/postbleach images were processed using ImageJ (http://rsb.info.nih.gov/ij/, U.S. National Institutes of Health). The FRET images were calculated using the FRETcalc ImageJ plugin (http://rsb.info.nih.gov/ij/plugins/fret/fret-calc.html).[40] Pre/postbleach images of the donor channel were background corrected and subjected to 2 x 2 median filtering. An intensity threshold was applied to circumvent erroneous FRET efficiency calculations for pixels with very low intensity. The “blue-orange-icb” look-up-table was used for false-color representation of the FRET efficiencies.

β-Elimination

3 Million HEK293T cells were seeded in 10 cm dishes and treated with 100 µM Ac_4GlcNAc, Ac_4GlcNCyoc, Ac_4GalNCyoc, or Ac_4ManNCyoc 6 h later. After 20 h, cell lysates were prepared as described above. DAinv reaction was performed with 150 µM biotin-Tz for 90 min at room temperature.[1] The pH-value was adjusted to pH = 12 by the addition of 1 M NaOH to a final concentration of 55 mM.[3] An equal amount of water was added to control samples. Subsequently, lysates were incubated under constant mixing at 37 °C for 90 min. SDS sample buffer was added and samples were incubated for 10 min at 98 °C. They were stored at -25 °C until usage.

Statistics

For cell experiments, sample size was chosen to fit the assumptions of D'Agostino & Pearson omnibus normality test. Statistic calculations were performed with the GraphPad Prism 5.0 software. The confidence interval was set to 95% for all calculations. Pairwise data were not significantly different for p-values > 0.05 (ns), but significant with * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.
For Figures 2C and 4, statistical significance was calculated with a Two-Way ANOVA and a Bonferroni posttest for selected columns (all data compared to the ones of EGFP).
For Figure S6B, the data were transformed by extracting the square root to achieve normal distribution. The normal distribution of the transformed data was tested with D'Agostino & Pearson omnibus normality test. Bartlett’s test was applied to test for equal variances. Afterwards, statistical significance was calculated with a One-Way ANOVA and the Tukey-Kramer posttest.
For Figure S9B, normal distribution of data was tested with D'Agostino & Pearson omnibus normality test. P-values were determined using two-tailed t-tests for unpaired observations.

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