Appendix B

DIRECTING NEURONAL SIGNALING THROUGH CELL-SURFACE GLYCAN ENGINEERING

Pulsipher, A., Griffin, M. E., **Stone, S. E.**, Brown, J. M., Hsieh-Wilson, L. C. "Directing neuronal signaling through cell-surface glycan engineering." J. Am. Chem. Soc. 2014, 136 (19), 6794-6797. DOI: 10.1021/ja5005174

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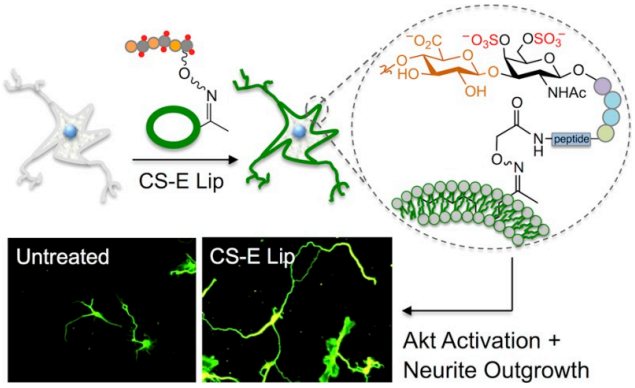

Abstract

The ability to tailor plasma membranes with specific glycans may enable the control of signaling events that are critical for proper development and function. We report a method to modify cell surfaces with specific sulfated chondroitin sulfate (CS) glycosaminoglycans using chemically modified liposomes. Neurons engineered to display CS-E-enriched polysaccharides exhibited increased activation of neurotrophin-mediated signaling pathways and enhanced axonal growth. This approach provides a facile, general route to tailor cell membranes with biologically active glycans and demonstrates the potential to direct important cellular events through cell-surface glycan engineering.

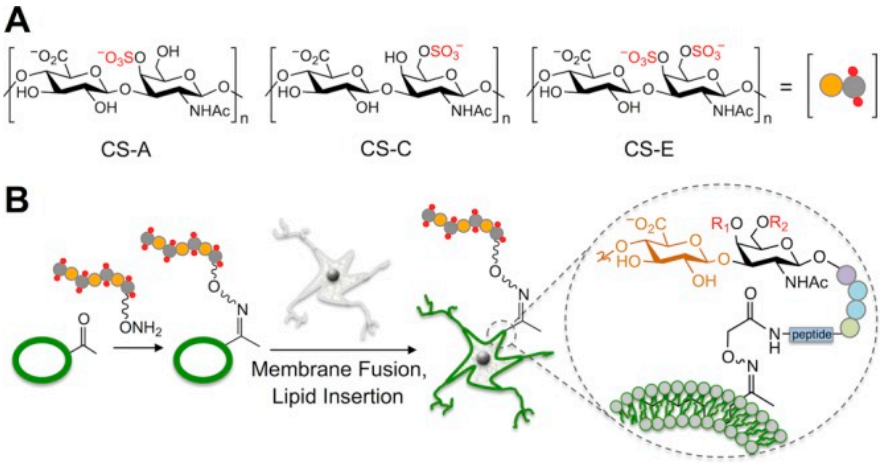

Figure 1: (A) CS polysaccharide structures used in this study. $n \approx 110$. (B) Strategy to remodel cell surfaces with CS GAGs and control signaling pathways.

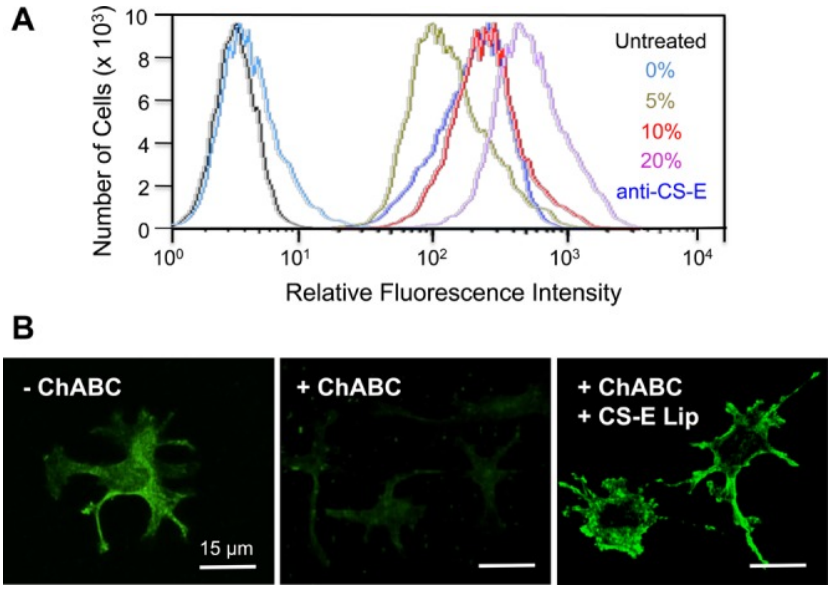

Figure 2: Controlled cell-surface display of CS polysaccharides and fluorophores. (A) FACS analysis of PC12 cells treated with liposomes presenting varying amounts (0–20%) of AF488-hyd. (B) Immunofluorescence detection of CS-E (green) on PC12 cells treated with or without chondroitinase (ChABC) and CS-E-functionalized liposomes as indicated.

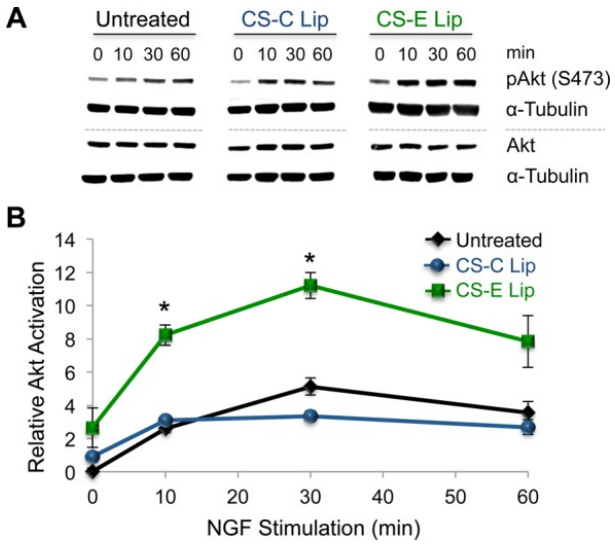

Figure 3: Presentation of CS-E polysaccharides on neuronal cell surfaces enhances NGF-mediated Akt activation. (A) Representative Western blots and (B) quantitation of Akt activation in neurons displaying CS-C- or CS-E-enriched polysaccharides. Akt activation was normalized against total Akt levels at each time point and compared to untreated neurons. Data represent mean ± SEM (*$P < 0.05$) from at least three experiments.

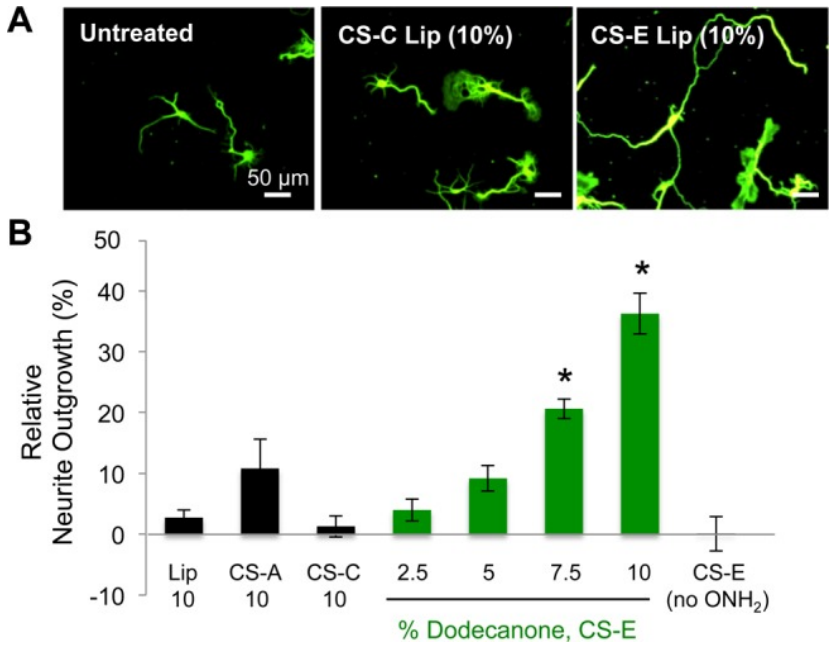

Figure 4: Presentation of CS-E polysaccharides on neuronal cell surfaces stimulates neurite outgrowth. (A) Representative images and (B) quantification of neurite outgrowth for neurons treated with unmodified liposomes or liposomes displaying CS-A-, CS-C-, or CS-E-enriched polysaccharides. Neurite outgrowth was normalized and plotted relative to untreated neurons. Data represent mean ± SEM (*$P < 0.05$) from at least three experiments.

Appendix C

LONG-LIVED ENGINEERING OF GLYCANS TO DIRECT STEM CELL FATE

Pulsipher, A., Griffin, M. E., **Stone, S. E.**, Hsieh-Wilson, L. C. "Long-lived engineering of glycans to direct stem cell fate." Angew. Chem. Int. Ed. **2015**, 54, 1466-1470. DOI: 10.1002/anie.201409258

Abstract

Glycans mediate many critical, long-term biological processes such as stem cell differentiation. However, few methods are available for the sustained remodeling of cells with specific glycan structures. We report a new strategy that enables the long-lived presentation of defined glycosaminoglycans on cell surfaces using HaloTag proteins (HTPs) as anchors. By controlling the sulfation patterns of heparan sulfate (HS) on pluripotent embryonic stem cell (ESC) membranes, we demonstrate that specific glycans cause ESCs to undergo accelerated exit from self-renewal and differentiation into neuronal cell types. Thus, the stable display of glycans on HTP scaffolds provides a powerful, versatile means to direct key signaling events and biological outcomes such as stem cell fate.

Keywords: embryonic stem cells, heparan sulfate, cell-surface engineering, stem cell differentiation, cell signaling

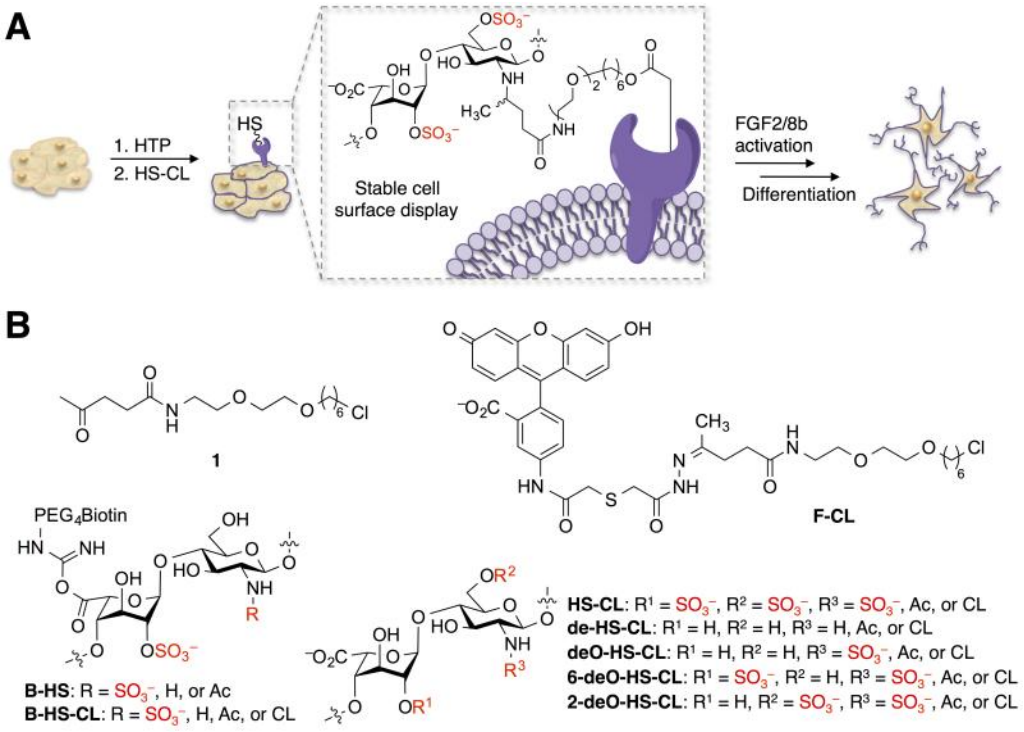

Figure 1: A) Strategy for presenting HS GAGs on cell membranes using HTP anchors to direct stem cell differentiation. B) Molecules used in this study.

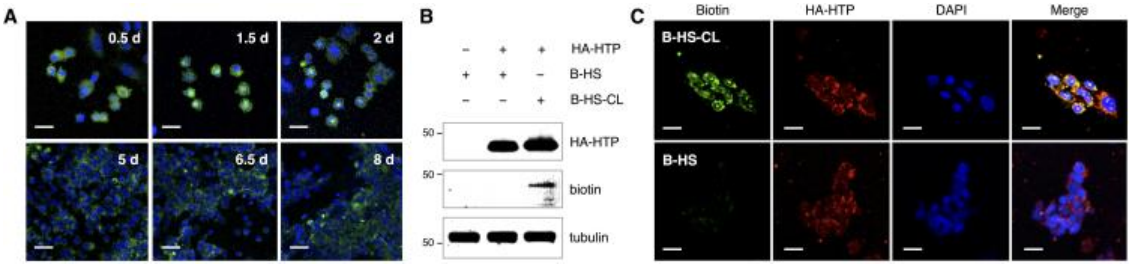

Figure 2: Extended cell-surface display by HTP anchoring. A) CHO cells stably expressing HTP were functionalized with a single treatment of F-CL (shown in green) and imaged over 8 days. Cell nuclei were co-stained at each time point with DAPI (shown in blue). B) Western blot detection and C) fluorescence imaging of hemagglutinin (HA)-tagged HTP and biotinylated HS. Stably transfected CHO cells were labeled with biotinylated HS with or without the chloroalkane linker (B-HS-CL or B-HS, respectively). Tubulin was used as a control for equal protein loading in B. Scale bars represent 20 μm.

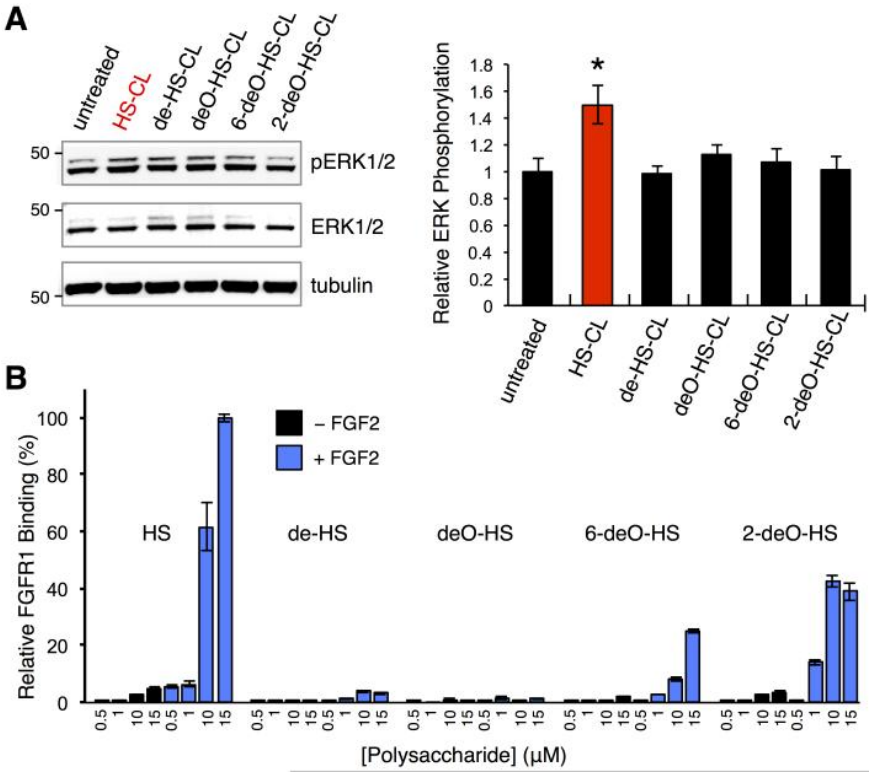

Figure 3: Cell-surface presentation of HS GAGs on ESCs induces FGF2-mediated ERK1/2 activation. A) Representative immunoblots (left) and quantification (right) of ERK1/2 phosphorylation levels in ESCs remodeled with the indicated HS GAGs and stimulated with FGF2. Phospho-ERK levels were normalized with respect to total ERK levels for each condition and compared to untreated ESCs. Tubulin was used as a control for equal protein loading. Data represent the mean ± S.E.M. (*$P < 0.05$) from three experiments. B) FGFR1-Fc binding to glycan microarrays in the presence (blue bars) or absence (black bars) of FGF2. Data represent the mean ± S.E.M. from ten replicate microarray spots.

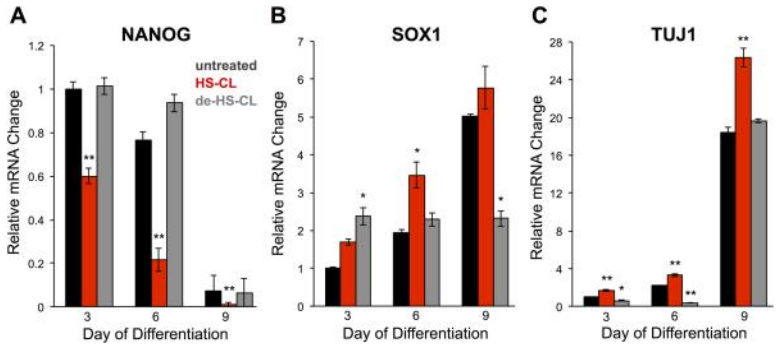

Figure 4: Remodeling the glycocalyx of ESCs with highly sulfated HS induces accelerated self-renewal exit, neural lineage commitment, and differentiation into mature, neuronal cells. qRT-PCR quantification of mRNA levels for A) pluripotent marker NANOG, B) neuroectoderm marker SOX1, and C) neuronal marker TUJ1. Data points were normalized to the housekeeping genes GAPDH and SDHA for cross comparison and to the untreated condition at day 3 for data presentation. Values represent the mean ± S.E.M. (*$P < 0.05$, **$P < 0.01$ when compared to the untreated control at each time point) from two independent experiments.

Appendix D

COMPREHENSIVE MAPPING OF *O*-GLCNAC MODIFICATION SITES USING CHEMICALLY CLEAVABLE TAG

Griffin, M. E., Jensen, E. H., Mason, D. E., Jenkins, C. L., **Stone, S. E.**, Peters, E. C., Hsieh-Wilson, L. C. "Comprehensive mapping of *O*-GlcNAc modification sites using a chemically cleavable tag." Mol. Biosys., **2016**, 12, 1756-1759. DOI: 10.1039/c6mb00138f

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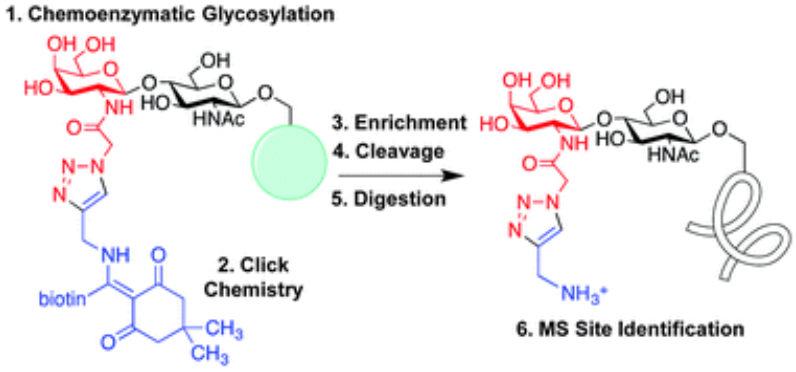

Abstract

The post-translational modification of serine or threonine residues of proteins with a single *N*-acetylglucosamine monosaccharide (*O*-GlcNAcylation) is essential for cell survival and function. However, relatively few *O*-GlcNAc modification sites have been mapped due to the difficulty of enriching and detecting *O*-GlcNAcylated peptides from complex samples. Here we describe an improved approach to quantitatively label and enrich *O*-GlcNAcylated proteins for site identification. Chemoenzymatic labelling followed by copper(I)-catalysed azide–alkyne cycloaddition (CuAAC) installs a new mass spectrometry (MS)-compatible linker designed for facile purification of *O*-GlcNAcylated proteins from cell lysates. The linker also allows subsequent quantitative release of *O*-GlcNAcylated proteins for downstream MS analysis. We validate the approach by unambiguously identifying several established *O*-GlcNAc sites on the proteins α-crystallin and *O*-GlcNAc transferase (OGT), as well as discovering new, previously unreported sites on OGT. Notably, these novel sites on OGT lie in key functional domains of the protein, underscoring how this site identification method may reveal important biological insights into protein activity and regulation.

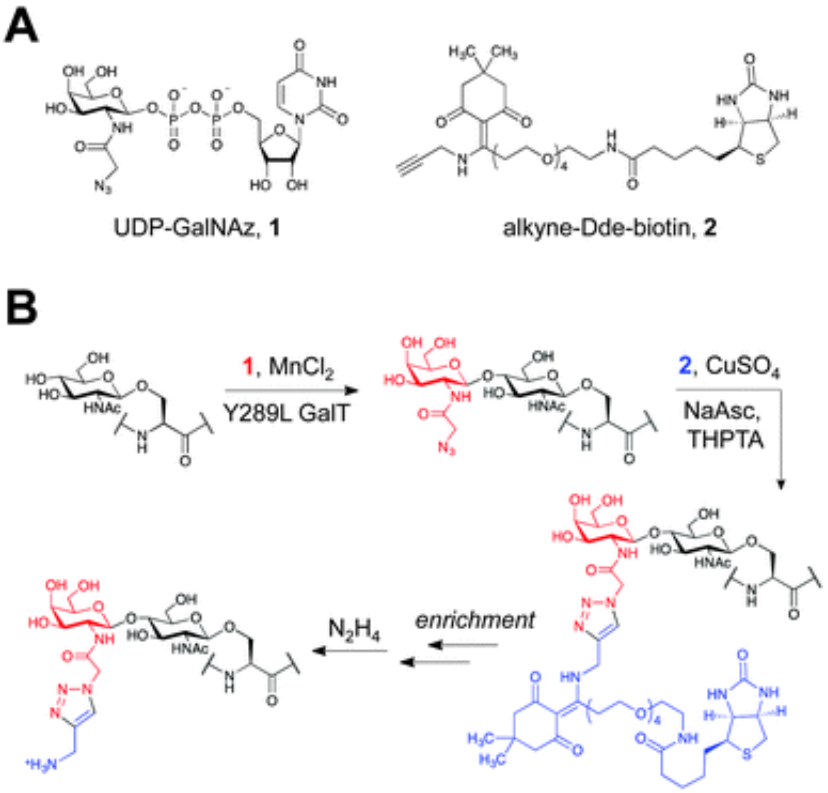

Figure 1: (A) Chemicals used in the labelling protocol. (B) Schematic of *O*-GlcNAc protein enrichment and elution using the two-step chemoenzymatic/CuAAC labelling protocol.

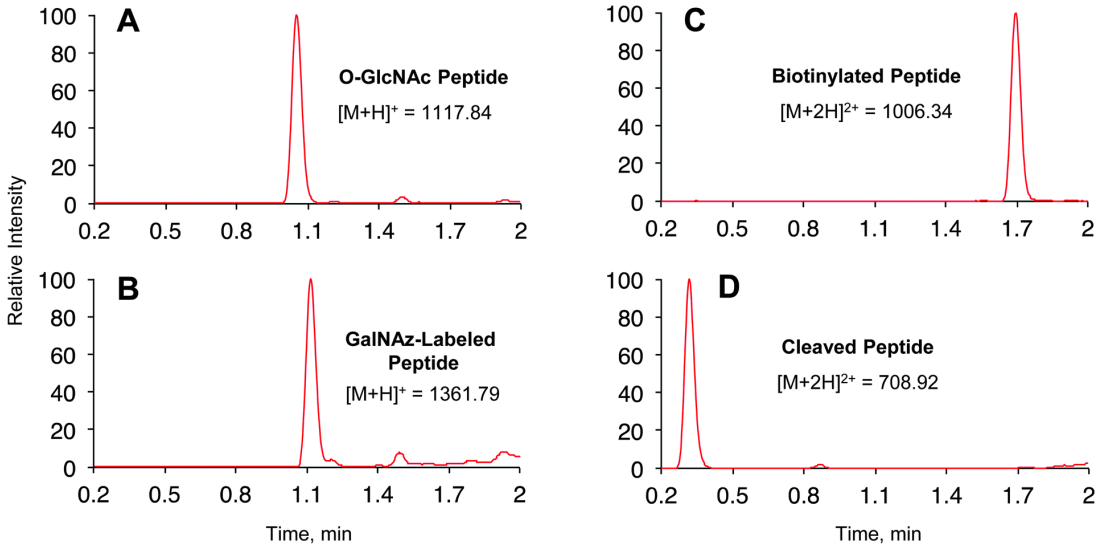

Figure 2: Labelling and cleavage reactions proceed quantitatively. Reverse phase LC-MS analysis of *O*-GlcNAc peptide labelling reactions at (A) time 0, (B) 16 h after addition of **1** and Y289L GalT, (C) 1 h after CuAAC with **2**, and (D) 1 h after cleavage with 2% aqueous hydrazine. See ESI† for

experimental details. (A) and (B) show base peak chromatograms. (C) and (D) show extracted ion chromatograms of the starting material and product within ±1 *m*/*z* of calculated values.

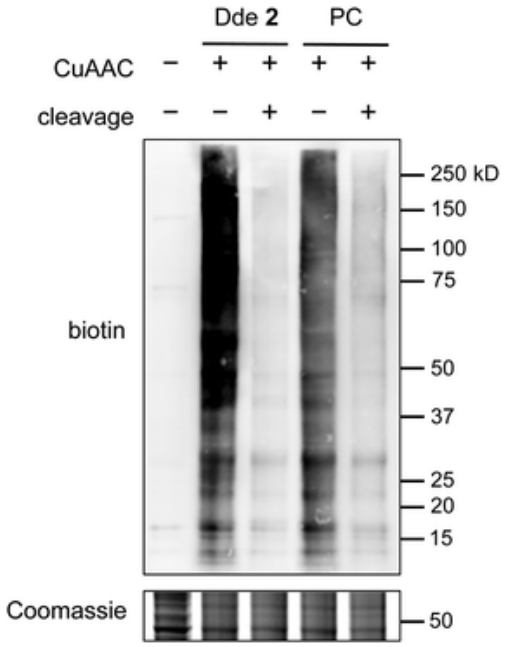

Figure 3: Alkyne-Dde-biotin linker **2** outperforms a widely used alkyne-photocleavable-biotin linker (PC). Protein lysates labelled with **2** show higher biotin signal after labelling (lane 2 *vs*. lane 4) and lower residual signal after cleavage (lane 3 *vs*. lane 5) compared to the PC linker. See Fig. S2 (ESI†) for full Coomassie gel.

Protein	Peptide sequence	Site(s)	Mascot ion score	Mascot delta ion score	Method
α-Crystallin A	AIPVSREEKPSSAPSS	Ser-162	24.9	23.5	ETD
sOGT	ISPTFADAYSNMGNTLK	Ser-10*/Thr-12*	46.5	—	ETD
sOGT	ISPTFADAYSNMGNTLK	Ser-20*	21.6	13.6	ETD
sOGT	ISPTFADAYSNMGNTLK	Ser-10*/Thr-12*, Ser-20*	38.4	—	ETD
sOGT	EMQDVQGALQCYTR	Thr-38	41.8	35.0	CID
sOGT	AIQINPAFADAHSNLASIHKDSGNIPEAIASYR	Ser-52*	53.5	7.9	ETD
sOGT	AIQINPAFADAHSNLASIHKDSGNIPEAIASYR	Ser-56*	56.8	15.7	ETD
sOGT	LYLQMWEHYAAGNKPDHMIKPVEVTESA	Thr-662	33.1	8.0	ETD

Table 1: *O*-GlcNAc sites identified following labelling with **2**, Neutravidin affinity purification, and hydrazine-mediated elution. Sites and regions of modification are denoted in red or red underline, respectively. Novel site identifications are marked by an asterisk.

Appendix E

BIOORTHOGONAL NONCANONICAL AMINO ACID TAGGING (BONCAT) ENABLES TIME-RESOLVED ANALYSIS OF PROTEIN SYNTHESIS IN NATIVE PLANT TISSUE

Glenn, W. S., **Stone, S. E.**, Ho, S. H., Sweredoski, M. J., Moradian, A., Hess, S., Bailey-Serres, J., Tirrell, D. A. "Bioorthogonal noncanonical amino acid tagging (BONCAT) enables time-resolved analysis of protein synthesis in native plant tissue." Plant Phys. **2017**, 173 (3), 1543-1553. DOI: 10.1104/pp.16.01762

Summary

Pulsing the noncanonical amino acid azidohomoalanine into *Arabidopsis thaliana* seedlings enables in-gel visualization, physical enrichment, and identification of newly synthesized proteins.

Abstract

Proteomic plasticity undergirds stress responses in plants, and understanding such responses requires accurate measurement of the extent to which proteins levels are adjusted to counter external stimuli. Here, we adapt bioorthogonal noncanonical amino acid tagging (BONCAT) to interrogate protein synthesis in vegetative *Arabidopsis thaliana* seedlings. BONCAT relies on the translational incorporation of a noncanonical amino acid (ncAA) probe into cellular proteins. In this study, the probe is the methionine surrogate azidohomoalanine (Aha), which carries a reactive azide moiety in its amino acid side chain. The azide handle in Aha can be selectively conjugated to dyes and functionalized beads to enable visualization and enrichment of newly synthesized proteins. We show that BONCAT is sensitive enough to detect *Arabidopsis* proteins synthesized within a 30-min interval defined by an Aha pulse, and that the method can be used to detect proteins made under conditions of light stress, osmotic shock, salt stress, heat stress and recovery from heat stress. We further establish that BONCAT can be coupled to tandem liquid chromatography-mass spectrometry (LC-MS) to identify and quantify proteins synthesized during heat stress and recovery from heat stress. Our results are consistent with a model in which, upon the onset of heat stress, translation is rapidly reprogrammed to enhance the synthesis of stress mitigators and is again altered during recovery. All experiments were carried out with commercially available reagents, highlighting the accessibility of the BONCAT method to researchers interested in stress responses as well as translational and post-translational regulation in plants.

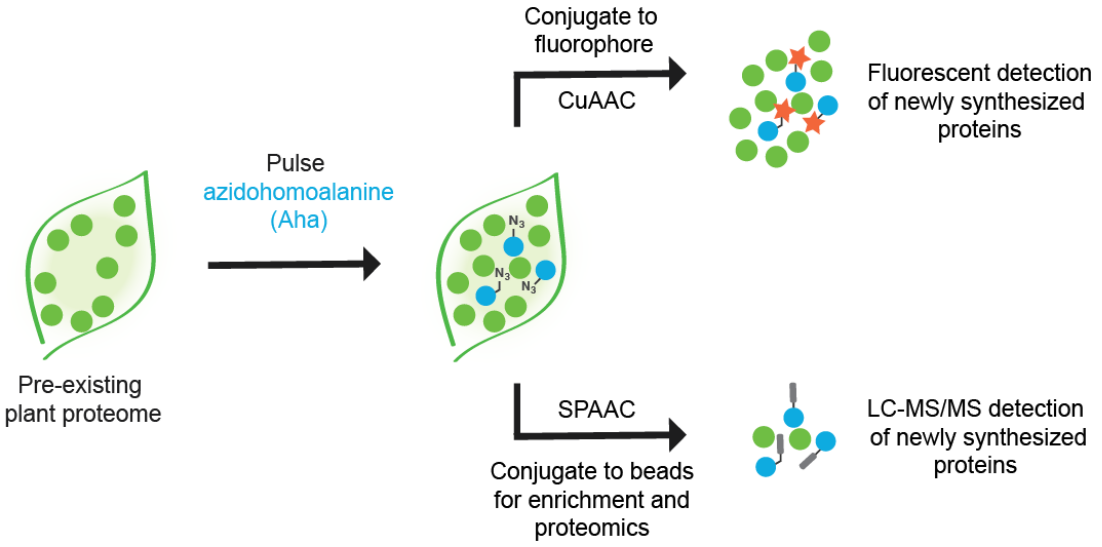

Figure 1: Scheme of BONCAT in native plant tissues. Azidohomoalanine (Aha) is pulsed into aerial tissues where it can be incorporated into nascent proteins. The azide enables conjugation to fluorophores or beads for visualization or enrichment, respectively. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) was used to conjugate TAMRA alkyne to nascent proteins. Strain-promoted azide-alkyne cycloaddition (SPAAC; a biocompatible 'click reaction') was employed to conjugate nascent proteins to beads for enrichment.

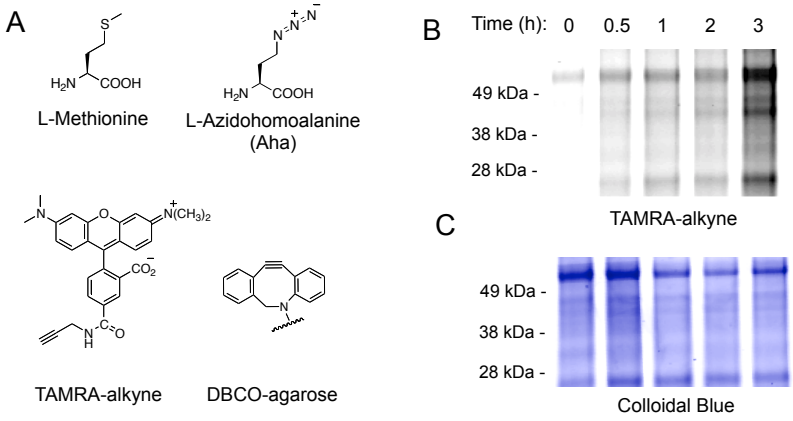

Figure 2: **A**. Probes used in this study. L-Azidohomoalanine (Aha) is a methionine surrogate replete with an azide moiety, which renders labeled proteins amenable to cycloaddition with fluorescent alkynyl probes (5,6-TAMRA alkyne) and strained cyclooctyne reagents (DBCO-agarose). **B**. Time course of Aha incorporation into nascent proteins of aerial tissues in *A. thaliana* seedlings. TAMRA-alkyne was conjugated to newly synthesized Aha-tagged proteins to render them fluorescent. The gel was visualized with an excitation laser at 532 nm and an emission band pass filter at 580 nm. **C**. Loading control. After measuring fluorescence, the gel was stained with

colloidal blue to confirm equal loading. Abbreviations: TAMRA – tetramethylrhodamine; DBCO – dibenzoazacyclooctyne.

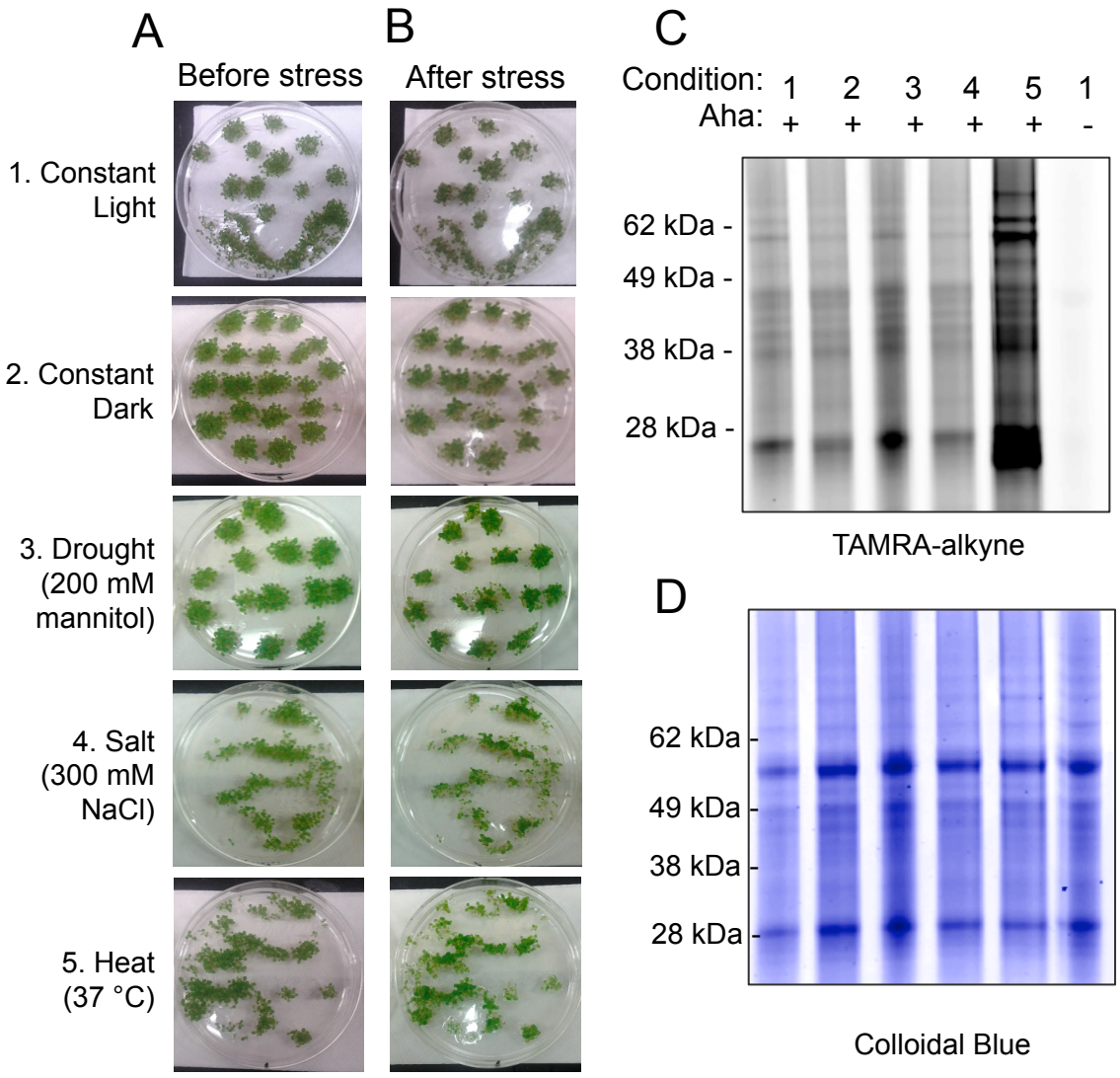

Figure 3: Labeling five separate stress conditions in *A. thaliana.* **A**. *A. thaliana* seedlings prior to Aha pulse and stress exposure. **B.** Seedlings 3 h after Aha pulse (1 mM) and constant exposure to stress conditions 1-5 (defined at left of figure). **C.** In-gel fluorescence assay to demonstrate labeling under stress conditions. Newly synthesized proteins incorporate Aha; TAMRA-alkyne is conjugated to proteins containing Aha. Labeling is observed under each condition. Little background signal is observed in a negative control, where plants were not exposed to Aha. **D.** Colloidal Blue loading control to demonstrate equal loading across lanes. Gels are representative examples of at least 3 biological replicates.

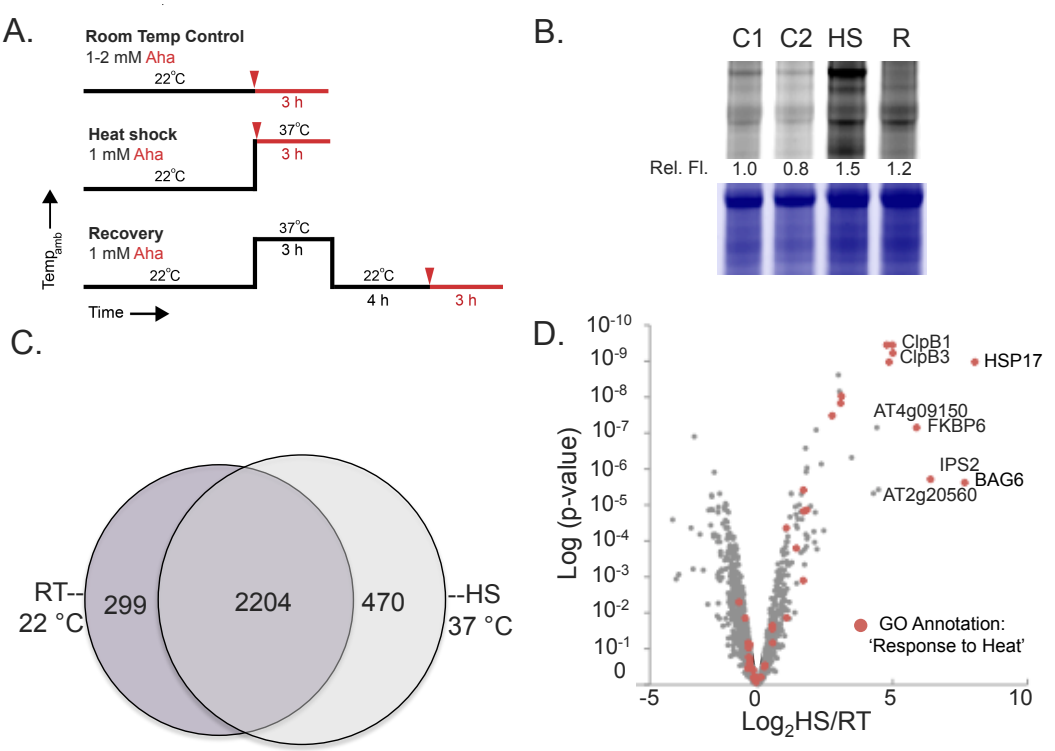

Figure 4: Enrichment of newly synthesized proteins for proteomics. **A.** Treatments used for this study. Arrowheads indicate time of introduction of Aha. **B.** Labeling under various conditions. **C.** Venn diagram of proteins identified in control conditions versus heat shock conditions. **D.** Volcano plot of ratios of expression levels of proteins shared between heat shock and control conditions. Proteins with higher average expression in RT samples fall on the left side of the plot, whereas proteins with higher average expression in HS samples are displayed on the right. To construct the plot, LFQ values were averaged for each condition. Then, the HS average was divided by the RT average and the Log_2 value was taken. Each point represents a protein. Proteins shown in red have the GO annotation "Response to Heat". Abbreviations: HS – heat shock, RT – room temperature, LFQ – label free quantitation, avg – average.

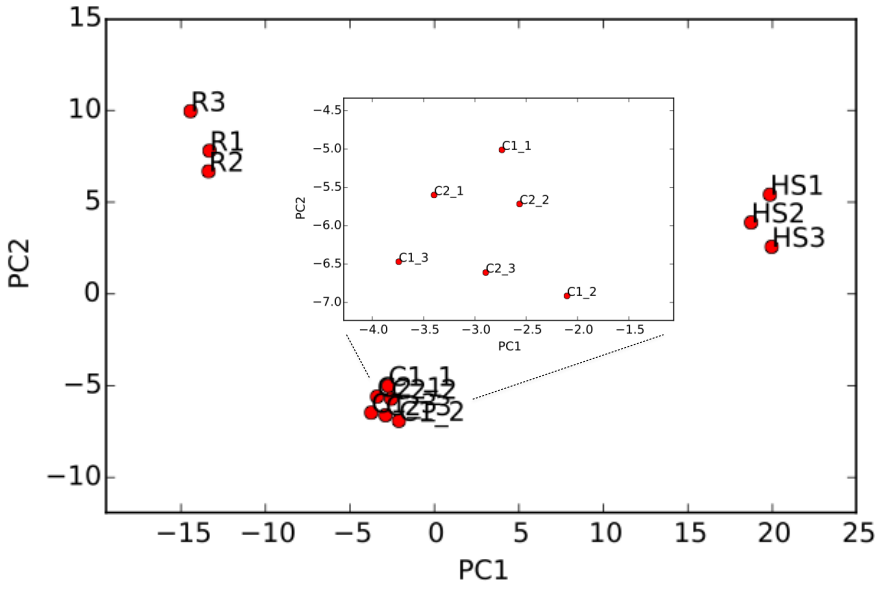

Figure 5: Principal component analysis of mass spectrometry results based on LFQ values. This analysis shows clear separation of control samples, heat shock samples and recovery samples. Inset shows zoom-in of controls cluster.

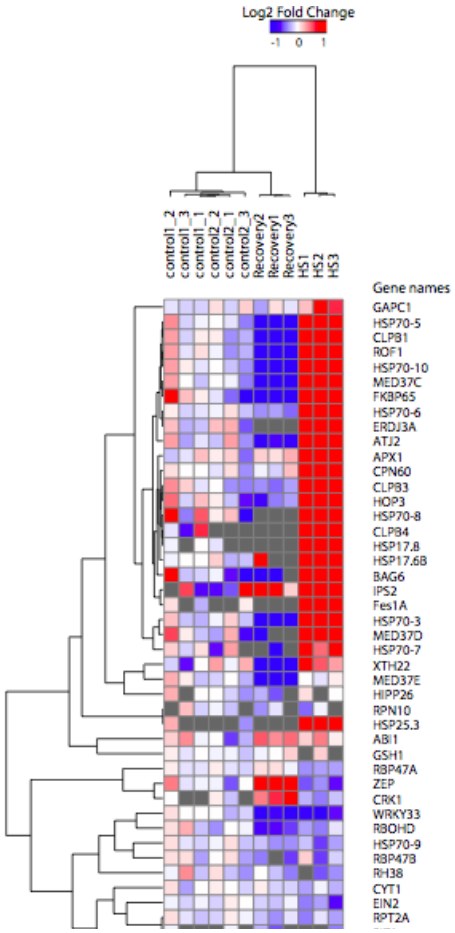

Figure 6: Partial heat map of proteins with GO annotation 'response to heat' found in this study. Significance of each fold change was calculated using the R package limma. Heat maps were created using GENE-E where the sample clustering was performed using the average linkage and Euclidean distance and the gene clustering was performed using the average linkage and 1-Pearson correlation coefficient. For heat map visualization, proteins had to be quantified in at least two control samples and two "treated" samples (either heat shock or recovery). Relative protein expression was normalized individually for each protein so that the average control expression was zero.

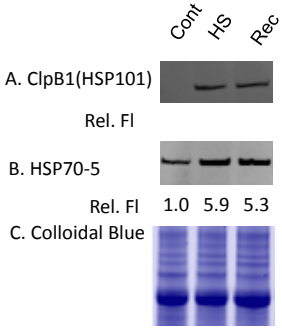

Figure 7: Immunoblotting analysis of select proteins shown in BONCAT screen to be up-regulated in response to heat stress. **A.** ClpB1(HSP101) and **B**. Heat Shock Protein 70-5 (HSP70-5) were found to be highly up-regulated in response to heat stress. These proteins are not synthesized at high levels during the recovery period. Neither are they rapidly degraded during the recovery period. Steady state ClpB1 levels during recovery are 0.95 ± 0.08 when the fluorescent signal of heat shock samples is normalized to 1.00. Relative fluorescence values are provided for the control (room temperature), heat shock and recovery for HSP70-5. **C.** Loading control. All fluorescence signals were normalized to Colloidal Blue staining. Abbreviations: Rel. Fl – relative fluorescence, Cont – control (room temperature), HS – Heat Shock, Rec – Recovery.