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The Role of Glycogen Synthase 1

in Brown Adipose Tissue

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Stacy Alina Tletlepantzi Lara

2024

ABSTRACT OF THE THESIS

The Role of Glycogen Synthase 1

in Brown Adipose Tissue

by

Stacy Alina Tletlepantzi Lara

Master of Science in Physiological Science

University of California, Los Angeles, 2024

Professor Claudio Javier Villanueva, Chair

Brown adipocytes are thermogenic cells activated during cold exposure. When activated, they generate heat through uncoupling the mitochondrial electron transport chain. Brown adipose tissue (BAT) activity is associated with a decreased risk of developing type 2 diabetes and other cardiometabolic diseases. Upon activation, pyruvate, and tricarboxylic acid (TCA) cycle intermediates increase, therefore, glucose oxidation may serve an essential role in BAT activation. Glucose is stored in glycogen through glycogen synthase (GYS1), a central enzyme that controls cytosolic glucose levels. Preliminary findings suggest that GYS1 protein expression is upregulated during thermogenesis. However, this upregulation is not due to an increase in transcription levels. Thus, we hypothesized post-translational regulation may be driving the elevated levels of GYS1 in BAT in response to thermogenesis. To test our hypothesis, brown

pre-adipocytes were isolated and immortalized from male C57BL/6L mitochondrial pyruvate carrier (MPC1) floxed mice, generating two cell lines: pBABE (control) and HA-TurboID-GYS1. These cell lines were then treated with and without CL-316, 243, a beta-3-adrenergic agonist. As a result, we expected to see an upregulation in GYS1 protein expression in both the pBABE and HA-TurboID-GYS1when treated with CL-316, 243. In the end, we found GYS1 protein expression increased under CL-316, 243, insulin, and insulin in combination with essential amino acids, suggesting GYS1 may also play a role in acute thermogenesis and the mTOR signaling pathway.

The thesis of Stacy Alina Tletlepantzi Lara is approved.

Amy Catherine Rowat

David William Walker

Claudio Javier Villanueva, Committee Chair

University of California, Los Angeles

2024

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ACKNOWLEDGEMENTS

I also want to highlight some of my previous mentors who strongly influenced me to pursue research, Dr. Rudy Ortiz and Dr. Yolanda A. Pineda-Vargas from the University of California, Merced.

Lastly, I deeply thank my family for always believing in me and constantly encouraging to be my best.

Introduction

Obesity

Obesity is a serious, costly chronic disease that affects millions of people from all age groups. According to the Centers for Disease Control and Prevention, from 1999-2000 through 2017-2020, United States (US) obesity prevalence increased from 30.5% to 41.9%. Furthermore, obesity led to the development of secondary chronic diseases, including heart disease, stroke, certain types of cancer, and type 2 diabetes. The estimated annual medical cost of obesity in the U.S. was close to $173 billion in 2019. Medical costs for obese adults were $1,861 higher than people with a healthy weight. Notably, more than 37 million Americans were diagnosed with diabetes (about 1 in 10) each year, and approximately 90-95% of them had type 2 diabetes.

These diseases were caused by various factors, one of them being the impairment of energy homeostasis (Cheng et al., 2021; Saltiel, 2016). Brown adipose tissue (BAT) has the unique ability to maintain mammalians core body temperature independent of the environmental temperature (Vianna et al., 2001). This response is also known as "adaptive thermogenesis," in which physiological responses maintain the core body temperature to preserve biological functions (Stocks et al., 2004). As a result, the rapid activation and inactivation of the uncoupling protein 1 (UCP1) heat dissipation, discussed later in this chapter, may serve as a key mechanism to increase the metabolic rate via non-shivering thermogenesis to promote survival (Celi, 2009). Due to these findings, researchers have found adults with a high number of BAT correlated with a healthy metabolism (Cypress et al., 2009; Virtanen et al., 2009; van Marken Lichtenbelt et al., 2009; Saito et al., 2009). On the other hand, obesity, diabetes, and ageing correlated with a lower

number of BAT (Pfannenberg et al., 2010). More recent studies have discovered, short-term cold exposure was sufficient to increase BAT activity, measured by the increased uptake of the glucose analog ^{18}F-fluorodeoxyglucose on positron emission tomography (^{18}F FDG-PET). This protocol was then repeated under long term exposure, which resulted in a significant fat mass reduction (Yoneshiro et al., 2013; Spiegelman, B., 2018). Therefore, regulating energy balance may serve as a possible treatment for metabolic diseases.

As a result, understanding the role of glycogen synthase in BAT is essential to better comprehending this mechanism. Glycogen synthase (GYS1) is the central enzyme in charge of synthesizing muscle glycogen. Glycogen serves as a carbohydrate and energy reserve (McCorvie et al., 2022). The compact structure of glycogen granules allows cells to store glucose when it is available in excess and make it readily available when needed (Nelson et al., 2021). In summary, GYS1 is an enzyme that catalyzes blood glucose into glycogen and regulates glucose homeostasis (Nutrition, 1990, Markussen et al., 2018). However, the role of glycogen synthase during thermogenesis remains unclear. Here we propose to study and analyze the function of glycogen synthase in BAT during cold exposure conditions.

Although glycogen synthase has been mainly studied in muscle tissue and beige adipocytes, the function of glycogen synthase in BAT remains unknown. In order, to identify which proteins are interacting with glycogen synthase during thermogenesis, enzyme-catalyzed proximity labeling will be used. Proximity labeling is a new approach to study the spatial and interaction characteristics of proteins in living cells. This approach has been used to define the interaction network of cellular organelles, such as ER, mitochondria, plasma membrane, cytosol, and other

organelles (Branon et al., 2018; Cho et al., 2020). However, previous enzymes used for labeling require over 18-hour labeling times, such as BioID and APEX2 (Branon et al., 2018). To overcome these limitations, a new biotin ligase was used, known as TurboID. This biotin ligase is a variant of E. coli biotin ligase (BirA), also known as BioID. This enzyme uses ATP to convert biotin into biotin-AMP, a reactive intermediate that covalently labels proximal proteins. What makes TurboID innovative is its ability to label proximal proteins within 10 minutes, rather than 18 hours. To test if TurboID is an effective tool to label proximal proteins, two cell lines were generated. The control cell line versus the TurboID- GYS1. The control cell line contained an empty vector in BAT pre-adipocyte immortalized cells, allowing only the endogenous GYS1 to be expressed. For our second cell line, TurboID was directly fused to the protein or enzyme of interest. In this case, the TurboID was directly fused with GYS1 and inserted as a vector in BAT preadipocyte immortalized cells. In order to detect any changes in GYS1 expression, BAT pre-adipocyte cells were treated with CL-316,243 (CL), a β3-adrenergic agonist. CL treatment has been shown to increase the expression of uncoupling protein 1 (UCP1), a key marker for thermogenesis by activating the same pathways as cold exposure (Liu et al., 1998).

Background and Preliminary Data:

Adipose tissue is a highly dynamic organ that can be classified based on the cellular composition of different depots and distinct anatomic localization (Koenen et al., 2021). In our body we have three different types of adipose tissue: white, brown, and beige. Together they serve as an energy reservoir for the body (Sakers et al., 2022). Each type of fat serves a specific function. For my research project we will be examining the function of BAT. Prolonged cold exposure activates BAT to produce heat through a process known as non-shivering thermogenesis, this helps

maintain body temperature in newborn humans and small rodents, and more recently in human adults (Elia et al., 2019). Previous studies have found BAT transform energy from food into heat production through various metabolic pathways, this includes an increase in glucose production, lipid reuptake, fatty acid oxidation, and glycogen storage (Carmean et al., 2016; Nedergaard, 2004). Thus, for this research project we will be homing in on the role of GYS1 in BAT.

Per Farkas and his lab reported during short term cold exposure, in a male Wistar rat model, there was a decrease in glycogen repletion and an increase in Hexokinase II (HKI), an insulin-responsive isoform enzyme that is involved in glucose metabolism. Glucose 6-phosphate (G-6-P) concentration decreased due to short term cold exposure, along with glycogen-synthesizing enzyme (GS) and glycogen-degrading enzyme (GP). However, after 24 hours when rats returned to room temperature there was an increase in GS and GP, which led to enhanced glycogen synthesis. Therefore, providing one possible pathway which may be involved in regulating glycogen homeostasis (Farkas et al., 1999). More recent studies have found non-shivering thermogenesis to be mediated by the stimulation of β3-adrenergic receptors in BAT. This is done via UCP1, found in mitochondria, which serves to decouple the mitochondrial respiratory chain from ATP production and dissipate that energy into heat (Mayeuf-Louchart, 2021). For UCP1 to become activated, humans are exposed to the cold or CL-316,243, this activates the sympathetic nervous system which is in charge our fight or flight response. Resulting in the release of norepinephrine, which then binds to a β3-adrenergic receptor, activating adenylate cyclase (AC) producing cyclic adenosine monophosphate (cAMP). This activates protein kinase A (PKA) and p38, leading to a signaling cascade ultimately promoting UCP1 transcription and translation

(Tabuchi & Sul, 2021). Preliminary data from the Villanueva lab showed when primary brown adipocytes were treated with CL-316,243 there was an increase in GYS1 protein expression.

mTOR Signaling Pathway

The mechanistic target of rapamycin (mTOR) is a kinase that is activated by anabolic signals. It also plays a major role in regulating lipid biosynthesis and metabolism in response to nutrition. This can be seen in mammals, when amino acids and insulin levels are high following food intake this drives the synthesis of phospholipids and sterols, which are required for membrane synthesis, cell growth, and cell proliferation. mTOR is a serine/threonine kinase that is part of the phosphatidylinositol 3-kinase (PI3K)- related kinases (PIKKs) family. This kinase is made up of two large protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is involved in protein synthesis, lipid metabolism, lysosome and mitochondrial biogenesis, energy metabolism, and autophagy. This complex is also inhibited by rapamycin, an antifungal produced by the bacterium Streptomyces hygroscopicus. On the other hand, mTORC2 promotes cell survival, metabolism, cytoskeletal organization and is insensitive to the inhibition of rapamycin (Caron et al., 2015).

   When insulin is present, it binds to specific receptor tyrosine kinases of mTORC1, which activate PI3K and Ras. This is then leads to the activation of protein kinase B/Akt (Akt), extracellular-signal-regulated kinase 1/2, and ribosomal S6 kinase 1. These kinases deactivate tuberous sclerosis complex 2 (TSC2), resulting in the activation of mTORC1. In contrast, amino acids activate a different pathway that promote the activity of mTORC1. mTORC1 requires the Ras-related GTP-binding protein (Rag) family of small GTPases. RagA/B recruits mTORC1 on the lysosomes where the mTORC1 activator Rheb resides. Less in known about the mTORC2

activation (Caron et al., 2015). However, Olsen et al. found mTOR complex 2 had a novel role in β3-adrenergic stimulated glucose uptake in BAT. This involved the cAMPK pathway and GLUT1 transcription (Olsen et al., 2014). However, GYS1 may also be involved in this pathway by regulation glucose homeostasis, however, this mechanism remains unknown.

Approach:

This preliminary data suggest that post-translational regulation may be driving the elevated levels of glycogen synthase. To identify proteins that associate with GYS1, we will use proximity labeling using the turboID system as previously introduced. By using TurboID, we will be able to identify the proteins interacting with GYS1 during normal and acute thermogenic conditions. In order to carry this out, we developed two aims:

Aim 1. Use proximity labeling approach to identify interacting partners of glycogen synthase 1.

Expected Outcomes: We expect to see an increase in GYS1 protein expression in both the pBABE and HA-TurboID-GYS1 cell lines when they are stimulated with CL-316,243.

Aim 2. Validate the interacting proteins identified by using co-immunoprecipitation experiments.

Expected Outcomes: Be able to isolate GYS1 and neighboring proteins

Materials & Methods

Animals

Three-month-old male C57BL/6J mice were obtained from Jackson Laboratories. The C57BL/6J mitochondrial pyruvate carrier floxed (MPC1 F/F) mice were generated as described by (Birsoy et al.,2015). Mice were housed in an environment maintained at 22–23°C with a 12-hour light/12-hour dark cycle. They were fed a standard Chow diet (2920x-030917M) and had continuous access to water. Food was withheld only during experimental procedures (Panic et al., 2020).

Cell Culture

Brown preadipocytes were isolated from 6-week-old MPC1 F/F mice (Rodriguez-Cuenca et al, 2007). Intrascapular BAT were removed, minced, and digested in buffer containing 1% collagenase, DMEM, antibiotics-50 IU Penicillin/mL, 50 μg Streptomycin/ mL, and Primocin 100 μg/mL. Samples were incubated in the shaking water bath at 37°C for 45 min after which they were allowed to cool on ice for 20 min. Infranatant was filtered through a 100 mm filter and centrifuged for 5 min at 500xg. The digestion buffer was removed, and pellet was washed twice with DMEM with antibiotics. After the last spin pellet was resuspended in 1 mL of DMEM containing 10% FBS (Cat# FB-01, Omega Scientific, Inc) and antibiotics. Cells were then plated into a six-well plate and the next day they were immortalized by retroviral expression of SV40 Large T-antigen (Cat# 13970, Adgene) using hygromycin for selection (Panic et al., 2020). Brown preadipocytes MPC 1 F/F cells were then transiently transfected with pBABE or HA-TurboID-GYS1 plasmids, positive cells were selected in DMEM supplemented with puromycin. For gene expression experiments, cells were plated in 10 cm plates (300,000 cells/ well) in

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DMEM containing 10% FBS, penicillin Streptomycin, 1 nM T3 and 20 nM insulin. When cells reached 100% confluency, they were given a differentiation cocktail containing 10% FBS, penicillin Streptomycin, 1 nM T3, 20 nM insulin, 1 µM rosiglitazone, 0.5 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 0.125 mM indomethacin. After 2 days of differentiation, media was changed to DMEM containing 10% FBS, 1nM T3, 20nM insulin, and 1 µM rosiglitazone. Cells were collected on day 0, day 6, and 8 of differentiation for experimental analyses.

Cell Treatments

On day 0, day 6, and day 8, media containing DMEM and 10% FBS, 1nM T3, 20nM insulin, and 1 µM rosiglitazone was changed to DMEM and 1% FBS. Cells were then incubated overnight at 37˚C with 5% CO_2. Media was then removed, and cells were washed with 1X PBS and then starved for 1 hour with HBSS 1X. Cells were treated with and without 500 µM biotin and 0.5% DMSO for 1 hour or 3 hours, and with and without 100nM CL-316,243 (1mg/mL), 100nM insulin, 1x or 3x MEM Amino Acids (50X), 6.5 mM Glucose Solution (200g/L), and 1x Glutamax for 1 hour treatment.

Western Blots

Cells were lysed using Radioimmunoprecipitation assay (RIPA) buffer plus a protease inhibitor cocktail. Cell lysates were passaged through a 25-gauge needle ten times. Then vortexed and centrifuged at maximum speed (17,000rmp) for 20 minutes at 4˚C. A syringed was used to transfer the cells to a new tube without the lipid layer, cells were then sonicated. Protein concentrations were measured using a Pierced BCA Protein Assay Kit. 10 µg of total protein was

denatured using Laemmli buffer and sampled were heated at 95˚C for 10 min. Protein was loaded onto 10% SDS-PAGE gels and transferred to a nitrocellulose membrane for 1 hour at 100V. Membranes were blocked in 5% blocking buffer for 1 hour and then incubated with primary antibodies (1:1000 dilution) in 1% milk overnight at 4˚C. Horse radish peroxidase conjugated secondary antibodies (1:5000 dilution) were incubated for 1 hour. Western blots were developed using Radiance Plus Chemiluminescent substrate and detected by Azure Imaging System.

Antibodies and reagents

Glycogen synthase antibody (3893), Phospho-HSL Rabbit (4139S), HSL Rabbit (4107S) were purchased from Cell Signaling Technologies, Monoclonal Anti-Vinculin antibody produced in mouse (V9131) was purchased from Millipore Sigma.

Data Analysis

Mean (± Standard error) were calculated and plotted using Prism 7 comparative variable testing.

Results

HA-TurboID-GYS1 fusion protein did not hinder the function of endogenous GYS1 protein

To determine if the HA-TurboID-GYS1 fusion was successfully expressed in MPC1 F/F brown pre-adipocytes, whole cell lysate was analyzed by western blot (WB) analysis with and without the addition of 500 µM biotin and 100nM CL-316,243 (1mg/mL), a ß3-adrenergic receptor agonists, for 1 hour to induce acute thermogenesis (figure **1a**). In the absence of biotin, the pBABE cell line showed two bands that highlight endogenous self-biotinylation. However, cells that expressed the HA-TurboID-GYS1 showed a smear, indicating there were multiple proteins being biotinylated in response to biotin. An HA-Tag was used to locate ligase expression seen in the HA-TurboID-GYS1 cell line (figure **1b**). When cells were treated with biotin and in combination with CL-316,243, biotinylation did not change.

CL-316,243 treatment increased GYS1 protein expression

On day 6 of cell differentiation, glycogen synthase expression was measured to determine its role in acute thermogenesis (figure **2a,b**). Overall, when MPC1 F/F brown pre-adipocytes were treated with CL-316,243 there was an increase in GYS1 protein expression, but it was not significant. To address the variability in protein expression and determine if the CL-316,243 treatment was properly working, we measured the phosphorylation status of Hormone Sensitive Lipase (HSL). Thus, we measured Phospho-HSL and total HSL levels (Figure **3a**). In the presence of CL-316,243, Phospho-HSL protein expression increased. UCP1 protein expression was also measured but did no change under CL-316,243 treatment, Vinculin was used as a loading control (figure **3a**).

CL-316,243 and essential amino acids plus insulin increased GYS1 protein expression independently from each other

To further induce glycogen synthase expression, MPC1 F/F brown pre-adipocyte cells were differentiated for a longer period of time, day 8. GYS1 protein expression was reblotted under different treatment groups known to activate thermogenesis and the MTOR signaling pathway (Figure **4a-c**). When cells were treated with CL-316,243 and 100nM of Insulin plus 3X of essential amino acids (EAA) for 1 hour, GYS1 protein expression drastically increased. These finding suggests, GYS1 serve an important function during CL-316,243 treatment and the MTOR pathway, however, further studies need to be conducted. Biotinylation was then measured for the cells treated with insulin and essential amino acids, however, there was a lot of endogenous self-biotinylation in the pBABE cell line when compared to the HA-TurboID-GYS1 cell line (Figure **5a**). As a result, we decided to look at GYS1 protein expression on day 0 of cell differentiation.

GYS1 protein expression began to appear on day 0

When the cells were collected on day 0, GYS1 protein expression increased under insulin and EAA treatment (figure **6a**). A biotinylation time course was then conducted to reduce the amount of endogenous biotinylation (figure **7a**). Based on the results, we decided to add biotin for 3 hours and blotted for Streptavidin-HRP. Biotinylation decreased when insulin and EAA were added suggesting proteins may be dissociating their interactions with GYS1 (figure **8a**). To determine if insulin or EAA was inducing this dissociation, cells were treated with insulin, EAA, and insulin plus EAA. Biotinylation was then measured again, and it seems like when cells were

treated with insulin, proteins began to dissociate from interacting with GYS1. This dissociation

continued in the EAA, and combination treated groups (Figure **8b**). To determine if insulin or

EAA was inducing the over expression of GYS1, glycogen synthase expression was measured.

The cell lines exhibited an increase in GYS1 protein expression on the insulin treated groups and

combination treatment, when cells were treated with and without biotin (figure **8c, d**). GYS1

protein expression also increased when cells were treated with insulin and in combination with

EAA in both cell lines (figure **8c, d**).

Discussion

Obesity is a nationwide epidemic. It is predicted that by 2030 obesity prevalence will exceed 50% in 29 states and not fall below 35% in any state (Ward et al., 2019). Obesity has also been a major contributor to metabolic and cardiovascular disease. Therefore, thermogenic fat may serve as a potential therapeutic treatment for metabolic diseases. More recent studies have found individuals with BAT had lower prevalences of cardiometabolic diseases, this was also independently correlated with lower levels of type 2 diabetes (T2D), dyslipidemia, coronary artery disease, cerebrovascular disease, congestive heart failure, and hypertension. In addition to this, individuals exhibited lower glucose and triglyceride levels and increased high-density lipoprotein values (Cohen & Kajimura, 2021; Becher et al., 2021). However, the mechanism by which BAT regulates these functions remains unknown.

Therefore, our study focused on understanding the role of GYS1 in BAT under thermogenic conditions and the MTOR signaling pathway. Based on these results the ligase, HA-TurboID, was successfully fused to the gene of interest, GYS1. This fusion was also successfully transfected into MPC1 F/F mice brown pre-adipocytes. GYS1 also seemed to play an essential role during CL-316,243 treatment due to its increase in protein expression, however, its biotinylation levels did not change. These findings suggest GYS1 protein expression may be upregulated due other mechanisms, independent of protein interactions. Two possible explanations for these results could be due to the increase in ribosome biogenesis, leading to an increase in protein synthesis (Jiao et al., 2023). Or GYS1 could undergoing deubiquitination causing less proteins to be ubiquitinated for protein degradation (Suresh et al., 2016). However, further studies need to be conducted to understand how GYS1 is regulated in BAT during acute

thermogenic conditions. Furthermore, UCP1 levels did not change, therefore, this could not be used as an indicator of thermogenesis. However, previous studies have found the activation of the sympathetic nervous system stimulates parallel processes, such as thermogenesis and glucose uptake. Thus, thermogenesis coincides with glucose uptake. However, when this was tested on UCP1 (-/-) mice, with CL-316,243 treatment for one hour, glucose uptake increased. Therefore, Olsen et al. showed adrenergically-induced glucose uptake and thermogenesis are two separately regulated processes in BAT, and that acute uptake may occur via mTOR mediation (Olsen et al., 2017).

We also found GYS1 may also play a role in the mTOR pathway by disassociating its interactions with other proteins leading to an increase in its protein expression. However, further studies need to be conducted to validate these findings. Insulin may serve as a key driver of GYS1 protein expression and may have an additive effect when combined with essential amino acids, which are both involved in the mTOR signaling pathway. However, future studies need to be conducted to verify if the mTOR pathway is activated during these treatments by measuring S6K1 protein expression and Rapamycin, an mTOR inhibitor. However, previous studies have found a connection between glucose uptake and mTOR complex 2-promoted GLUT1 translocation (Olsen et al., 2014). However, based on our findings GYS1 may also be involved in glucose uptake but the mechanism remains unknown.

Although we were able to find some interesting findings, there were various limitations. One of them being that our findings were very broad. Based on the data that was collected, we are unable to provide a solid mechanism of why we see GYS1 expression increase under acute

thermogenic conditions. And although we did see a decrease in protein biotinylation under insulin and essential amino acid conditions, protein enrichment and proteomics must be carried out. These cells were also genetically modified by the cre/lox system; therefore, this could have modified the cells themselves causing them to respond differently.

Regarding future studies, the protocol from figure **4b** needs to be repeated and determine if biotinylation stays the same as in figure **1a**, or whether we do see a difference in protein interactions. We also need to remeasure the HA tag for all days to verify the ligase is still present. And need to measure ubiquitination and ribosomal expression under acute thermogenic conditions. For the insulin and essential amino acid treatment, S6K1 protein expression needs to be measured and cells must also be treated with Rapamycin. Also, protein enrichment and proteomics need to be carried out to identify which proteins are disassociating with GYS1 causing its overexpression. Glucose levels can also be measured to see if GYS1 is also increasing glucose uptake or decreasing.
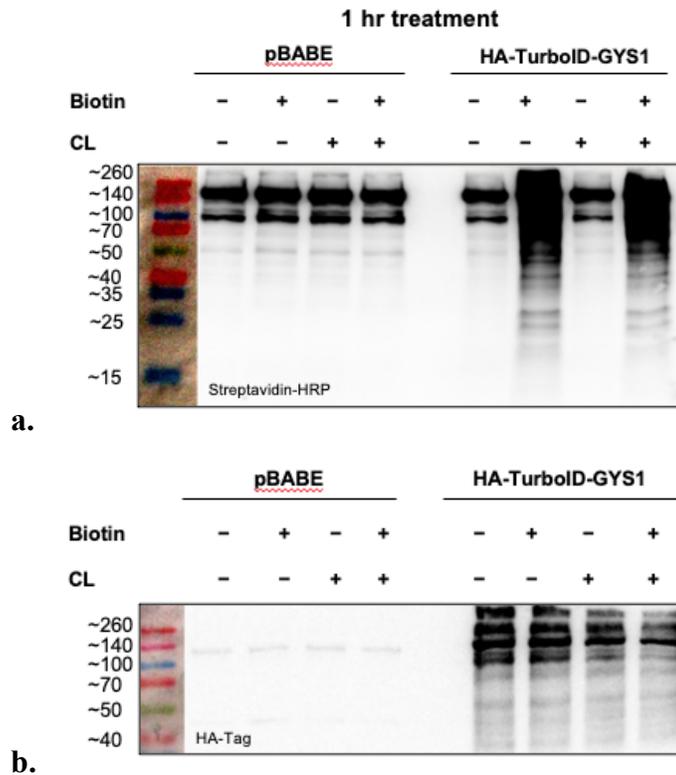
Figures

a.

b.

Figure 1. HA-TurboID-GYS1 fusion protein did not hinder the function of endogenous GYS1 protein

 pBABE and HA-TurboID-GYS1 cells were differentiated up to day 6 and treated with 100nM CL-316,243 (1mg/mL) and 500 µM biotin for 1 hour (**a**) Whole cells lysates were blotted with Streptavidin-HRP to visualize biotinylated proteins. (**b**) HA-Tag detects ligase expression (western blots done by Ezequiel Delgado).
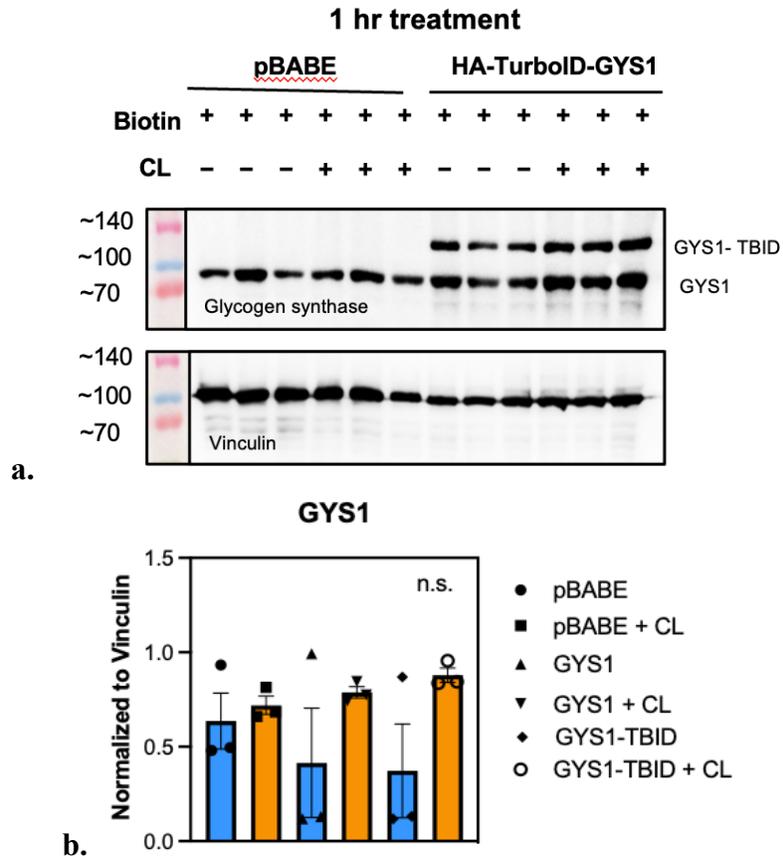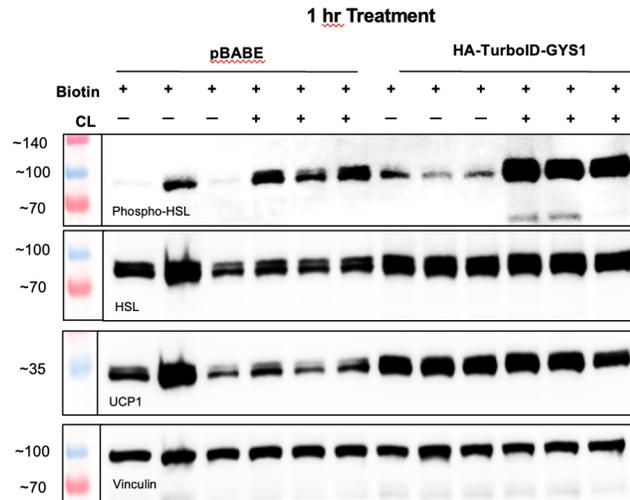
Figure 2. CL-316,243 treatment increased GYS1 protein expression but it was not significantly significant

pBABE and HA-TurboID-GYS1 cells were differentiated up to day 6, starved, and treated with 100nM CL-316,243 (1mg/mL), 1x MEM Amino Acids (50X), 6.5 mM Glucose Solution (200g/L), and 1x Glutamax for 1 hour treatment. (**a**) Whole cells lysates were blotted with Glycogen Synthase to visualize GYS1 and GYS1-TurboID (GYS1TBID) protein. (**b**) GYS1 protein expression was calculated using Vinculin as the loading control.

1 hr Treatment

Figure 3. Phospho-HSL was used as a positive control for CL-316,243 treatment

pBABE and HA-TurboID-GYS1 cells were differentiated up to day 6, starved, and treated with 100nM CL-316,243 (1mg/mL) and 500 µM biotin for 1 hour. (**a**) Phospho-HSL served as positive control for CL-316,243 treatment. HSL, UCP1, and vinculin were also blotted (western blots done by Jeslyn Zhang).
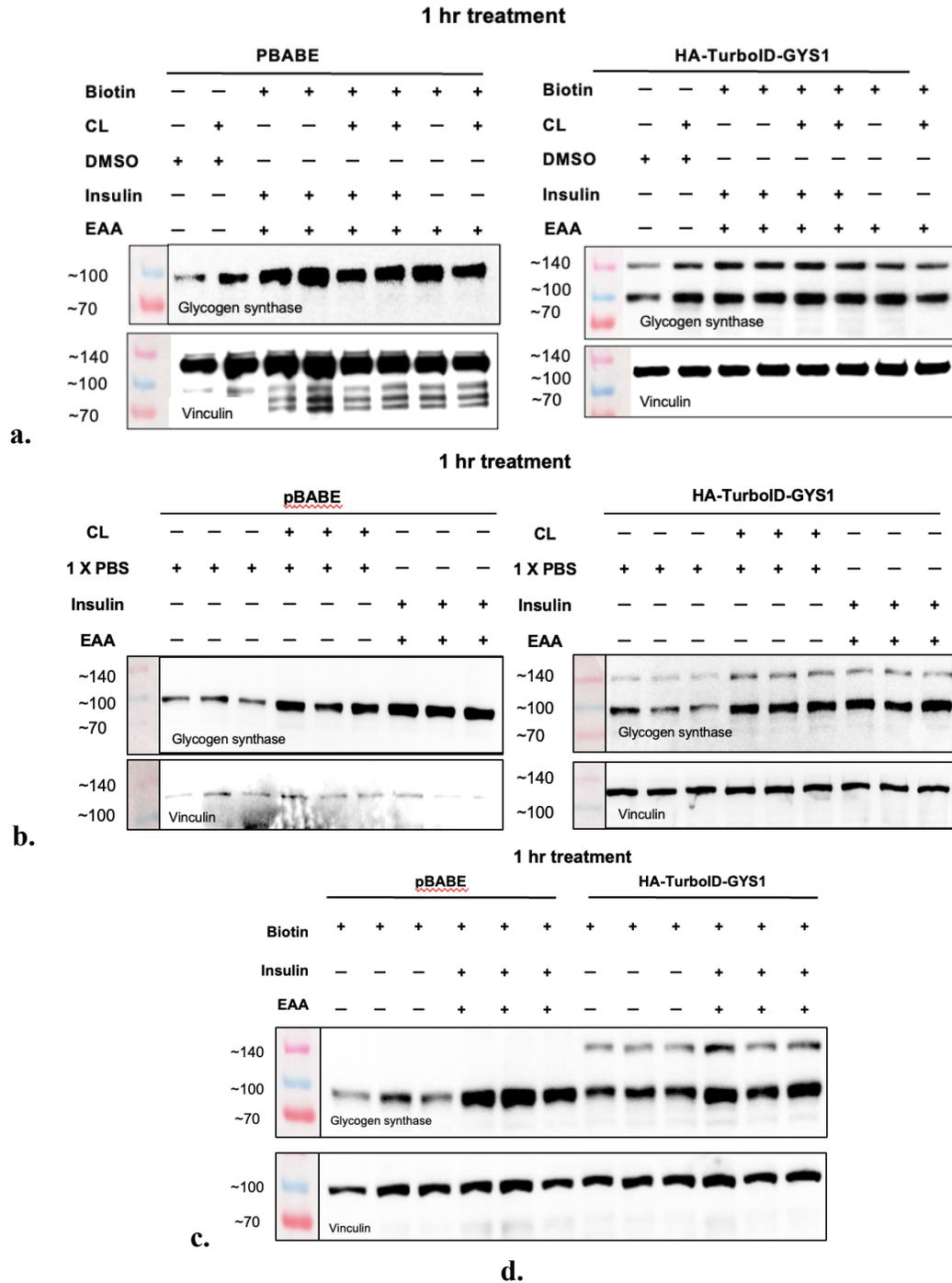
Figure 4. CL-316,243 and essential amino acids plus insulin increased GYS1 protein expression independently from each other

pBABE and HA-TurboID-GYS1 cells were differentiated up to day 8, starved, and treated with 100nM CL-316,243 (1mg/mL) and 500 µM biotin, 0.5% DMSO, 100nM of Insulin, and 3X of

essential amino acids for 1 hour. (**a, b, c, d**) Whole cells lysates were blotted with Glycogen

Synthase to visualize GYS1 and GYS1-TurboID (GYS1TBID) protein (c, western blot done by
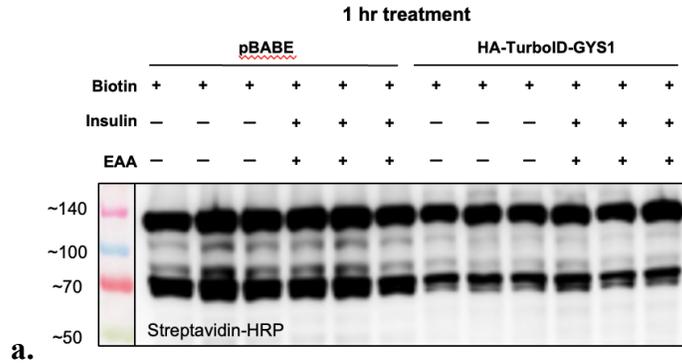
Jeslyn Zhang).

1 hr treatment

	pBABE						HA-TurboID-GYS1					
Biotin	+	+	+	+	+	+	+	+	+	+	+	+
Insulin	−	−	−	+	+	+	−	−	−	+	+	+
EAA	−	−	−	+	+	+	−	−	−	+	+	+

~140
~100
~70
~50

Streptavidin-HRP

a.

Figure 5. On day 8 of cell differentiation there was an increase in endogenous self-biotinylation

pBABE and HA-TurboID-GYS1 cells were differentiated up to day 8, starved, and treated with 100nM CL-316,243 (1mg/mL) and 500 μM biotin, 100nM of Insulin, and 3X of essential amino acids for 1 hour. (**a**) Whole cells lysates were blotted with Streptavidin-HRP to visualize biotinylated proteins (western blot done by Jeslyn Zhang).
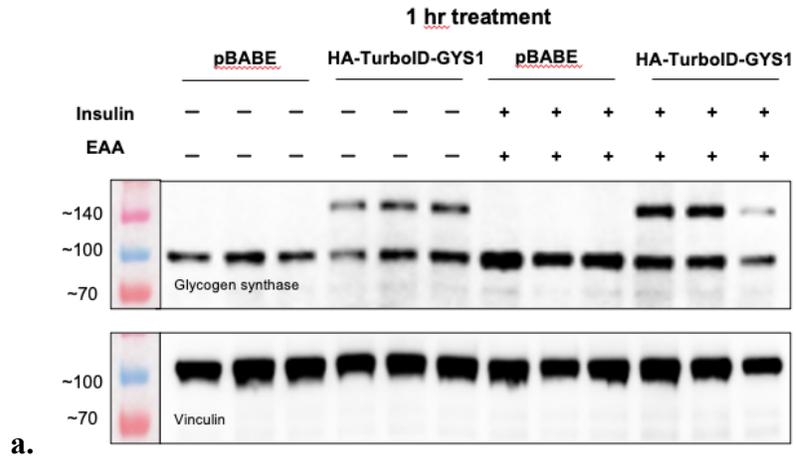
Figure 6. On day 0 of cell differentiation, GYS1 was also expressed

pBABE and HA-TurboID-GYS1 cells were grown to day 0, starved, and treated with 100nM of Insulin and 3X of essential amino acids for 1 hour. (**a**) Whole cells lysates were blotted with Glycogen Synthase to visualize GYS1 and GYS1-TurboID (GYS1TBID) protein (western blot done by Jeslyn Zhang).
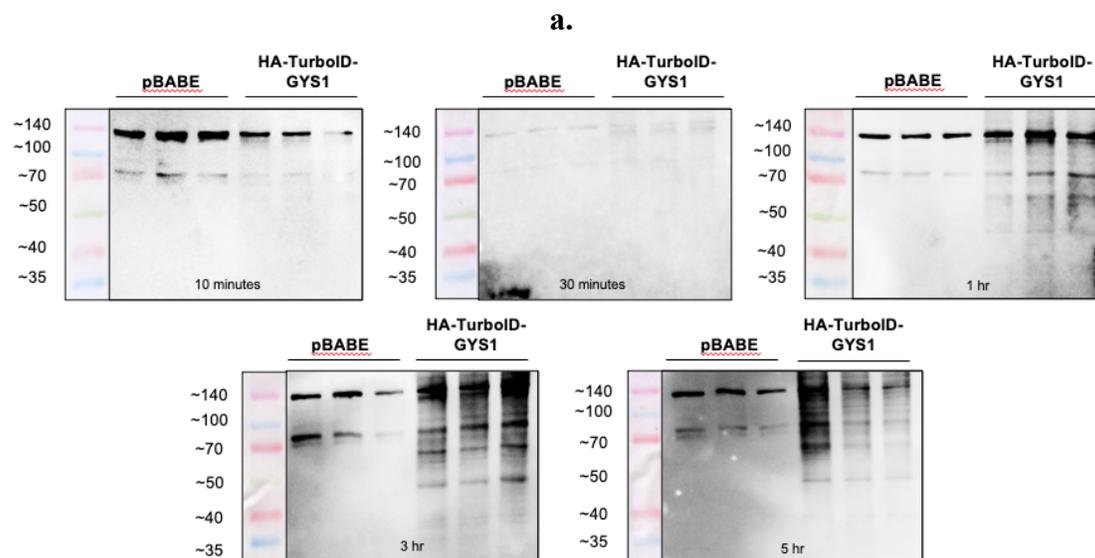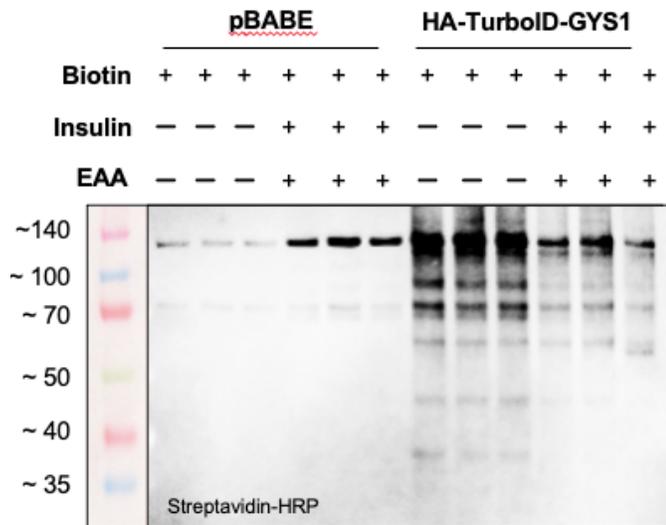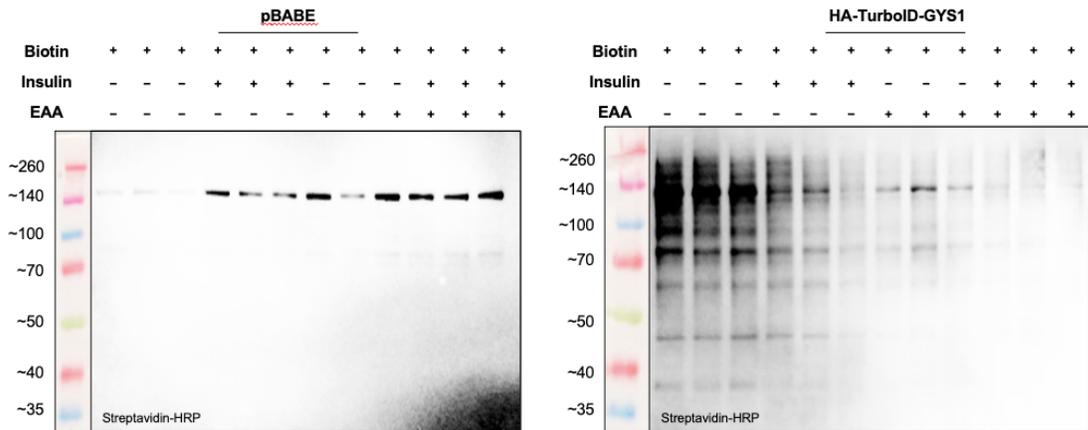
Figure 7. Biotinylayion time course on day 0

pBABE and HA-TurboID-GYS1 cells were differentiated up to day 0 and treated with 500 μM

biotin at different time points as indicated by the number on each blot. (**a**) Whole cells lysates

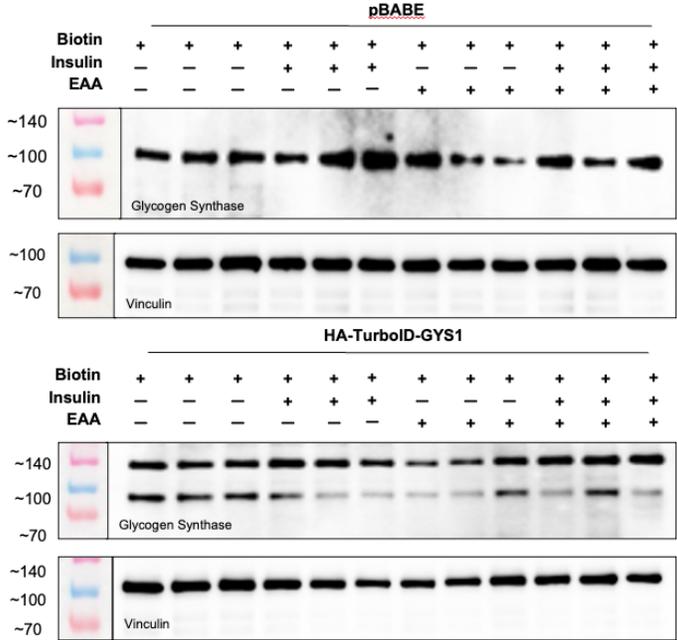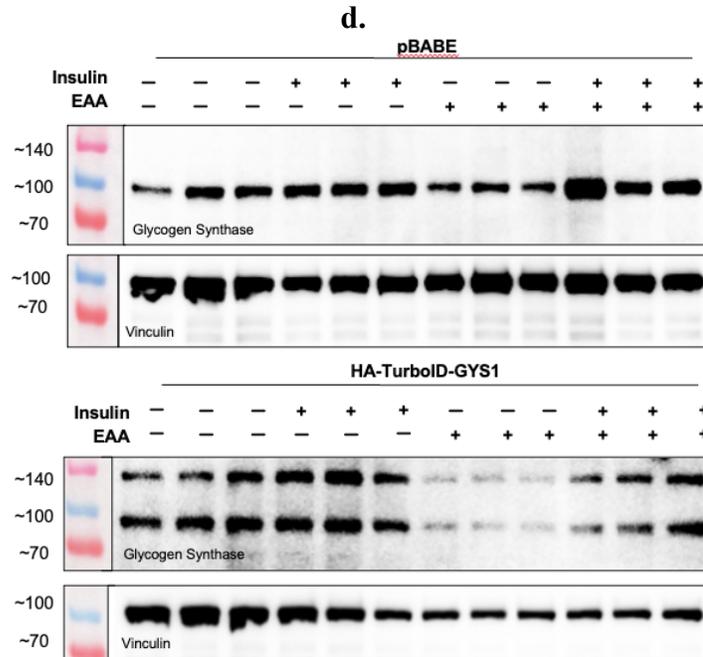were blotted with Streptavidin-HRP to visualize biotinylated proteins.

Figure 8. 3 hour Biotinylayion on day 0 under different treatment groups

pBABE and HA-TurboID-GYS1 cells were differentiated up to day 0 and treated with 500 µM

biotin for 3 hours and 100nM of Insulin, 3X of essential amino acids, and both 100nM of Insulin,

3X of essential amino acids in combination for 1 hour. (**a,b**) Whole cells lysates were blotted

with Streptavidin-HRP to visualize biotinylated proteins.(**c, d**) Whole cells lysates were blotted

with Glycogen Synthase to visualize GYS1 and GYS1-TurboID (GYS1TBID) protein (c and d,

western blot done by Jeslyn Zhang).

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