Metabolic-Labeling with O-GlcNAc

A. Introduction:

"Click-it" type reaction extends the scope of identifying the installation of unnatural sugars into the glycocalyx. "Alkyne-azide" type click reactions are reported in the literature extensively and in use. Here we are using cells metabolically labeled with GlcNAz and that unnatural sugar, in theory, should react with the azido group attached to Biotin. Such a reaction would enable us to enrich proteins by streptavidin-biotin affinity chromatography or to detect them by western blot.

B. Materials and equipment:

i) cells (MEFS OGT WT and Null) ii) Ac_4GlcNAz (C33367) iii) Methanol iv) Chloroform v) Milli Q Water vi) Biotin-Alkyne (Invitrogen B10185, MW 528.66): 'Click-it' kit (Invitrogen, Catalog no. C10276) vii) 1% v/v NP-40 in TBS ; viii) 2mM EDTA ix) SDS-PAGE/Western Blot x) Micro Centrifuge at room temperature xi) Speed Vac xi) Dry ice xii)Pierce 660 Reagent; xiii) BSA protein standard xiv) screw capped eppendorf tube xv)vortex

C. Preparatiopn of Cell:

The deletion of OGT was induced with 4-hydroxytamoxifen (Kazemi et al., 2010). 30h post-the induction of OGT deletion, cells were labeled overnight with 25μM Ac-GlcNAz. A subset of cells was also treated with 2μg/ml Tunicamycin. Cells were harvested 18h after the initiation of GlcNAz labeling. While it's ideal to label cells for longer with the GlcNAz reagent, O-GlcNAcase does not remove GlcNAz efficiently. As such, we were concerned that if we added the GlcNAz to soon there would be sufficient OGT expressed that that the label would build up in the OGT null cells. **Treatments:** 1) OGT WT; 2) OGT WT + Tunicamycin (2μg/ml); 3) OGT Null (@48h); 4) OGT Null (@48h) + Tunicamycin (2μg/ml); and 5) Unlabeled cells.

D. Protein extraction from treated cells:

Cells were extracted in 1% NP-40 in TBS, 2mM EDTA with appropriate protease, phosphatase and hexosaminidase inhibitors. (See auxiliary protocol). Extracts were diluted to 1mg/ml and then the proteins were precipitated as below:

To 75 μl of sample in a screw capped eppendorf tube add:

i) 600 μl of methanol, mix well by vortexing;

ii) 150 μl of chloroform, mix well by vortexing;

iii) 525 μl of water, mix well by vortexing.

⚠ *Critical step:* Mix well before the adding of the next reagent. Add reagents in order.

iv) Centrifuge at >12,000xg for 5min at room temperature.

v) The protein pellet forms at the interface – it will look like a white disc;

vi) Remove the aqueous layer (upper)

⚠ *Critical step: Sometimes it's possible to remove the lower layer as well. This is more likely if you have a lot of protein and you can flip the disk of protein onto the side of the tube.*

vii) Add 600 µl methanol, mix well by vortexing

viii) Centrifuge at >12,000xg for 5min at room temperature, the pellet should now be at the bottom of the tube.

ix) Remove the remaining liquid,

x) Dry samples briefly, do not over dry of the protein will be difficult to re-suspend.

E. Click-it labeling of cells:

i) Resuspend your protein pellet in: 50mM Tris-HCl, 0.5% SDS

a). 100 µg of protein was resuspended in 50µl of buffer

b). the approximate concentration is 2mg/ml

⚠ *Pause point:* samples can be stored at -80°C at this point

ii) For each reaction we need 100 µl **of reaction mix** (make 6)

a). To 600 µl of Component A (C10276)

b). Add 60 µl of the Alkyne reagent (C10276)

iii) To 50 µl of protein

a). Add 100 µl of reaction mix

b). Add 10 µl of Water

c). Cap tubes and vortex

iv) Add 10 µl of Copper Sulfate (Component B, C10276)

v) Add 10 µl of the reducing agent (Component C, C10276), vortex, incubate for 2minutes, but no longer than 5 minutes.

vi) Add 20 µl of Component D (C10276) , vortex for 5 minutes, the solution should turn bright orange.

vii) Mix for 20minutes at room temperature.

viii) Recover by Methanol/Chloroform Precipitation

a). Resuspend in 50ul of 1x SDS-PAGE sample buffer

b). Boil for 5min

⚠ *Pause point:* samples can be stored at -80°C at this point

ix) Analyze by samples

Note: In this experiment we have clicked on a biotin tag which can be used to detect proteins by SDS-PAGE and Western blot, Immunoprecipitation/Western Blot, or alternatively to enrich proteins by streptavidin affinity chromatography.

A) Western Blot: This will tell us if our labeling worked, and if our treatments altered cellular glycosylation. In this experiment we expect that deleting OGT (no O-GlcNAc) and treating cells with tunicamycin (no N-linked glycosylation) will profoundly reduce the about of Biotin incorporated into proteins.

B) IP/Western Blot: It is possible to resuspend proteins and then immunoprecipitate proteins of interest. Streptavidin-HRP can then be used to determine if your protein of interest if glycosylated. Note, you will have to run appropriate controls to determine if Biotin has been clicked to O-GlcNAc or another glycans.

Controls may include: Deletion of OGT (above), Treatment with Tunicamycin (Above), or inhibition of O-GlcNAcase.

C) Affinity Chromatography: Glycoproteins that have been resuspended can be enriched by Streptavidin affinity chromatography. As this enrichment is performed under denaturaing conditions (3M Urea), presence in the bound material would suggest that that protein (or peptide) if glycosylated. Again, appropriate controls should be performed to ensure which type of glycan one is studying.