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Identifying potentially O-GlcNAcylated proteins using metabolic labeling, bioorthogonal enrichment, and Western blotting

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Abstract

O-GlcNAcylation is a widespread posttranslational modification of intracellular proteins. Phenotypic and genetic experiments have established key roles for O-GlcNAc in development, mammalian cell survival, and several human diseases. However, the underlying mechanisms by which this modification alters biological pathways are still being discovered. An important part of this discovery process is the discovery of O-GlcNAcylated proteins, where chemical approaches have been particularly powerful. Here we describe how to combine one of these approaches, metabolic chemical reporters (MCRs), with bioorthogonal chemistry and Western blotting to identify potentially O-GlcNAcylated proteins.

1. Introduction

O-Linked β-*N*-acetylglucosamine glycosylation (O-GlcNAcylation) is the addition of the monosaccharide *N*-acetylglucosamine (GlcNAc) onto serine or threonine residues (Bond & Hanover, 2015; Yang & Qian, 2017). Since its discovery, O-GlcNAc has been shown to be an abundant post-transcriptional modification (PTM) and an integral part of cell survival. Unlike other forms of glycosylation which contains multiple saccharide subunits, O-GlcNAcylation is not further elaborated by additional carbohydrates. Additionally, this type of glycosylation is unique because it occurs on intracellular proteins and is dynamic because it is added by the enzyme O-GlcNAc transferase (OGT) and subsequently removed by O-GlcNAcase (OGA) (Vocadlo, 2012). Currently, multiple hundreds of proteins, ranging from transcription factors to central metabolic enzymes, have been identified as being potentially O-GlcNAc modified, suggesting that this modification affects multiple biological pathways. O-GlcNAc is required for development in both mice and *Drosophila* and conditional deletion of OGT in mouse embryonic fibroblasts is lethal (O'Donnell, Zachara, Hart, & Marth, 2004; Shafi et al., 2000; Sinclair et al., 2009; Wang, Jensen, Rexach, Vinters, & Hsieh-Wilson, 2016). Furthermore, global up-regulation or down-regulation of O-GlcNAcylation are associated with several diseases, including cancer and neurodegenerative diseases, respectively. O-GlcNAcylation has also been shown to affect other PTMs, especially phosphorylation, through both direct competition for serine/threonines or more remote

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effects (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011; Hu, Shimoji, & Hart, 2010). Despite the clear importance of O-GlcNAcylation in basic cellular biology and human health, characterization of specific modification events that control key proteins is still quite rare and a significant roadblock to understanding the presumably multitude of functions for O-GlcNAcylation.

One of the first steps toward the characterization of any PTM is the development of tools that allow for its visualization and enrichment. Toward this goal, several different antibody and lectin approaches have been created (Comer, Vosseller, Wells, Accavitti, & Hart, 2001; Snow, Senior, & Gerace, 1987; Teo et al., 2010; Zachara, Vosseller, & Hart, 2011). While many O-GlcNAcylated proteins have been identified using these techniques they are not without limitations. For example, pan-anti-O-GlcNAc antibodies have been shown to have preference for certain underlying peptide sequences and do not recognize the entire repertoire of O-GlcNAcylated proteins. In contrast, the GlcNAc-recognizing lectin wheatgerm agglutinin (WGA) can recognize other GlcNAc-containing glycans and has relatively low affinity. Because of these issues different chemical approaches have been created for the enrichment and characterization of potentially O-GlcNAcylated proteins (Banerjee, Hart, & Cho, 2013). One such powerful technique, which we will not discuss in detail here, involves the chemoenzymatic attachment of visualization or affinity tags onto O-GlcNAc modifications. (Clark et al., 2008; Thompson, Griffin, & Hsieh-Wilson, 2018) This method was pioneered by the Hsieh-Wilson lab and involves three main steps. Cell or tissue lysate is generated, and any O-GlcNAc modifications present in the lysate are then enzymatically elaborated through the action of a mutant galactosyltransferase GalT(Y289L) (Ramakrishnan & Qasba, 2002). Specifically, this enzyme will use the azide-containing uridine-diphosphate (UDP) sugar donor UDP-GalNAz to generate a corresponding disaccharide GalNAz(β1-4)GlcNAc on any endogenous O-GlcNAc modifications. Bioorthogonal chemistries can then be used to react the azide with various tags, as described in greater detail below. This system is commercially available as the Click-IT O-GlcNAc Enzymatic Labeling System from Thermo Fisher Scientific; however, it is expensive and requires multiple handling steps that could prove challenging for non-experts.

A complementary chemical approach involves the development of monosaccharide analogs that directly bear bioorthogonal reactive groups like azides or alkynes (Chuh, Batt, & Pratt, 2016; Chuh & Pratt, 2015; Grammel & Hang, 2013). These analogs, which we have termed metabolic chemical reporters (MCRs), are “fed” to living cells or organisms. If they are similar enough to naturally occurring carbohydrates, MCRs will be biosynthetically converted to the corresponding donor sugars and then utilized by glycosyltransferases, resulting in their direct incorporation into glycoproteins. Again, bioorthogonal reactions can then be used for the installation of different tags. The first MCR aimed at O-GlcNAcylation was an azide-modified analog, Ac_4GlcNAz (Fig. 1A), developed by the Bertozzi lab (Vocadlo, Hang, Kim, Hanover, & Bertozzi, 2003). In most monosaccharide MCRs, *O*-acetate protecting groups are used to facilitate passive diffusion across the cell membrane and are subsequently removed by intracellular lipases to yield the free sugar. Using in vitro biochemistry and cell labeling, the Bertozzi lab demonstrated that GlcNAz can transit each step of the biosynthetic pathway, yielding UDP-GlcNAz, and that both OGT and OGA would readily tolerate the azide substitution. Ac_4GlcNAz was originally thought to be

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largely selective for O-GlcNAcylation, but we and others have demonstrated that it can be also be incorporated into cell-surface glycans. In retrospect this result is not surprising, as GlcNAc is found in the branches of both N-linked and mucin O-linked glycans of cell surface and secreted proteins. Additionally, different monosaccharides can be interconverted by the metabolic machinery of living cells. In fact this interconversion was exploited by the Bertozzi lab to label O-GlcNAcylated proteins upon treatment with the 4-epimer of Ac_4GlcNAz, Ac_4GalNAz (Fig. 1A) (Boyce et al., 2011). Notably, our lab demonstrated that subtle changes to the MCR structure could bias the types of glycosylation that were labeled. For example, we showed that Ac_4GlcNAlk (Fig. 1A) has preference for O-GlcNAcylation and N-linked glycosylation with little labeling of mucin O-linked glycans (Zaro, Yang, Hang, & Pratt, 2011). Using this observation as a starting point, we were then able to create two azido-MCRs, $Ac_3$6AzGlcNAc and $Ac_4$2AzGlc (Fig. 1A), with high selectivity for O-GlcNAcylation over other types of glycosylation (Chuh, Zaro, Piller, Piller, & Pratt, 2014; Zaro, Batt, Chuh, Navarro, & Pratt, 2017). Gratifyingly, the selectivity of $Ac_4$2AzGlc was independently confirmed by the Vocadlo lab (Shen et al., 2017) and our approach was subsequently used by the Wang lab to create yet another O-GlcNAcylation selective MCR $Ac_3$4dGlcNAz (Fig. 1A) (Li et al., 2016).

In addition to the selectivity of a given MCR for one type of glycosylation over another, another important consideration is the bioorthogonal reaction. The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is typically the reaction of choice for cell lysates as the corresponding strain-promoted reaction displays relatively high levels of background reactivity under these conditions. However, the orientation of the CuAAC reaction is also important (Speers & Cravatt, 2004). The use of alkyne-tags to match azido-MCRs results notably higher background labeling of proteins than the reverse orientation, presumably due to the excess copper-activated alkyne-tag. This can be clearly seen in Fig. 2 by comparing cell lysates that were not treated with MCR but reacted with either azido- or alkyne-TAMARA fuorescent tags. In fact, this signal-to-noise difference prompted our synthesis of $Ac_3$6AlkGlcNAc (Fig. 1A), which displays the best O-GlcNAcylation selectivity and signal-to-noise of any MCR to date (Chuh et al., 2017). Another often overlooked variable is the choice of buffer during cell lysis and subsequent CuAAC reaction. Although the reason is not completely clear, certain buffers can interfere with the CuAAC reaction. One of the best buffers for this reaction is HEPES, which is our buffer of choice when using an alkyne-MCR and azide-tag. However, the increased reactivity in the reaction when using HEPES causes even higher background in the opposite CuAAC orientation. Therefore, a less reactive buffer, like triethanolamine (TEA), is often a better choice.

Unbiased approaches to identifying O-GlcNAcylated proteins typically combine one of the enrichment techniques described above with proteomic mass spectrometry. In the case of MCRs, this usually involves the bioorthogonal installation of a biotin-tag, followed by enrichment with streptavidin beads. After washing, the labeled proteins can then be subjected to on-bead trypsinolysis and the released peptides identified using a proteomic workflow. This method has been extremely powerful; however, it requires either access to an advanced mass spectrometer or utilization of a fee-for-service proteomics facility. Additionally, low abundance proteins may be missed by this type of analysis because they are masked by more prevalent proteins. Therefore, biotin-enrichment followed by SDS-

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PAGE and Western blotting is an important technique for both hypothesis driven assessment of protein O-GlcNAcylation and confirmation of proteomics data. Unfortunately, the biotin-streptavidin interaction is so strong that the elution of the enriched proteins can be quite difficult. To overcome this issue, a variety of cleavable tags have been developed (Fig. 1B). In general, these tags contain biotin on one end and either an azide or alkyne at the other. In between these groups is a chemical functionality that is stable to the CuAAC reaction and protein enrichment but can be cleaved through a handful of reactions ranging from simple acid to ultraviolet light. This “cleavage” reaction results in the mild release of the enriched proteins that can then be readily applied to SDS-PAGE and visualized using Western blotting.

The following protocol describes the combination of MCRs with the azo-bond containing cleavable linker that can be cleaved by reduction with sodium dithionite ($Na_2S_2O_4$). We have found that this linker performs the best in the CuAAC reaction, enabling the robust enrichment of potentially O-GlcNAcylated proteins followed by a simple cleavage reaction to retrieve these proteins from streptavidin beads.

2. Materials

All solutions and buffers should be prepared with 18MΩ H_2O at 25°C. Reagents should be stored at room temperature unless otherwise noted. Dispose of hazardous waste appropriately.

3. Materials for metabolic incorporation of chemical reporters

1. 200m*M* of $Ac_3$6AzGlcNAc (Click Chemistry Tools, 74.47mg/mL), $Ac_3$6AlkGlcNAc (71.07mg/mL), Ac_4GlcNAz (Click Chemistry Tools, 86.08mg/mL), or Ac_4GlcNAlk (Click Chemistry Tools, 84.5mg/mL) in DMSO. Store at −20°C.
2. 1 × solution of DPBS (Thermo Scientific): Combine 9.6 g of DPBS powder with 1L of H_2O and autoclave.
3. Cell line of interest, corresponding media, and tissue culture flask or plate.
4. H1299 or NIH3T3 can be used as an established control cell-line for MCR labeling and enrichment.

4. Buffers for azide-containing MCRs

1. 4% SDS buffer: 4% SDS, 150m*M* NaCl, 50m*M* TEA, pH 7.4. Add 20 g SDS, 4.38 g NaCl, and 3.73 g TEA to 400mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 500mL. Readjust pH to 7.4 if needed.
2. 1.25% SDS buffer: 1.25% SDS, 150m*M* NaCl, 50m*M* TEA pH 7.4. Add 6.25 g SDS, 4.38 g NaCl, and 3.73 g TEA to 400mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 500mL. Readjust pH to 7.4 if needed.

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3. 0% SDS buffer: 150m*M* NaCl, 50m*M* TEA pH 7.4. Add 4.38 g NaCl, and 3.73 g TEA to 400mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 500mL. Readjust pH to 7.4 if needed.

5. Buffers for alkyne-containing MCRs

1. 4% SDS buffer: 4% SDS, 150m*M* NaCl, 50m*M* HEPES pH 7.4. Add 20 g SDS, 4.38 g NaCl, and 5.96 g HEPES to 400mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 500mL. Readjust pH to 7.4 if needed.
2. 1.25% SDS buffer: 1.25% SDS, 150m*M* NaCl, 50m*M* HEPES pH 7.4. Add 20 g SDS, 4.38 g NaCl, and 5.96 g HEPES to 400mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 500mL. Readjust pH 7.4 if needed.
3. 0% SDS buffer: 150m*M* NaCl, 50m*M* HEPES pH 7.4. Add 20 g SDS, 4.38 g NaCl, and 5.96 g HEPES to 400mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 500mL. Readjust pH to 7.4 if needed.

6. Materials for CuAAC, biotin enrichment, elution, and preparation of samples

1. 5m*M* stock of azido-diazo-biotin (Click Chemistry Tools, 3.56mg/mL) or alk-diazo-biotin (Click Chemistry Tools, 3.98mg/mL) in DMSO. Can be stored in −20°C for short term storage (~6 month) or −80°C for long term storage.
2. 50m*M* TCEP (Thermo Fisher Scientific, 14.34mg/mL) freshly prepared in H_2O and used for up to 6h.
3. 10m*M* TBTA (Alfa Aesar, 5.31mg/mL) in DMSO. Can be stored in −20°C for short term storage (~6 month) or −80°C for long term storage.
4. 50m*M* $CuSO_4 \cdot 5H_2O$ (Sigma, 12.47mg/mL) freshly prepared in water and used for up to 6h.
5. High Capacity NeutrAvidin Agarose (Thermo Fisher Scientific). Stored at 4°C.
6. 1% SDS in DPBS: Add 10 g SDS and 9.6 g DPBS powder to 900mL of H_2O. Mix and adjust pH to 7.4. Add additional H_2O to a final volume of 1L. Readjust pH to 7.4 if needed.
7. 0.2% SDS, 150m*M* NaCl, 50m*M* TEA, pH 7.4: Add 1 g SDS, 4.38 g NaCl, and 5.96 g TEA to 400mL of H_2O. Mix and adjust pH to 7.4. Add an additional H_2O to a final volume of 500mL. Readjust pH to 7.4 if needed.
8. Sodium dithionite ($Na_2S_2O_4$) solution: freshly prepared 25m*M* $Na_2S_2O_4$ (Sigma Aldrich, 4.36mg/mL) in 1% SDS in DPBS and used for up to 2h. Solid sodium dithionite should be discarded and replaced after 3 months.
9. 2 × SDS-free loading buffer (20% glycerol, 0.2% bromophenol blue, pH 6.8). As needed, add 14μL of β-mercaptoethanol (BME) to a 1mL aliquot. Store the loading buffer containing BME at −20°C.

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7. Metabolic incorporation of chemical reporters and preparation of cell lysates

1. Replace media on cells at ~80% confluency with fresh media containing 200 μ*M* MCRs or DMSO vehicle. H1299 and NIH 3T3 require one 150mm dish to produce enough protein for a 1mg enrichment, the cell line of interest might require more or less.
2. After 16h, cells are collected using trypsin and DPBS for adherent cells or by pelleting in original media for suspension cells. Centrifuge at 4°C for 2 min at 2500 × *g*.
3. Remove media by aspiration, resuspend each pellets with 1mL of DPBS, and then transfer then into new microcentrifuge tubes. Centrifuge at 4°C for 2 min at 2500 × *g*. Remove DPBS by aspiration. Repeat wash, centrifugation, and aspiration one time. *At this time cells can be frozen (−20°C) and stored.*
4. Add 200μL of 4% SDS buffer containing 5mg/mL cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich).
5. Sonicate using a tip sonicator for 10s on.
6. Pellet any debris by centrifugation at 10,000 × *g* for 10min at 15°C.
7. Transfer the soluble fractions into new microcentrifuge tubes.
8. Conduct BCA assay (Thermo Scientific) by preparing Working Reagent (WR) by mixing 50 parts (1mL) of Reagent A with 1 part (20μL) of Reagent B. The amount of reagent needed is number of samples plus six (for standards). Aliquot 1mL of the WR into separate 1.5mL microcentrifuge tube. To separate tubes add 0, 1, 2, 4, and 8μL (0, 2, 4, 8, and 16μg, respectively) of albumin standard (provided with kit) to the WR. The microcentrifuge tubes are then placed in a heating block at 60°C for 30 min. Upon completion, read each sample using a UV spectrophotometer set to 562nm using the 0μg as a blank.
9. Dilute each sample to 4μg/μL in 4% SDS buffer containing 5mg/mL cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich).
10. Prepare inputs by taking 20μL of each sample and diluting it with 20μL 2 × SDS-free loading buffer. This scale can be adjusted as needed.

8. CuAAC (1mg of total protein)

Typically, enriching from 1mg of total protein will be sufficient to detect potentially O-GlcNAcylated proteins. However, this will depend on MCR used, cell line, and incubation period. If desired, scale according.

1. Dilute each sample by taking 250μL of each sample (4μg/μL in 4% SDS) and adding 130μL of 1.25% SDS buffer followed by 550μL 0% SDS buffer resulting in a final concentration of 1mg/mL in 1.25% SDS buffer in a 15mL centrifuge tube.

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2. Add 70μL CuAAC cocktail per sample of protein.
    - CuAAC cocktail is prepared as a master mix. First multiply the mg of total protein by 1.1. The amount of each reagent needed is this number times 20μL of azido/alk-azo-biotin tag, 20μL of TCEP, 10μL of TBTA, and 20μL of $CuSO_4 \cdot 5H_2O$. Vortex in between the additional of each reagent.
3. Incubate the CuAAC cocktail with proteins for 1h at room temperature in the dark.
4. Add 10μL of 0.5 *M* EDTA to each sample.
5. Precipitate proteins using MeOH/$CHCl_3$ as follows:
    i. Add 3000μL of MeOH, vortex.
    ii. Add 750μL of $CHCl_3$, vortex.
    iii. Add 2000μL volume of H_2O, vortex.
    iv. Centrifuge 6000 × *g* for 5 min at 15°C and discard upper layer carefully.
    v. Add 2250μL volume of MeOH, vortex.
    vi. Centrifuge 6000 × *g* for 10 min at 15°C and carefully decant supernatant.
        - At this time the supernatant can be removed and fresh MeOH (1mL) can be added and stored at −20°C. When resuming, centrifuge at 6000 × g for 10min at 15°C and decant supernatant once again.
6. Air-dry pellets for 5 min.

9. Enrichment and elution (1mg scale)

Scale according to the amount of proteins used above. If background is observed during Western blotting, increase the number of 1% SDS in DPBS washes to 10.

1. Resuspend each protein pellet with bath sonication in 100μL of 4% SDS Buffer (TEA).
2. Add 1900μL of 0% SDS Buffer (TEA) to each sample.
3. Add 50μL of high-capacity Neutravidin beads (Thermo Fisher Scientific) into a fresh 2mL dolphin nose tube.
4. Add 1mL of 0.2% SDS to each dolphin nose tube containing the beads, vortex, and centrifuge at 2500 × *g* for 2 min at 15°C.
5. Remove supernatant by aspiration and repeat steps 4 and 5 twice.
6. Add resuspended proteins to their respective beads and incubate with full rotation for 1.5 h at room temperature.

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7. Collected beads by centrifugation at 2500 × *g* for 2 min at 15°C. Remove supernatant by aspiration.
8. Add 1mL of 1% SDS in PBS to the beads, vortex, and centrifuge 2500 × *g* for 2 min at 15°C.
9. Remove supernatant by aspiration and repeat steps 8 and 9 six times.
10. Incubate beads with 50μL of $Na_2S_2O_4$ buffer for 30 min with gentle rocking.
11. Collect beads using centrifugation: 2500 × *g* for 2 min at 15°C.
12. Transfer supernatant into fresh microcentrifuge tubes.
13. Repeat steps 10–12 and pool corresponding supernatants together.
14. Precipitate proteins in the supernatant by adding 500μL MeOH to each sample, vortexing, and incubating the samples for 2–24 h at −20°C.

10. SDS-PAGE and Western blot (do not scale)

1. Pellet proteins by centrifugation for 10 mins at 10,000 × *g* at 4°C.
2. Air-Dry pellets for 5 mins.
3. Resuspend enriched proteins in 15μL 4% SDS (TEA) buffer with bath sonication.
4. Add 15μL of 2× SDS-free loading buffer (with BME) and boil for 5 mins.
5. Load 20μL for each input, and load complete sample from enrichment.
6. Preform SDS-Page and Western blotting.

Controls

1. Postive controls of known O-GlcNAcylated proteins.
   - Anti-CREB (Cell Signaling Technology 9104S)
   - Anti-Nup62 BD (Biosciences 610497)
   - Anti-NEDD4 (EMD Millipore 07-049)
   - Anti-PK (Abcam ab118499)
2. Negative control
   - Anti-Actin (Sigma A2066-.2ML)

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Abbreviations

$Ac_3$6AlkGlcNAc	1,3,4-tri-*O*-acetyl-2-deoxy-2-*N*-acetyl-6-deoxy-alkynyl-glucopyranose
$Ac_3$6AzGlcNAc	1,3,4-tri-*O*-acetyl-2-deoxy-2-*N*-acetyl-6-deoxy-azido-glucopyranose
Ac_4GalNAz	1,3,4,6-tetra-*O*-acetyl-*N*-azidoacetylgalactoseamine
Ac_4GlcNAz	13,4,6-tetra-*O*-acetyl-*N*-azidoacetylglucosamine
Ac_4GlcNAlk	13,4,6-tetra-*O*-acetyl-*N*-4-pentynylglucosamine
Ac_4ManNAz	1,3,4,6-tetra-*O*-acetyl-*N*-azidoacetylmannoseamine
Azido-diazo-biotin	1*H*-thieno[3,4-d]imidazole-4-pentanamide, *N*-[13-[4-[(1*E*)-2-[5-(2-azidoethyl)-2-hydroxyphenyl]diazenyl]phenyl]-13-oxo-3,6,9-trioxa-12-azatridec-1-yl]hexahydro-2-oxo-, (3aS,4S,6aR)-, (cas # 1339202-33-3)
Alk-diazo-biotin	1*H*-thieno[3,4-d]imidazole-4-pentanamide, hexahydro-*N*-[1-[4-[2-[2-hydroxy-5-[2-[[1-oxo-3-(2-propyn-1-yloxy)propyl]amino]ethyl]phenyl]diazenyl]phenyl]-1-oxo-5,8,11-trioxa-2-azatridec-13-yl]-2-oxo-, (3aS,4S,6aR)-, (cas # 1884349-58-9)
BCA	bicinchoninic acid assay
BME	β-mercaptoethanol
$CHCl_3$	chloroform
CREB	cAMP response element binding
DMSO	dimethyl sulfoxide
DPBS	Dulbecco’s phosphate-buffered saline, no calcium, no magnesium
EDTA	ethylenediaminetetraacetic acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
H_2O	water
MeOH	methanol
NaCl	sodium chloride
NEDD4	E3 ubiquitin-protein ligase
Nup62	nucleoporin 62

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PK	pyruvate kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate—polyacrylamide gel electrophoresis
TBTA	tris[(1-benzyl-1-*H*-1,2,3-triazol-4-yl)methyl]amine
TEA	triethanolamine
TECP	tris(2-carboxyethyl)phosphine hydrochloride

References

Banerjee PS, Hart GW, & Cho JW (2013). Chemical approaches to study O-GlcNAcylation. Chemical Society Reviews, 42, 4345–4357. 10.1039/c2cs35412h. [PubMed: 23247267]

Bond MR, & Hanover JA (2015). A little sugar goes a long way: The cell biology of O-GlcNAc. The Journal of Cell Biology, 208, 869–880. 10.1083/jcb.201501101. [PubMed: 25825515]

Boyce M, Carrico IS, Ganguli AS, Yu S-H, Hangauer MJ, & Hubbard SC (2011). Metabolic cross-talk allows labeling of O-linked beta-N-acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. Proceedings of the National Academy of Sciences of the United States of America, 108, 3141–3146. 10.1073/pnas.1010045108. [PubMed: 21300897]

Chuh KN, Batt AR, & Pratt MR (2016). Chemical methods for encoding and decoding of posttranslational modifications. Cell Chemical Biology, 23, 86–107. 10.1016/j.chembiol.2015.11.006. [PubMed: 26933738]

Chuh KN, Batt AR, Zaro BW, Darabedian N, Marotta NP, & Brennan CK (2017). The new chemical reporter 6-alkynyl-6-deoxy-GlcNAc reveals O-GlcNAc modification of the apoptotic caspases that can block the cleavage/activation of Caspase-8. Journal of the American Chemical Society, 139, 7872–7885. 10.1021/jacs.7b02213. [PubMed: 28528544]

Chuh KN, & Pratt MR (2015). Chemistry-enabled methods for the visualization of cell-surface glycoproteins in metazoans. Glycoconjugate Journal, 32, 443–454. 10.1007/s10719-015-9589-3. [PubMed: 25913724]

Chuh KN, Zaro BW, Piller F, Piller V,& Pratt MR (2014). Changes in metabolic chemical reporter structure yield a selective probe of O-GlcNAc modification. Journal of the American Chemical Society, 136, 12283–12295. 10.1021/ja504063c. [PubMed: 25153642]

Clark PM, Dweck JF, Mason DE, Hart CR, Buck SB, & Peters EC (2008). Direct in-gel fluorescence detection and cellular imaging of O-GlcNAc-modified proteins. Journal of the American Chemical Society, 130, 11576–11577. 10.1021/ja8030467. [PubMed: 18683930]

Comer FI, Vosseller K, Wells L, Accavitti MA, & Hart GW (2001). Characterization of a mouse monoclonal antibody specific for O-linked N-acetylglucosamine. Analytical Biochemistry, 293, 169–177. 10.1006/abio.2001.5132. [PubMed: 11399029]

Grammel M, & Hang HC (2013). Chemical reporters for biological discovery. Nature Chemical Biology, 9, 475–484. 10.1038/nchembio.1296. [PubMed: 23868317]

Hart GW, Slawson C, Ramirez-Correa G, & Lagerlof O (2011). Cross talk between O-GlcNAcylation and phosphorylation: Roles in signaling, transcription, and chronic disease. Annual Review of Biochemistry, 80, 825–858. 10.1146/annurev-biochem-060608-102511.

Hu P, Shimoji S,& Hart GW (2010). Site-specific interplay between O-GlcNAcylation and phosphorylation in cellular regulation. FEBS Letters, 584, 2526–2538. 10.1016/j.febslet.2010.04.044. [PubMed: 20417205]

Li J, Wang J, Wen L, Zhu H, Li S, & Huang K (2016). An OGA-resistant probe allows specific visualization and accurate identification of O-GlcNAc-modified proteins in cells. ACS Chemical Biology, 11, 3002–3006. 10.1021/acschembio.6b00678. [PubMed: 27622469]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

O'Donnell N, Zachara NE, Hart GW, & Marth JD (2004). Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. Molecular and Cellular Biology, 24, 1680–1690. [PubMed: 14749383]

Ramakrishnan B,& Qasba PK (2002). Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: Point mutation broadens beta 4Gal-T1 donor specificity. The Journal of Biological Chemistry, 277, 20833–20839. 10.1074/jbc.M111183200. [PubMed: 11916963]

Shafi R, Iyer SP, Ellies LG, O'Donnell N, Marek KW, & Chui D (2000). The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. Proceedings of the National Academy of Sciences of the United States of America, 97, 5735–5739. 10.1073/pnas.100471497. [PubMed: 10801981]

Shen DL, Liu T-W, Zandberg W, Clark T, Eskandari R, & Alteen MG (2017). Catalytic promiscuity of O-GlcNAc transferase enables unexpected metabolic engineering of cytoplasmic proteins with 2-azido-2-deoxy-glucose. ACS Chemical Biology, 12, 206–213. 10.1021/acschembio.6b00876. [PubMed: 27935279]

Sinclair DAR, Syrzycka M, Macauley MS, Rastgardani T, Komljenovic I, & Vocadlo DJ (2009). Drosophila O-GlcNAc transferase (OGT) is encoded by the polycomb group (PcG) gene, super sex combs (sxc). Proceedings of the National Academy of Sciences of the United States of America, 106, 13427–13432. 10.1073/pnas.0904638106. [PubMed: 19666537]

Snow CM, Senior A, & Gerace L (1987). Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. The Journal of Cell Biology, 104, 1143–1156. [PubMed: 2437126]

Speers A, & Cravatt B (2004). Profiling enzyme activities in vivo using click chemistry methods. Chemistry & Biology, 11, 535–546. [PubMed: 15123248]

Teo CF, Ingale S, Wolfert MA, Elsayed GA, Nöt LG, & Chatham JC (2010). Glycopeptide-specific monoclonal antibodies suggest new roles for O-GlcNAc. Nature Chemical Biology, 6, 338–343. 10.1038/nchembio.338. [PubMed: 20305658]

Thompson JW, Griffin ME, & Hsieh-Wilson LC (2018). Methods for the detection, study, and dynamic profiling of O-GlcNAc glycosylation. Methods in Enzymology, 598, 101–135. 10.1016/bs.mie.2017.06.009. [PubMed: 29306432]

Vocadlo DJ (2012). O-GlcNAc processing enzymes: Catalytic mechanisms, substrate specificity, and enzyme regulation. Current Opinion in Chemical Biology, 16, 488–497. 10.1016/j.cbpa.2012.10.021. [PubMed: 23146438]

Vocadlo D, Hang H, Kim E, Hanover J, & Bertozzi C (2003). Achemical approach for identifying O-GlcNAc-modified proteins in cells. Proceedings of the National Academy of Sciences of the United States of America, 100, 9116–9121. [PubMed: 12874386]

Wang AC, Jensen EH, Rexach JE, Vinters HV, & Hsieh-Wilson LC (2016). Loss of O-GlcNAc glycosylation in forebrain excitatory neurons induces neurodegeneration. Proceedings of the National Academy of Sciences of the United States of America, 113, 15120–15125. 10.1073/pnas.1606899113. [PubMed: 27956640]

Yang X, & Qian K (2017). Protein O-GlcNAcylation: Emerging mechanisms and functions. Nature Reviews. Molecular Cell Biology, 18, 452–465. 10.1038/nrm.2017.22. [PubMed: 28488703]

Zachara NE, Vosseller K, & Hart GW (2011). Detection and analysis of proteins modified by O-linked N-acetylglucosamine. Current Protocols in Molecular Biology. 10.1002/0471142727.mb1706s95. Chapter 17 Unit17.6.

Zaro BW, Batt AR, Chuh KN, Navarro MX, & Pratt MR (2017). The small molecule 2-azido-2-deoxy-glucose is a metabolic chemical reporter of O-GlcNAc modifications in mammalian cells, revealing an unexpected promiscuity of O-GlcNAc transferase. ACS Chemical Biology, 12, 787–794. 10.1021/acschembio.6b00877. [PubMed: 28135057]

Zaro BW, Yang Y-Y, Hang HC, & Pratt MR (2011). Chemical reporters for fluorescent detection and identification of O-GlcNAc-modified proteins reveal glycosylation of the ubiquitin ligase NEDD4-1. Proceedings of the National Academy of Sciences of the United States of America, 108, 8146–8151. 10.1073/pnas.1102458108. [PubMed: 21540332]

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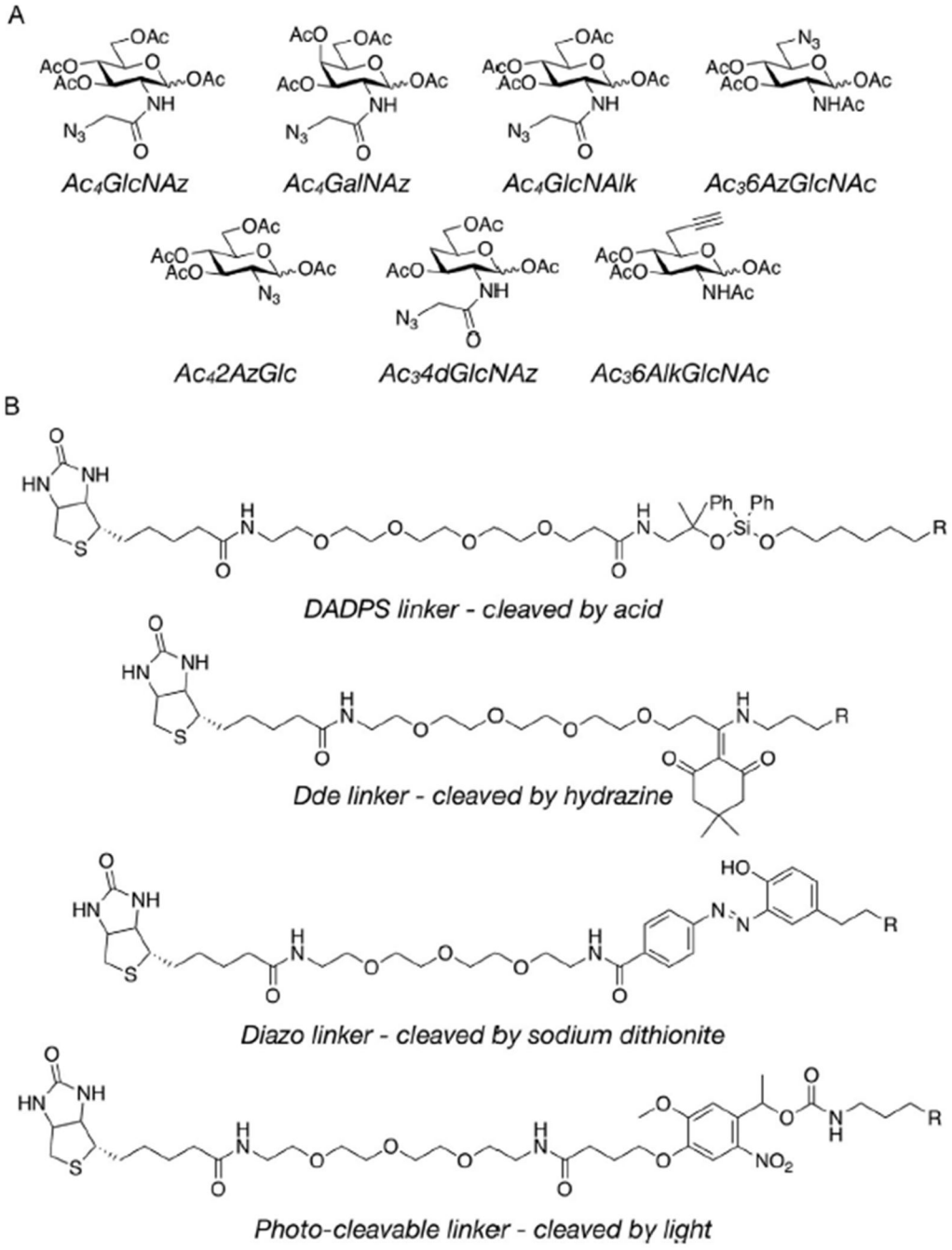

Fig. 1.

Identifying O-GlcNAcylated proteins using metabolic chemical reporters (MCRs). (A) Overview of different MCRs that can label O-GlcNAcylated proteins but with different efficiencies and selectivities. (B) Commercially available cleavable-linkers that can be used in combination with MCRs for the enrichment and subsequent elution of labeled proteins. R = azide or alkyne.

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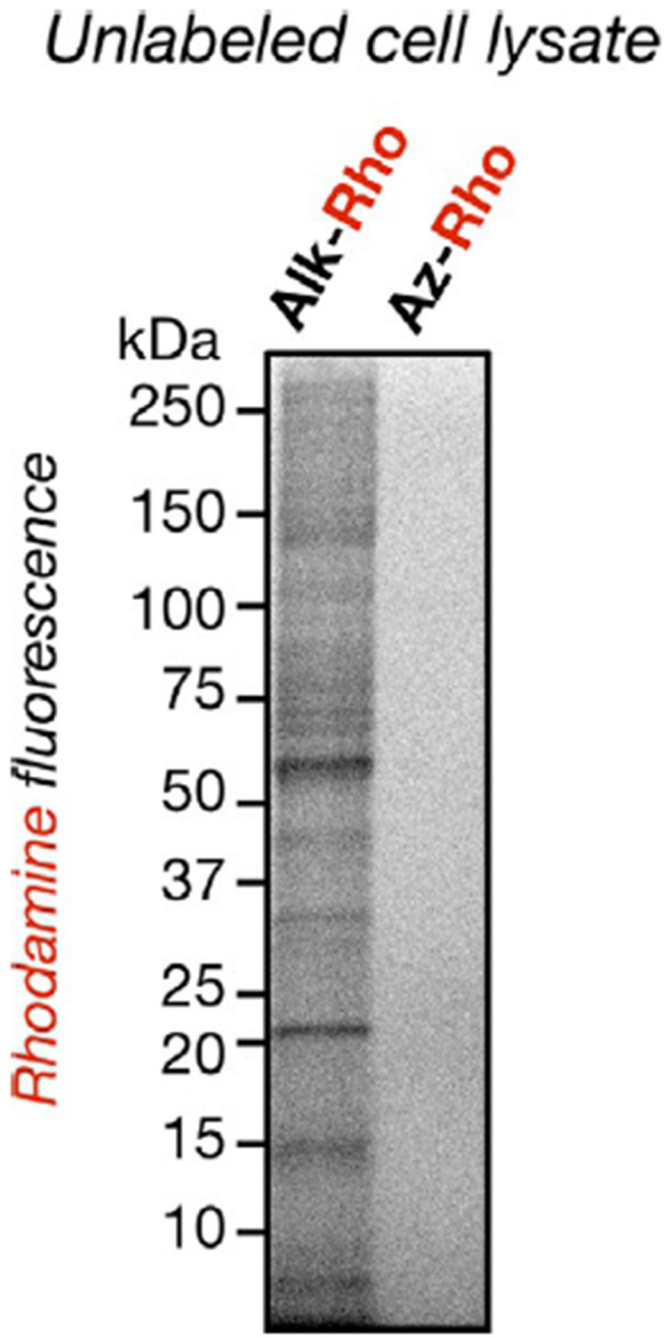

Fig. 2.

Alkyne-bearing MCRs and azide-tag display better signal-to-noise compared to the reverse orientation. Unlabeled NIH3T3 cell lysates were subjected to CuAAC conditions with either azido- or alkyne-rhodamine before SDS-PAGE and analysis by in-gel florescence.