



Investigating the Impact of O-GlcNAc Regulatory Elements on the Gene Expression of ATF4

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Regulation of mitochondrial structure and function plays a vital role in cellular processes. The organelle facilitates control of energy metabolism, immune signaling, and apoptosis with the help of post-translation modifications (PTMs). PTMs are covalent modifications that occur during or after protein synthesis and can rapidly alter protein activity, localization, stability, or interactions. Mitochondrial stress can activate the integrated stress response (ISR), a cellular mechanism that regulates protein synthesis to maintain homeostasis. If mitochondria are impaired by stress it can lead to cascading effects linked to diseases such as neurodegenerative disorders, cancer, and others. During the ISR, eukaryotic translation initiation factor 2 alpha (eIF2 α) is phosphorylated, regulating the downstream synthesis of numerous proteins. Among these, eIF2 α phosphorylation promotes the translation of stress-related proteins, including activating transcription factor 4 (ATF4). Chronic ATF4 upregulation is observed in several disease states, many of which are also associated with mitochondrial stress and dysregulated O-GlcNAcylation, the covalent attachment of O-linked N-acetylglucosamine (O-GlcNAc). O-GlcNAcylation is an example of a PTM that occurs on the serine or threonine residues of thousands of proteins. O-GlcNAc transferase (OGT) is responsible for the addition of the sugar while O-GlcNAcase (OGA) removes the same sugar, potentially changing the activity of the protein. This dynamic maintains the cellular O-GlcNAc homeostasis, as well as regulation of mitochondrial function. Critically, O-GlcNAc homeostasis is believed to be indispensable to maintaining a healthy cell and preventing diseases such as cancer and neurodegenerative disorders. Our aim is to use a 'dead' CRISPR Cas9, 'dCas9', construct with either OGT or OGA fusions to target their activity specifically to promoter-associated proteins or chromatin at the ATF4 promoter. The SH-SY5Y neuroblastoma cell line will be used to determine how targeted OGT or OGA activity affects ATF4 expression during the ISR. This creates a model with the ability to observe the interaction between O-GlcNAc and ATF4 and how it may influence changes in gene expression. This study aims to understand the role that OGT and OGA play during a stress response and how the disruption of O-GlcNAc can impact mitochondrial function.

Current models of cellular function emphasize the dynamic interplay among metabolism, environmental conditions, and regulatory pathways. Within this complexity lies the mitochondrion, a crucial organelle in biological processes for maintaining cellular homeostasis. It is known for its role in generating adenosine triphosphate (ATP) and involvement in metabolic pathways. It also has a highly coordinated protein import and assembly where the majority of mitochondrial protein are encoded by nuclear DNA.¹ Defects in mitochondrial gene

expression can lead to dysfunction in diseases affecting muscular and neuronal tissues.²

Mitochondrial proteins participate in regulating apoptosis, cellular signaling pathways, and homeostasis, which is important for maintaining cellular health and responding to stress.¹ The mitochondrial integrated stress response (ISR) is a fundamental cellular adaptation mechanism with protective and pathological roles.³ It functions to modulate protein synthesis, convert energy, and direct resources during times of metabolic stress. While this process decreases production of housekeeping proteins, it also increases synthesis of stress-response proteins. A multi-protein factor, eukaryotic initiation factor 2 (eIF2 α), is central to the

ISR for regulating protein synthesis.⁴ When eIF2 α is phosphorylated by kinases such as Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK) and General Control Nonderepressible 2 (GCN2), translation of most mRNAs is repressed, while translation of activating transcription factor 4 (ATF4) is increased.⁵ This transcription factor is vital for regulating genes related to metabolism and facilitates a coordinated stress response by control of a range of adaptive genes involved in amino acid transport and redox homeostasis to promote cell survival. However, under persistent stress ATF4 promotes a switch to cell-cycle arrest and potentially apoptosis.⁶ The balance between this transcription factor's pro-survival and pro-apoptotic effects

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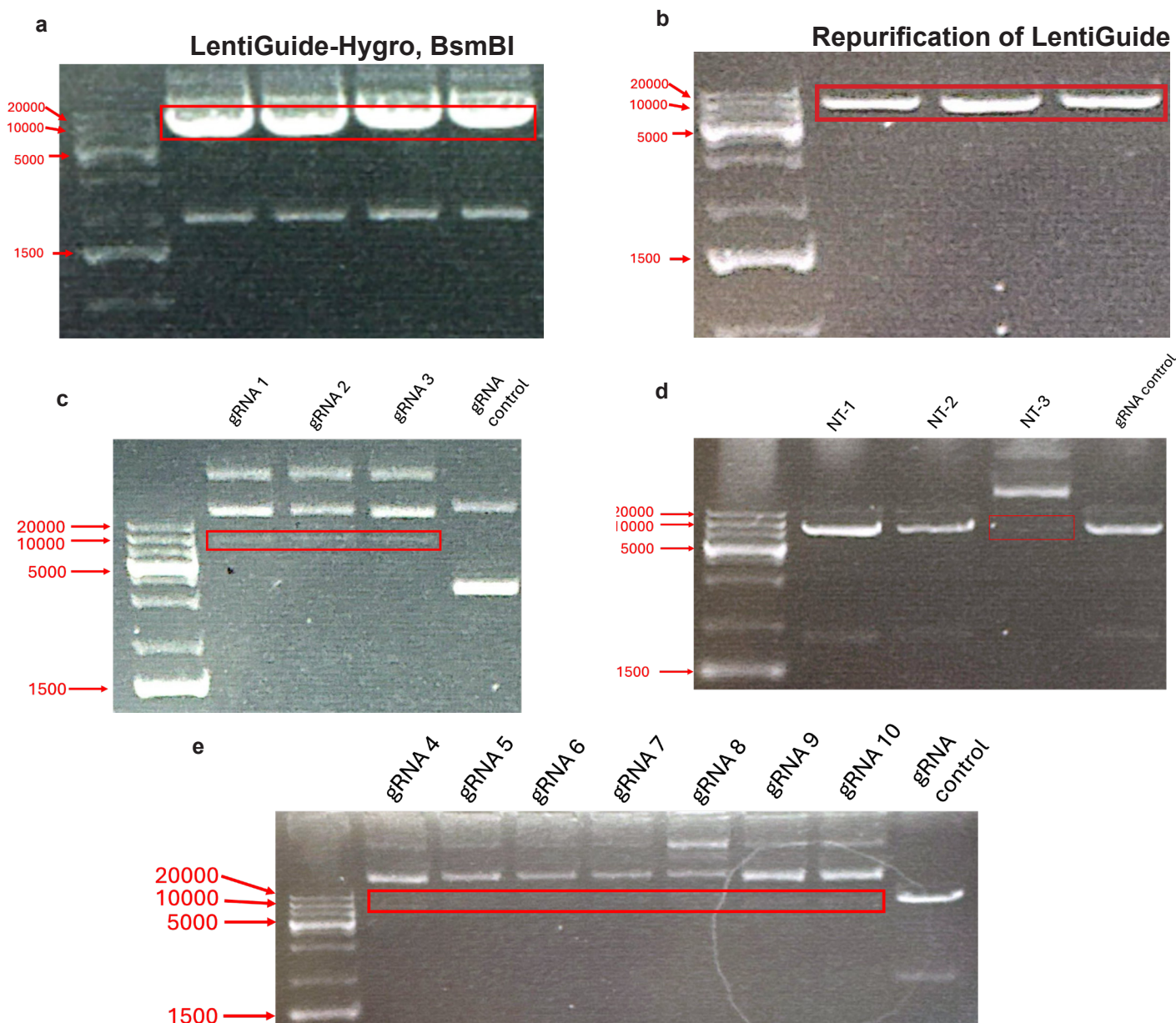


Figure 1 | Target guide sequence cloning using a lentiviral vector. **a**, The LentiGuide vector was digested with the restriction enzyme, BsmBI V2 and products were run on a 1% agarose gel. All lanes are the same sample. The top band (highlighted in red) was gel purified. **b**, The purified sample from Fig.1a was re-cut with BsmBI V2 and re-purified. **c-e**, Target guide sequence cloning plasmids, gRNA 1-10, NT 1-3, and gRNA control, were isolated by miniprep, digested with BsmBI V2 and run on a 1% agarose gel. Successful clones were identified by restriction pattern including the (very faint) ~8,000 bp band.

depends on regulation by post translational modifications (PTMs).⁷

PTMs are chemical changes that occur during protein synthesis that can change the stability, activity, and structure of proteins. Mitochondrial PTMs are used to regulate cellular homeostasis, respond to dynamic metabolic and environmental changes, and their dysregulation has been associated with several diseases.⁸ They also regulate a number of processes including epigenetics, gene expression, translation, and protein degradation.⁹ Glycosylation is a kind of PTM that involves the addition of a sugar to

a protein. A specific kind of glycosylation PTM involves O-linked N-acetylglucosamine modification (O-GlcNAcylation). O-GlcNAcylation occurs intracellularly and is catalyzed by two enzymes. O-GlcNAc transferase (OGT) adds O-GlcNAc to a serine or threonine amino acid residue of a protein whereas O-GlcNAcase (OGA) removes it.^{10,11} This PTM has the ability to modify thousands of proteins, including those directly within the mitochondria. Additionally, it functions directly as a cellular nutrient and stress sensor that has impacts on cellular physiology and disease progres-

sion. This sugar has been linked to diseases such as neurodegeneration, cancers, diabetes, and has been associated with mitochondrial dysfunction.⁹ Studies involving O-GlcNAc can be quite difficult because complete knockout of OGT or OGA can be lethal.¹²

To approach this obstacle, ATF4 can serve as a model target to observe what is occurring with O-GlcNAc during a stress response and as a model for genetic regulation via O-GlcNAc. ATF4 and O-GlcNAc both share metabolic sensor roles, where they are involved in metabolism,



stress response, cellular homeostasis, and mitochondrial function. ATF4 promotes transcription of activating transcription factor 5 (ATF5), which is also regulated by eIF2 α and includes an ATF4 promoter. 13–15. ATF5 transcription may provide an additional mechanism of measuring ATF4 activity. This construct will carry enzymes OGT or OGA to the ATF4 promoter region during a stress response, where they may interact with the transcription factor or other cis-regulatory elements. Regulation of ATF4 will be assessed by qPCR and Western blot as a measure of the effect O-GlcNAcylation has during a stress response.

Materials and Methods

Target guide sequence cloning

To insert the sgRNA sequence into the LentiGuide-Puro (lentiGuide-Puro was a gift from Feng Zhang (Addgene plasmid # 52963 ; <http://n2t.net/addgene:52963> ; RRID:Addgene_52963)), the Target Guide Sequence Cloning Protocol by Zhang Lab was used.^{16,17} The puromycin resistance region in the vector was previously replaced with hygromycin resistance via Gibson assembly. Gel purification was performed on the 8000 bp band after the LentiGuide plasmid was cut with BsmBI V2 (NEB, Cat No. R0739L Lot No. 10315738) in NEBuffer r3.1 (NEB, Cat No. B6003S Lot No. 10281919). The Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat No. D4007 Lot No. 267352) was used for the gel purifications. To phosphorylate the oligos, 1 μ L of 10X T4 DNA Ligase buffer (NEB, Cat No. B0202S) containing 1 mM ATP was added to oligonucleotides and T4 Polynucleotide Kinase (NEB, Cat No. M0236S). The LentiGuide backbone was ligated with the oligos using T4 DNA Ligase (NEB, Cat No. M0202S) and transformed into High Efficiency NEB 5-alpha Competent *E. coli* using their protocol (NEB, Cat No. C2987H).

Plasmid DNA isolation from bacterial cultures

Single colonies were selected from transformed bacteria on Luria-Bertani (LB) Miller agar plates (Fisher Scientific, Cat No. BP1425-500 Lot No. 106557) with 100 μ g/mL of ampicillin. Colonies were inoculated in LB liquid media with ampicillin overnight in a shaking incubator at 250 rpm at 37 °C. Plasmid DNA was isolated from

bacterial cultures using either a QIAprep Spin Miniprep Kit (Qiagen, Cat No. 27106 Lot No. 56903375) or ZymoPURE II Plasmid Maxiprep Kit (Zymo Research, Cat No. D4203-A Lot No. 269176).

Gel electrophoresis and restriction enzyme digests

All agarose gels used were made at 1% with TopVision Agarose (Thermo Scientific, Cat No. R0491 Lot No. 3374282) and 0.5x Tris/Borate/EDTA (TBE) buffer. All gel electrophoresis utilized GeneRuler 1 kb Plus Ladder (Thermo Scientific, Cat No. SM1333 Lot No. 00835607) and Gel loading Dye Purple 6x (NEB, Cat No. B7024A Lot No. 10308510) for samples. Gels were run for approximately 1–1.5 hrs. at 80 volts. OGT- and dOGT-dCas9 plasmids were digested with ClaI (NEB, Cat No. R0197S Lot No. 0361101) and KpnI (NEB, Cat No. R0142S Lot No. 0531010) with CutSmart Buffer (NEB, Cat No. B6004S Lot No. 10021126). OGA and dOGA constructs were digested with EcoRV (NEB, Cat No. R0195S Lot No. 0441101) and NEBuffer r3.1 (NEB, Cat No. B7003S Lot No. 0151502).

Cell culture

The SH-SY5Y neuroblastoma cell line was cultured in DMEM with 4 mM of D-(+)-Glucose (Sigma, Cat No. G7021-1KG Lot No. 080M01759V), 15 mg/L phenol red (Acros Organics, Cat No. 143-74-8 Lot No. A0266455), 44 mM sodium bicarbonate (Fisher Scientific Cat No. S233-3 Lot No. 170369), 1% GlutaMAX 100x (Gibco, Cat No. 35050-061 Lot No. 2360316), 10% Fetal Bovine Serum (GeminiBio, Cat No. 100-106 Lot No. A324000), and 1% penicillin–streptomycin (Gibco, Cat No. 15140-122 Lot No. 250552). Additional reagents for passaging cells included PBS (phosphate buffered saline) and 0.25% Trypsin-EDTA 1x (Gibco, Cat No. 25200-056 Lot No. 3136922).

Results

Target guide sequence cloning using a lentiviral vector

To deliver and express the single guide RNA (sgRNA) for the CRISPR-dCas9, a lentiviral vector, LentiGuide-hygromycin, was used.¹⁷ The LentiGuide backbone was isolated by digestion with BsmBI V2 fol-

lowed by running the entire reaction across several wells of an agarose gel (**Fig. 1a**). The digest produced two bands, one ~8,000 bp and one ~2,000 bp piece, where the 2 kb piece was an inconsequential portion of the vector and was discarded. The linearized ~8,000 bp band was extracted and gel purified. The isolated fragment was re-digested with BsmBI V2 to ensure that the initial digestion was complete and run on a second gel and gel-purified a second time (**Fig. 1b**). The target guide sequence cloning was preceded by phosphorylation and annealing of the oligos. In parallel reactions, the oligo duplex, a control duplex, and a water control were ligated to the digested LentiGuide and transformed into DH5 α . Single colonies were selected and grown overnight in LB supplemented with antibiotic and miniprep the following day. gRNA-LentiGuide samples were digested with BsmBI V2 (**Fig. 1c–e**). The large bands running slower than the 20,000 bp ladder band, represent uncut, supercoiled forms of the plasmid. The smaller, faint band, can be seen at the expected ~8,000 bp size. The ‘no-gRNA’ control sample included no oligo duplex during the ligation step. Colonies that grew from the control samples were a result of a re-ligated plasmid since there was no oligo duplex. Ten different gRNA samples were cloned and sequenced to ensure that the gRNA was successfully inserted into the LentiGuide-hygromycin.

Linear plasmid map of dCas9 constructs and restriction enzyme digests

The CRISPR-dCas9 constructs were previously designed and constructed by Dr. Matt Parker (manuscript in preparation and **Fig. 2a**). Constructs were made on a PUC19 backbone with a puromycin resistance selection marker. OGT-dCas9 had a control, dOGT-dCas9, which mirrored OGT but included a single amino acid change at the amino acid 558 position, in the OGT region, resulting in a histidine-to-phenylalanine substitution. OGA-dCas9 also had a control, dOGA-dCas9, with a modification at the amino acid 174 position, in the OGA region, of an aspartate-to-alanine substitution. To verify the constructs a restriction digest using ClaI and KpnI was performed. OGT -and dOGT -dCas9 plasmids showed the expected bands at 8055, 2757, and 1794 bp. OGA- and dOGA -dCas9 plasmids had expected bands at 7431, 3303, and 1512 bp which were seen on the gel as well (**Fig. 2b**).



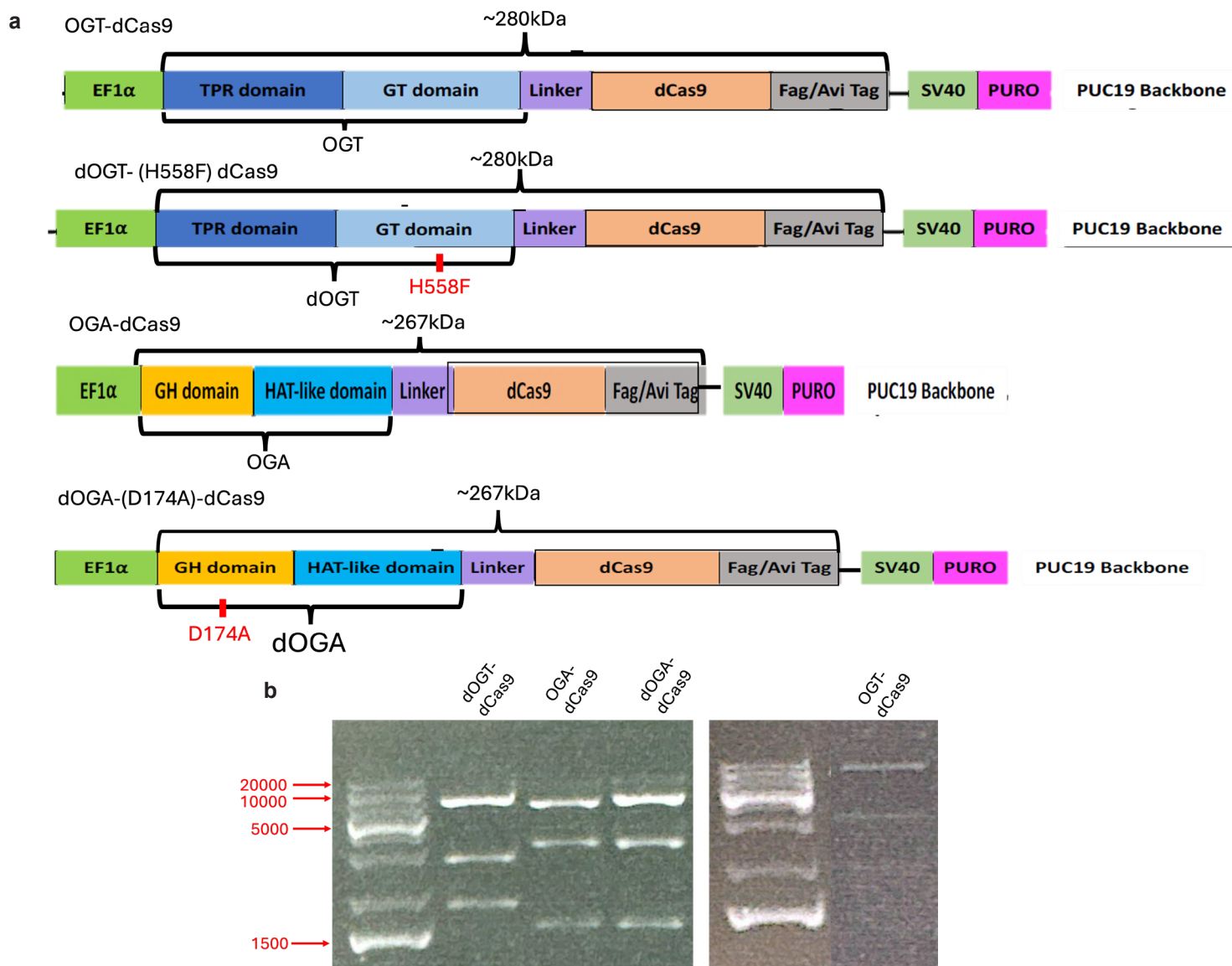


Figure 1 | Linear plasmid map of dCas9 constructs and restriction enzyme digest. a, Linear maps of plasmid dCas9 constructs OGT, dOGT, OGA, and dOGA. **b,** dOGT-dCas9 was cut with enzymes Clal and KpnI with expected bands at 8055, 2757 and 1794 bp. OGA-dCas9 and dOGA-dCas9 were cut with EcoRV with expected bands at 7431, 3303, and 1512 bp. OGT-dCas9 was also cut with Clal and KpnI restriction enzymes, with expected bands at 8055, 2757 and 1794 bp.

Sanger sequencing of dCas9 plasmid constructs

Restriction digests were performed on putative positive clones to rule out obviously flawed constructs. However, the OGX/dOGX constructs each produced identical restriction digests, therefore, to further verify that the CRISPR dCas9 plasmid constructs had the correct mutations and DNA sequence, they were sent for Sanger sequencing. An alignment was performed on the OGA portion of OGA-dCas9 against the wild type enzyme. Both constructs should have had a GAT, aspartic acid, at the 174th amino acid, which was confirmed by direct sequencing. At the same position, the

dOGA-dCas9 was constructed to include an inactivating mutation, GCT, encoding alanine, which sequencing also confirmed (Fig. 3).

Similarly, OGT-dCas9 includes a CAT codon encoding histidine at the 558th amino acid position of the OGT region. The alignment of sequencing results showed that the OGT-dCas9 had a TTT codon encoding phenylalanine at this position, meaning the 'OGT-dCas9 construct' was a mislabeled dOGT-dCas9 construct. dOGT-dCas9 alignment also included a TTT at the mutation site, confirming that the dOGT-dCas9 construct was labeled correctly (Fig. 3). Another mutation was found upstream of both OGT and dOGT -dCas9 at the 498th

amino acid where a second histidine to alanine mutation was found (Fig. 3). To confirm, multiple aliquots of the same construct were sequenced, and the same mutation was found. This 'mutated' 498th amino acid of OGT matched the OGT RefSeq from NCBI, revealing that the difference found from the original sequence data was due to an allelic variant. Given this, the 498th amino acid was accepted and not changed.

Discussion

Previous research of OGT and OGA have examined the cellular effects of its deletion. Complete OGA knockouts have shown re-

duced OGT expression and undetectable OGA levels.¹⁸ Knockout of OGT in mice have found substantial defects in termination of liver regeneration with increased liver injury.¹⁹ CRISPR-Cas9 has been utilized to create these knockouts and investigate substrate function. Using a CRISPR-dCas9 system provides a new tool for observing O-GlcNAcylation in cells. This project was intended to provide preliminary data to measure the influence of O-GlcNAc regulatory elements' effect on the gene expression of ATF4. The gRNA was successfully inserted into the LentiGuide-hygro vector and is ready for transfection. To transfect with the gRNAs, the CRISPR -dCas9 plasmid constructs must also be set. Based on the restriction enzyme digest of the CRISPR -dCas9 constructs, the resulting band sizes are as predicted, and the expected components are present. From the sequencing of the O-GlcNAc regulatory elements in the CRISPR -dCas9 plasmid constructs, OGA and dOGA have the correct sequences at the mutation site. The construct labeled as OGT-dCas9 was determined to be mislabeled dOGT-dCas9 constructs and a correct OGT-dCas9 was not located. An additional base mismatch at the 498th amino acid was also discovered but was indicative of a 'normal' allelic variant. Therefore, the dOGT-dCas9s is ready for transfecting, but

the OGT version will need to be remade. This verification was crucial to ensure differences in gene expression are truly based on the regulatory element. Although this prevented the project from proceeding with transfection, it was necessary to verify these constructs before they could produce confounding results. There are several methods available to rectify the mutation or recreate the construct entirely. With the validated OGT construct in hand, transfection of SH-SY5Y cells may proceed.

Future Directions

To continue this project, a new OGT construct must be made via Gibson assembly or site-directed mutagenesis. Either method will correct the error to generate a new CRISPR-dCas9 construct. When the construct is verified by full plasmid sequencing, H-SY5Y cells can then be transfected by electroporation. To select transformants after electroporation, media will be supplemented with both hygromycin and puromycin will be used. The LentiGuide vector contains hygromycin resistance, which took the place of the puromycin resistance gene in the original construct. Using both antibiotics, only cells that have both plasmids will survive. Additionally, direct confirmation of O-GlcNAc labeling will

be necessary to confirm that the model is performing as expected. After selection of stable cell lines, samples can be collected for comparative qPCR and western blots. qPCR of the ATF4 transcripts will allow for an initial quantification of the impact of O-GlcNAc-mediated regulation. Western blots will be used to assay the relative changes in protein abundance of OGT, OGA, and ATF4. Through these two assays, differences in gene expression can be measured based on the regulatory element being integrated into the cell. The possibility that these constructs may lead to unexpected gross alterations in cellular health remains, although ATF4^{-/-} and ATF5^{-/-} animals are viable, suggesting that dysregulation of this pathway will not be toxic to cells.

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OGA-dCas9

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501>ATCATTGCTTTGCTTTTGGATGATATAGACCATAATATGTGTGCAGCAGACAAAGAGGTATTCAGTTCTTTTGCTCATGCCCAAGTCTCCATCACAAAT>600
677>|||||
677>ATCATTGCTTTGCTTTTGGATGATATAGACCATAATATGTGTGCAGCAGACAAAGAGGTATTCAGTTCTTTTGCTCATGCCCAAGTCTCCATCACAAAT>776
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dOGA(D174)-dCas9

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501>ATCATTGCTTTGCTTTTGGATGATATAGACCATAATATGTGTGCAGCAGACAAAGAGGTATTCAGTTCTTTTGCTCATGCCCAAGTCTCCATCACAAAT>600
679>|||||
679>ATCATTGCTTTGCTTTTGGATGATATAGACCATAATATGTGTGCAGCAGACAAAGAGGTATTCAGTTCTTTTGCTCATGCCCAAGTCTCCATCACAAAT>778
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OGT-dCas9

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11101>CATCCAAAAGACTTGAAGCTCAGTGATGGTCGGCTGCGTGTAGGATATGTGATTCGACTTTGGGAATTCCTACTTCTCACCTTATGCAGTCTATTC>11200
347>|||||
347>CATCCAAAAGACTTGAAGCTCAGTGATGGTCGGCTGCGTGTAGGATATGTGATTCGACTTTGGGAATTCCTACTTCTCACCTTATGCAGTCTATTC>446
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dOGT(H558F)-dCas9

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11101>CATCCAAAAGACTTGAAGCTCAGTGATGGTCGGCTGCGTGTAGGATATGTGATTCGACTTTGGGAATTCCTACTTCTCACCTTATGCAGTCTATTC>11200
347>|||||
347>CATCCAAAAGACTTGAAGCTCAGTGATGGTCGGCTGCGTGTAGGATATGTGATTCGACTTTGGGAATTCCTACTTCTCACCTTATGCAGTCTATTC>446
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Both OGT and dOGT(H558F)-dCas9

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10901>ATTGGACAGACTATGATGAGCGAATGAAGAAGTTGGTCAGTATTGTGGCTGACCAGTTAGAGAAGAATAGGTGCTTCTGTGCATCCTTTTCATAGTAT>11000
147>|||||
147>ATTGGACAGACTATGATGAGCGAATGAAGAAGTTGGTCAGTATTGTGGCTGACCAGTTAGAGAAGAATAGGTGCTTCTGTGCATCCTTTTCATAGTAT>246
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Figure 3 | Sanger sequencing of dCas9 plasmid constructs. Primers were made previously for sanger sequencing and OGT or OGA regions were sequenced. OGT and dOGT -dCas9 had an additional mutation found at the 498th amino acid within the OGT region.



Author Biography

Nathaly is an emerging scientist in the Biotechnology Program at KU Edwards. She has always had a passion for science and research, as well as strategic problem-solving. She is eager to continue learning and expand these interests.

Author Contributions

N.WP. contributed to the design & execution of the work and wrote the manuscript. M.P. providing reagents used in this project. C.C. provided mentoring, funding, reagents, and space. B.M., J.F.T. M.D., and S.T. contributed mentorship and writing.

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