ABSTRACT

Title of Thesis: BIOINFORMATIC ANALYSIS AND DESIGN OF A GLUT3 CONSTRUCT TO EXAMINE CELL SURFACE EXPRESSION

Sandra Ogunwole, Master of Science, December 2019

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Department of Biology

Glucose transporter type 3 (GLUT3) expression at cell surface plays a vital homeostatic function in neurons. However, the mechanism involved has yet to be fully examined. We devise a strategy to tag a rat GLUT3 cDNA clone. This will be used to assess GLUT3 localization at cell surface. To install the tag, we attempt to generate a point mutation at the first exofacial loop, which would result in a unique Hpa1 restriction site in GLUT3. In another strategy, to compare total versus surface GLUT3, we propose an HA-GLUT3-eGFP fusion protein. Bioinformatic analyses suggest that this protein would be functional. GLUT3 movement to plasma membrane is carried out through vesicles. These vesicles interact with many trafficking proteins that regulate GLUT3 surface expression. Data mining using the STRING database has identified potential GLUT3-interacting proteins. Future work will focus on expressing tagged-GLUT3 and examining interactions with these proteins.

BIOINFORMATIC ANALYSIS AND DESIGN OF A GLUT3 CONSTRUCT TO EXAMINE CELL SURFACE EXPRESSION

By

Sandra O. Ogunwole

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Chapter 1: Introduction

Neuronal Cells: Glucose Transport

Neurons are high energy consuming cells that express glucose transporter type 3 (GLUT3), the neuronal glucose transporter. The expression of GLUT3 increases at cell surface in response to neural activity. However, the mechanism by which GLUT3 expression at cell surface is regulated remains to be elucidated. The brain utilizes glucose as the obligatory energy substrate. The brain represents only 2% of the body by mass but consumes 25%-50% of its available glucose. (Amato & Man, 2011; Bélanger, Allaman, & Magistretti, 2011). We note that glucose is the primary and preferred substrate needed to satisfy the energy requirements of all cell types (Kvidera et al., 2016; Mergenthaler, Lindauer, Dienel, & Meisel, 2013; Oberdanner, Kiesslich, Krammer, & Plaetzer, 2002). Therefore, the regulation of glucose transport is an important biological question. We focus on GLUT3 function in neurons and expression at cell surface.

GLUTs are a protein family of facilitative transporters. The GLUT protein family is characterized by 12 transmembrane helices which anchor each protein isoform to the plasma membrane of their respective target cells. GLUT proteins are derived from the SLC2A (solute carrier family 2) gene family (Mueckler & Thorens, 2013). In humans, the genes coding for the various isoforms of these GLUT proteins are located on chromosomes 1, 3, 4, 6, 9, 12, 17, 20, and 22 (Zhao & Keating, 2007). There are 14 known isoforms, divided into three classes based on sequence similarity and substrate specificity. Class I GLUTs consist of GLUT1, 2, 3, 4, and 14, class II GLUTs comprise GLUT5, 7, 9, 11, and class III GLUTs include GLUT6, 8, 10, 12, and 13 (Cura & Carruthers, 2012). Studies on GLUTs have shown that along with the similarity in protein structure, about 50% of their sequence is conserved based on their class (Simpson et al., 2008).

Table 1 below shows class I glucose transporters which are associated with high energy dependent cells such as neurons and muscle cells. GLUT1, a class I glucose transporter possesses an N-glycosylation in its first extracellular loop. This N-glycosylation affects GLUT1 stability, substrate binding affinity, and possibly targeting (Asano et al., 1991, 1993). The conserved sequences and similarity between GLUTs of the same class leads us to infer that the presence of

the same N-glycosylation sites in GLUT3 and GLUT4, confer similar characteristics to these glucose transporters.

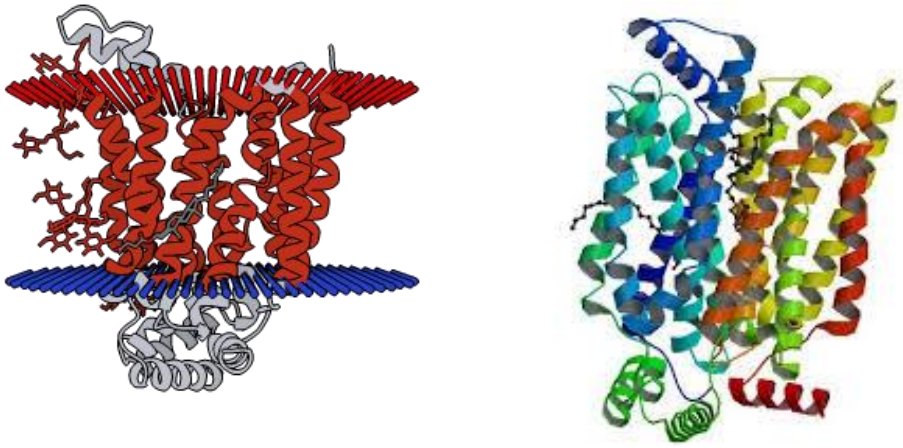
A study by Maher et al. shows that GLUT3 is a high-apparent-affinity, high-activity, glucose transporter (Maher, Davies-Hill, & Simpson, 1996). Due to its high binding affinity for glucose, GLUT3 is a very important transporter in the embryo as well as in the brain. In a study by Schmidt et al., knockout of GLUT3 in mice embryos caused rapid developmental regression and death (Schmidt et al., 2009). GLUT3 also "has a higher affinity for glucose than GLUT1, 2, or 4 and at least a fivefold greater transport capacity than GLUT1 and 4. This is particularly significant for the role of GLUT3 in neuronal transport, as the ambient glucose levels surrounding the neuron are only 1–2 mM compared with 5–6 mM in serum" (Simpson et al., 2008).

Table 1: Class 1 Glucose Transporters (Mueckler & Thorens, 2013) showing GLUT3 as the glucose transporter expressed in neurons and testis.

Human gene name	Protein name	Predominant substrates	Tissue distribution and cellular/ subcellular expression	Link to disease	Human gene locus	Sequence accession ID
SLC2A1	GLUT1	glucose, galactose, mannose, glucosamine	erythrocytes, brain, blood-brain barrier, blood-tissue barrier, many fetal	paroxysmal exertion-induced dyskinesia, dystonia-18, Glut1 deficiency syndrome	1p35-31.3	NM_006516
SLC2A2	GLUT2	glucose, galactose, fructose, mannose, glucosamine	liver, islet of Langerhans, intestine, kidney, brain	Fanconi-Bickel syndrome, (type 2 diabetes)	3q26.1-q26.2	NM_000340
SLC2A3	GLUT3	glucose, galactose, mannose, xylose	brain (neurons), testis		12p13.3	NM_006931
SLC2A4	GLUT4	glucose, glucosamine	adipose tissue (white and brown), skeletal and cardiac muscle	(type 2 diabetes)	17p13	NM_001042
SLC2A14	GLUT14		testis		12p13.31	NM_153449

GLUT3: The Protein

GLUT3 as the name implies, was the third glucose transporter to be cloned (Fukumoto et al., 1988). This glucose transporter is known to be expressed in cells with high energy demands such as white blood cells, cancer cells, preimplantation embryos, and neurons (Carayannopoulos et al., 2014; Schmidt et al., 2009; Simpson et al., 2008). GLUT3 is a ~481 amino acid transport protein, which weighs ~53KDa or 52,520 Dalton. The crystal structures for GLUT3 are shown below.



(a) (b)

Fig. 1: GLUT3 crystal structure (a) transmembrane view PDB: 5c65 (Pike et al., n.d.) (b) bound D-glucose in a binding pocket formed by Transmembrane (TM) 8, TM10a, and TM10b 4ZW9 (Deng et al., 2015).

GLUT3 Cell Surface Expression

Studies by Ferreira et al. (2011) and Weisová et al. (2009) have shown that synaptic activity increases the expression levels of GLUT3 at the cell surface in neurons. Increasing N-methyl-D-Aspartate Receptor (NMDAR) activity, a glutamatergic receptor, triggers the hydrolysis of ATP (Attwell & Laughlin, 2001; Ferreira, Burnett, & Rameau, 2011; Mori & Mishina, 1995; Weisová, Concannon, Devocelle, Prehn, & Ward, 2009). The increased expenditure of ATP in the cell creates a need for more ATP, stimulating glucose influx through GLUT3 in neurons.

Research by Ferreira et al. (2011) highlights the role of nitric oxide (NO) /cGMP kinase pathway on GLUT3 surface expression following NMDAR activation. Glutamate excitation, which activates the NMDAR, elevates intracellular free Ca^{2+}. Free Ca2+ forms a Ca2+/calmodulin complex that binds to neuronal nitric oxide synthase (nNOS) to stimulate NO production. However, optimal nNOS activity in cells requires post-translational modification through phosphorylation. NMDAR mediates Akt activation phosphorylates nNOS at Serine 1412, which seems to be required for increasing NO production in neurons. The increased NO stimulates cGMP and cGMP-dependent kinase pathway, leading to increase GLUT3 expression at neuronal cell surface. However, the mechanism by which that happens is unknown (Ferreira et al., 2011). Further experiments expressing a NOS phosphomimetic mutant (S1412D) shows an increase in GLUT3 surface expression in neurons. (Ferreira et al., 2011).

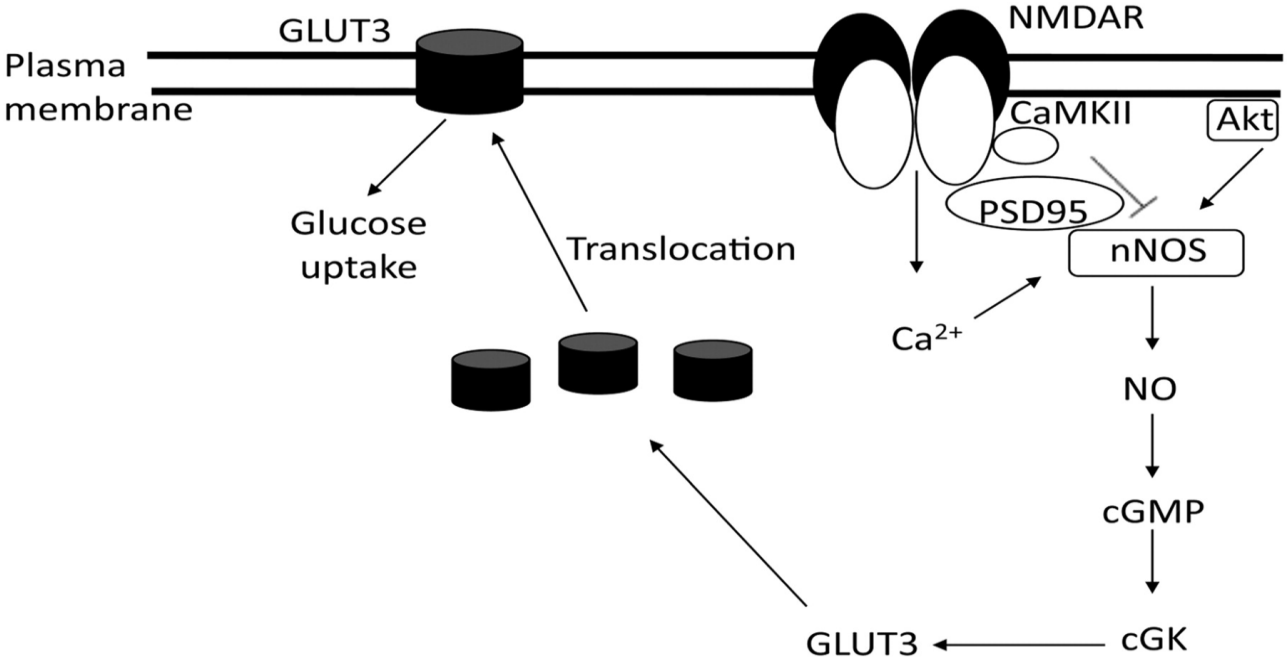


Fig. 2: The NO/cGK pathway to increased GLUT3 expression at neuronal cell surface (Ferreira et al., 2011)

Research by Khatri and Man also highlights that the activation of glutamatergic receptors allow sodium and calcium to flow into the cell. The influx of sodium is balanced by sodium pumps which are dependent on ATP for energy. It is thought that hydrolysis of ATP by these pumps cause alterations in the AMP: ATP levels in the cell (Khatri & Man, 2013). Increased AMP levels

activates AMP-dependent kinase (AMPK) which works to regulates energy homeostasis in the cell.

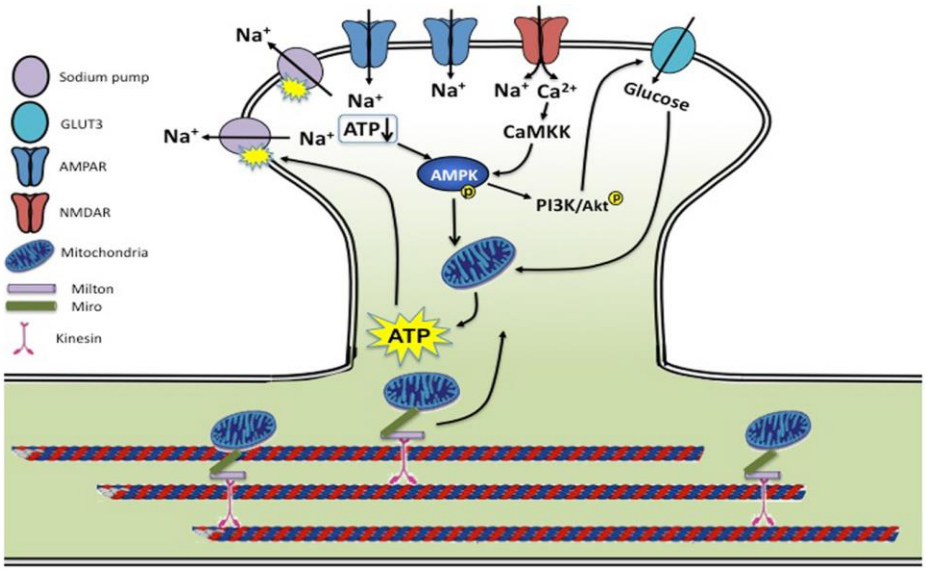


Fig. 3: an AMPK/PI3K pathway to increased GLUT3 expression at neuronal cell surface (Khatri & Man, 2013).

Cidad et al. showed that NO induces AMPK-Thr172 phosphorylation leading to increased GLUT3 activity in astrocytes and HEK293 cells in a NO dependent manner (Cidad, Almeida, & Bolaños, 2004).

Nitric Oxide (NO) / NOS

Nitric oxide (NO) is a free radical gas which was identified as a biological molecule over three decades ago. NO was first discovered as the Endothelial-Derived Relaxing Factor (EDRF) which acts via stimulation of soluble guanylate cyclase. In blood vessels, NO functions as the endothelium-derived relaxing factor; NO also works to inhibit platelet aggregation and vascular smooth muscle cell proliferation, playing a role as an endogenous nitro vasodilator and a uterine muscle relaxant (Ignarro, Byrns, Buga, & Wood, 1987; Loscalzo, 2013; Moncada, 1999; Moncada & Higgs, 2006; Palmer, Ferrige, & Moncada, 1987; Russwurm & Koesling, 2004). Scientists Robert F. Furchgott, Louis J. Ignarro and Ferid Murad won the Nobel Prize in physiology or medicine (1998) for their discoveries of nitric oxide as a signaling molecule in the cardiovascular system ("The Nobel Prize in Physiology or Medicine 1998," n.d.).

NO is synthesized biologically from the amino acid, L-arginine via the enzyme, NO synthase (Moncada, Palmer, & Higgs, 1989; Vincent, 2010). NO synthase (NOS) has three known isoforms: inducible - iNOS, endothelial – eNOS, and neuronal - nNOS with the last two NOS isoforms being activated in a calcium dependent manner (Contestabile, 2000; Moncada & Higgs, 2006, 2006). Fig. 4 below shows the different NO sources and their activators in a biological system.

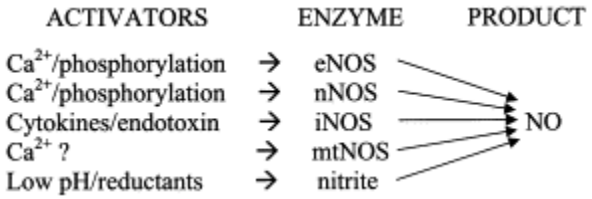


Fig. 4: Sources of Nitric Oxide (NO) in mammalian cells (Brown, 2001)

The NOS protein isoforms have about 50% homology between them however, their genes are expressed in different cell types and the isoforms are encoded in chromosomes 17, 7, and 12 respectively (Moncada, 1999; Prast & Philippu, 2001).

NO is known to bind rapidly with ferrous (Fe2+) heme prosthetic group in cGK that activates cGMP production in cells, including neurons to bring about neurotransmission, and smooth muscle relaxation as an EDRF (Arnold, Mittal, Katsuki, & Murad, 1977; Brown & Borutaite, 2001; Denninger & Marletta, 1999; Ignarro et al., 1987; Moncada et al., 1989; Russwurm & Koesling, 2004). Physiologic NO functions as a neurotransmitter and neuromodulator in neurons. It is synthesized on demand i.e. after nerve stimulation in a calcium dependent manner (Moncada & Higgs, 2006). NO has been implicated in pathologic conditions such as ischemia, hypoxia, and excitotoxicity.

NO interacts with Mitochondria to activate AMPK pathway

Sustained production of NO can inhibit mitochondrial complex IV (cytochrome C oxidase) by competing with oxygen binding at the complex. The competitive inhibition at the complex can lead to mitochondrial oxidative stress, leading to cell apoptosis. However, continued NO inhibition of mitochondrial complex IV can lead to cell protection from apoptosis (Beltrán, Mathur, Duchen, Erusalimsky, & Moncada, 2000; Brown & Borutaite, 2001). Inhibition of respiration in mitochondria can lead to increase in reactive oxygen species leading to increase AMP: ATP ratio through

inhibiting ATP production by mitochondria (Brown, 2001). The increase in AMP levels in cells lead to activation of the AMPK enzyme which activates an AMPK pathway to increase ATP production and reduce ATP consumption (Amato & Man, 2011).

AMPK & THE AMPK PATHWAY

AMPK is a heterotrimeric - 3 subunit (α, β, and γ) enzyme that is expressed in the cells of the liver, brain, skeletal muscle etc. AMPK is a heterotrimeric protein consisting of α1, α2 (Catalytic at N-terminus), β1, β2, and γ1, γ2, and γ3 (Bateman domain) subunit isoforms. Each of these isoforms are expressed in differential patterns across cell types. In neurons, the γ1 subunit is dominantly expressed along with α2 while either the β 1 or β 2 could be expressed (Amato & Man, 2011). AMPK can be regulated by AMPK kinases (AMPKK) some known upstream kinases: TAK1, LKB1, and CAMKKα/β are known to phosphorylate AMPK. AMPK can also be activated by pharmacological agents as well as by cellular stresses that elevates AMP/ADP (Amato & Man, 2011; Mihaylova & Shaw, 2011).

AMPK can sense changes in the energy status of the cell and send signals via its serine/threonine kinase activity to switch off ATP consuming pathways and switch on ATP producing pathways such as fatty acid oxidation, and glucose uptake in the cell (Khatri & Man, 2013). This enzyme is key in the energy signaling system. It acts as a bioenergy detector as the Bateman domains bind AMP or ATP exclusively in a cooperative manner to sense when AMP levels increase in relation to ATP. AMPK is activated in response to increased AMP and decreased ATP levels in the cell. The structure of AMPK allows for binding of two AMP molecules at the Bateman domains on the γ subunit in a cooperative manner. The binding of an AMP molecule at one Bateman domain of AMPK produces a conformational change in the enzyme complex that exposes Thr172 for phosphorylation by upstream kinases (AMPKKs) and inhibits protein phosphatase mediated dephosphorylating of Thr172. This activation mechanism enables AMPK to respond even at low levels (Amato & Man, 2011; Lipovka & Konhilas, 2015; Xiao et al., 2011).

In neurons, AMPK is activated because of the high energy consumption that occurs as a result of synaptic activity and the molecular events that accompany synaptic transmission. Synaptic

activity such as glutamate excitation involves the activation of receptors such as the NMDAR, sodium pumps, and other processes, some of which would require ATP hydrolysis thereby, increasing the level of AMP in neurons (Amato & Man, 2011). Glutamate excitation is usually accompanied by an influx of Ca++ into the cell and the mechanisms to remove Ca++ from the cell involve ATP depleting pumps (Gleichmann & Mattson, 2011). Therefore, when there is a substantial influx of Ca++, it can be expected that AMPK would be activated due to increased ATP hydrolysis to AMP (Zhang et al., 2008).

NO and the AMPK Pathway to GLUT3 Surface Expression

In astrocytes, increased NO levels have been shown to inhibit mitochondrial respiration, induce AMPK activity and promote PFK-2 activation to increase glycolysis. However, due to the low levels of PFK-2 in neurons, this pathway could not be fully established (Almeida et al., 2004). Increased NO levels in neurons has been associated with inhibition of mitochondrial respiration (Brown & Borutaite, 2001; Brown, 2001) which has been associated with induction of AMPK activity (Cidad et al., 2004). Nitric Oxide has also been shown to activate the AMPK pathway in vascular endothelial cells, (Zhang et al., 2008) in HEK-293T cells, (Cidad et al., 2004) and in neurons (Amato & Man, 2011; Weisova et al., 2009, Khatri & Man, 2013). Based on these studies, we expect that if NO inhibits mitochondrial respiration and activate AMPK, this could lead to increase glycolysis since AMPK increases cell surface GLUT3.

A research study done by Weisova et al. shows that GLUT3 expression at cell surface was regulated by the AMPK pathway. This study shows that transient glutamate excitation was associated with an increase in the AMP: ATP ratio which led to activation of AMPK. The activated AMPK may phosphorylate AMPK target molecules to increase surface GLUT3, while also targeting molecules that reduce ATP hydrolysis (Amato & Man, 2011; Lang & Föller, 2014; Weisová et al., 2009).

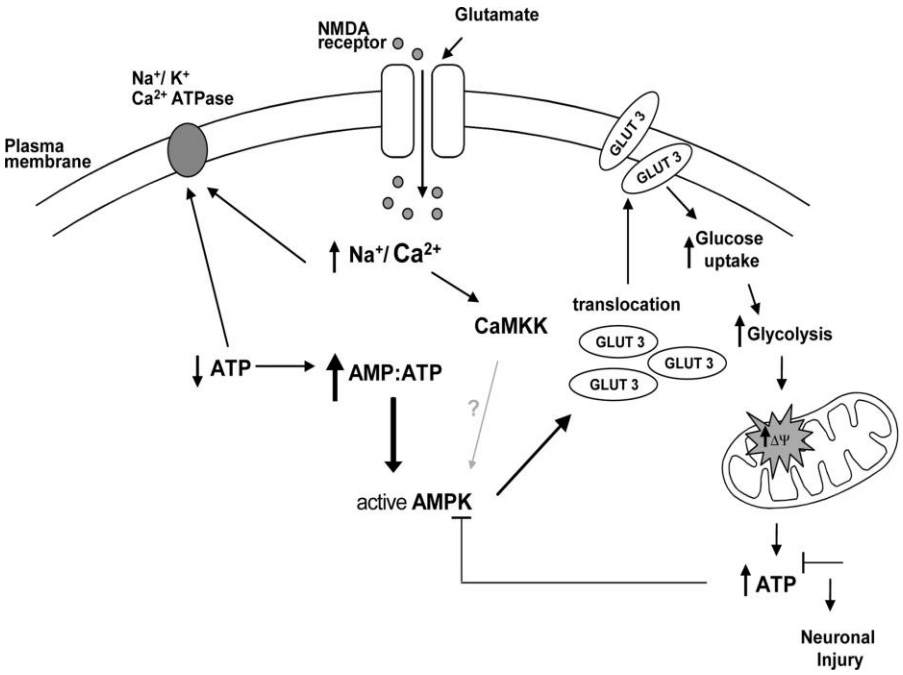


Fig. 5: Activity dependent AMPK pathway to increased GLUT3 surface expression in neurons (Weisová et al., 2009)

What Causes GLUT3 expression at Cell Surface?

Rameau et al. (2007) found that activity dependent NMDAR signaling pathways control the activity of neuronal nitric oxide synthase (nNOS) through phosphorylation. This activity also regulates the trafficking of the glucose transporter type-3 (GLUT3) at the cell surface (Ferreira et al., 2011; Rameau et al., 2007). The expression of GLUT3 at cell surface regulates the diffusion of glucose. A pertinent cellular pathway that is activated by usage of glucose and decline of ATP levels in the cell is the AMPK pathway which is activated by the enzyme, AMP-activated kinase (Hardie & Hawley, 2001). This kinase senses cellular energy insufficiency triggered by elevated AMP to ATP ratio (Haikala, Anttila, & Klefström, 2017).

The figures above (Fig. 3 & 5) show an AMPK pathway to increased GLUT3 expression at neuronal cell surface via energetic stress caused by glutamate excitation. The outline by Khatri and Man (2013) shows an AMPK/PI3K/Akt pathway to GLUT3 surface expression following activation of glutamatergic receptors. To examine an activity independent pathway to GLUT3 surface expression, we propose a NO pathway to increased GLUT3 expression at neuronal cell surface

building on the information derived from previous work done (Cidad et al., 2004; Ferreira et al., 2011; Lira et al., 2007; Weisová et al., 2009).

Khatri and Man (2013) highlight that hydrolysis of ATP causes alteration in ATP: AMP levels which is sensed by AMPK which then activates the PI3K/AKT pathway, leading to increased glucose uptake by GLUT3 in neurons. In a similar, yet contrasting light, Rameau et al. (2007) identify the action of PI3K/Akt pathway in regulating the activation of NOS upstream after NMDAR activation rather than downstream after AMPK activation, leading to GLUT3 surface expression. Also, a study by Amato et al., (2011) shows that in neurons, AMPK activates the PI3k/Akt pathway while leading to increased amount of glucose transporters at cell surface (Amato et al., 2011; Khatri & Man, 2013; Rameau et al., 2007).

The expression of nNOS phosphomimetic mutant S1412D triggers GLUT3 surface expression in cultured neurons (Ferreira et al., 2011). We hypothesize that NO increase surface GLUT3 constitutively in a mechanism dependent on AMPK → (PI3K/Akt) pathway (Amato et al., 2011). Testing this hypothesis would involve establishing that NO (via NOS mutants) stimulates AMPK activation and AMPK (via metformin or AICAR: an AMP analog that stimulates AMPK activity), activation leads to increased PI3K/AKT activity which would cause increased surface expression of GLUT3.

Specific Aims

We propose that Nitric Oxide via nNOS stimulation causes GLUT3 surface expression in an AMPK dependent manner. To address this, we have the following specific aims:

1. Construction of tagged GLUT3 to examine GLUT3 expression at the cell surface: To achieve this aim we perform a two-part project.

a. Bioinformatics analysis: using commercially available software, we compute and compare protein sequence of wild type GLUT3 and a GLUT3 construct (HA_GLUT3_EGFP) which we would possess a protein tag at its first exofacial loop and a luminescent protein at its C-terminal end.

b. Plasmid Construction: using commercially available molecular biology kits and plasmids, we construct HA_GLUT3_EGFP. To test viability of construct we will sequence commercially and then express the tagged GLUT3 in cultured cells.

2. Examine AMPK activation and surface GLUT3 expression after stimulation with nNOS mutant S1412D: stimulate NO production in cultured neurons and check for AMPK activation and GLUT3 surface expression via Western blot and/or Immunocytochemistry.

3. Test that AMPK activation (via metformin or AICAR) leads to increased PI3K/AKT activity and surface GLUT3 expression.

Chapter 2: Methods and Materials

Bioinformatics Analysis Methods

Sequence Alignment

Sequence alignment was conducted with Sequence Manipulation Suite software (Stothard, 2000). GLUT3 sequences were obtained from NCBI (*Rattus norvegicus solute carrier family 2 (facilitated glucose transporter), member 3 (Slc2a3), mRNA*, 2019; "Solute carrier family 2, facilitated glucose transporter member 3 [Hom—Protein—NCBI," n.d.) [See Appendix IV].

Physicochemical Analysis

To determine potential effects of mutagenesis on GLUT3 structure, We examine physicochemical properties and secondary structures of the wild type and mutant sequences using the PredictProtein Software (Yachdav et al., 2014) [See Appendix I]. Restriction site analysis was conducted using the Sequence manipulation Suite Software (Stothard, 2000) [See Appendix II].

STRING Analysis

Protein interactions maps were generated with STRING (Szklarczyk et al., 2015). The data were further segregated based on source of the data, such as literature reports, curated databases, co-expression, etc.

Plasmid Construction Methods

Strategy 1: Site-directed mutagenesis to Create Unique Restriction Enzyme Site

A pCMV6-Entry vector with a C-terminal Myc tagged GLUT3 was obtained from OriGene Technologies (RR202594). This plasmid construct was verified by 1.5% Agarose Gel Electrophoresis, restriction digest, and sequencing. The plasmid was then amplified by bacterial transformation in Top10 Competent cells (Invitrogen, C404003). In the absence of a unique restriction site at the first exofacial loop of GLUT3 cDNA clone, site-directed mutagenesis was carried out using a commercially available kit: QuikChange Lightning Site-directed mutagenesis Kit (Agilent Technologies, 210518) [See Appendix III]. The site-directed mutagenesis was performed to install a unique Hpa1 restriction site at the first extracellular loop via a point mutation at position

124 of GLUT3 cDNA (C→ G) using PCR. Sense and antisense primers for site-directed mutagenesis were designed using the Agilent technologies primer design software and synthesized by IDT Technologies [See Appendix V]. PCR was carried out as directed in kit protocol for mutagenesis.

Strategy 2: pcDNA3_HA_GLUT3_EGFP construction

GLUT3 cDNA was obtained by PCR (Qiagen, 203643) of the plasmid - RR202594 using the following forward and reverse primers respectively: 5' – AAGCTTATGGGGACAGCGAAG – 3' and 5' – GAATTCGGCATTGCCAGGGGT – 3' to install EcoRI and HindIII sites at 5' and 3' ends of GLUT3. The cDNA amplicon was analyzed by 1.5% agarose gel electrophoresis and purified using the QiAquick Gel Extraction kit (Qiagen, 28704) to extract the DNA product from the gel [See Appendix III].

Forward and reverse oligonucleotides were designed using the SnapGene software and the Serial Cloner software and synthesized by the Johns Hopkins Synthesis and Sequencing Facility. The Oligonucleotides to install HA tag at first exofacial loop of GLUT3: 5' – TACACGTTACCCATACGATGTTCCAGATTACGCTA – 3' and 5' – GTAGCGTAATCTGGAACATCGTATGGGTAACGTGTA – 3' were synthesized to be used [See Appendix V].

Fusion of GLUT3 cDNA amplicon with pcDNA-EGFP plasmid a gift from Doug Golenbock (Addgene plasmid # 13031; http://n2t.net/addgene:13031; RRID: Addgene_13031) was carried out after successful double restriction digest of insert and vector with EcoRI and HindIII restriction enzymes. We perform dephosphorylation of vector (Addgene, #13031) using Calf Intestinal Phosphatase (CIP), a commercially available kit from New England Bio labs (NEB, #M0290S) and phosphorylation of insert (GLUT3 cDNA) using a kit from New England Bio labs (NEB, #M0201S). The products from the phosphorylation treatments were ligated using a commercially available kit from New England Bio labs, T4 DNA ligase (NEB, #M0202S). Successful fusion was checked by agarose gel electrophoresis. [See Appendix III].

Chapter 3: Results

Sequence Alignment Results

For this project, we utilize GLUT3 cDNA clone from rat (Rattus norvegicus) to perform plasmid construction. Therefore, we perform sequence alignments of the protein sequences to ascertain functional and sequence similarity of the GLUT3 mRNA we obtained from OriGene Technologies (RR202594) with the GLUT3 expressed in humans. We do this by comparing the protein sequence of rat GLUT3 (NM_017102) from the cDNA clone with human GLUT3 protein sequence obtained from NCBI (NP_008862.1) in a pairwise sequence alignment using the Sequence Manipulation Suite (Stothard, 2000). We also highlight the conserved amino acid sequences using the same software.

>Rat (NM_017102) GLUT3
MGT-AKVTPSLVFAVTVATIGSFQFGYNTGVINAPETIIKDFLN-YTL-EER-LED--LP
SE-GLLTTLWSLCVAIFSVGGMIGSFSVGLFVNRFGRRNSMLLVNLIAI-LGGCLMGF-A
KIA-ESVEMLILGRLIIGIFCGLCTGFVPMYIGEVSPTALRGAFGTLNQLGIVVGILVAQ
VFGLDFILGSEELWP-GLLGLTIIPAILQSAALPFCPESPRFLLINRKEEDQATEILQRL
WGTPDV-IQEIQEMKDES-IRMSQEKQVTVLELFK-SPSY-FQPLLISVVLQLSQQFSGI
NAVFYYSTGIFQDAGVQEPIYATIGAGVVNTIFTVVSLFLVERAGRRTLHMIGLGGMA-V
CS-VFMTISLLLKDEYEAMSFVCI-VAILVYVAFFEIGPGPIPWFIVAELFSQGPRPAAM
AVAGCSNWTSNFLVGMFFPSAA-AYLGAYVFIIFAAFLV-FFL-IFTFFKVPETKGRTFE
DITRAFEGQAQ----SGKGSAGV-ELNSMQPVKE--TPGN-A

>Human (NP_008862.1) GLUT3
MGTQ-KVTPALIFAITVATIGSFQFGYNTGVINAPEKIIKEFINK-TLTD-KG--NAP-P
SEV-LLTSLWSLSVAIFSVGGMIGSFSVGLFVNRFGRRNSMLIVNLLAVT-GGCFMGLC-
KVAK-SVEMLILGRLVIGLFCGLCTGFVPMYIGEISPTALRGAFGTLNQLGIVVGILVAQ
IFGLEFILGSEELWPL-LLGFTILPAILQSAALPFCPESPRFLLINRKEEENAKQILQRL
WGTQDVS-QDIQEMKDESA-RMSQEKQVTVLELFRVS-SYR-QPIIISIVLQLSQQLSGI
NAVFYYSTGIFKDAGVQEPIYATIGAGVVNTIFTVVSLFLVERAGRRTLHMIGLGGMAF-
CSTL-MTVSLLLKDNYNGMSFVCIG-AILVFVAFFEIGPGPIPWFIVAELFSQGPRPAAM
AVAGCSNWTSNFLVGLLFPSAAH-YLGAYVFIIFTGFLITF-LA-FTFFKVPETRGRTFE
DITRAFEGQAHGADRSGKD--GVMEMNSIEPAKETTT--NV-

PAM (30) Alignment score: 2988

The sequences above are of Human and Rat GLUT3 protein: showing the optimal global alignment of both sequences.

Next, we highlight identical and similar amino acids in the alignments of rat versus human GLUT3 sequences using the color conservation scheme in the Sequence Manipulation Suite (Stothard, 2000).

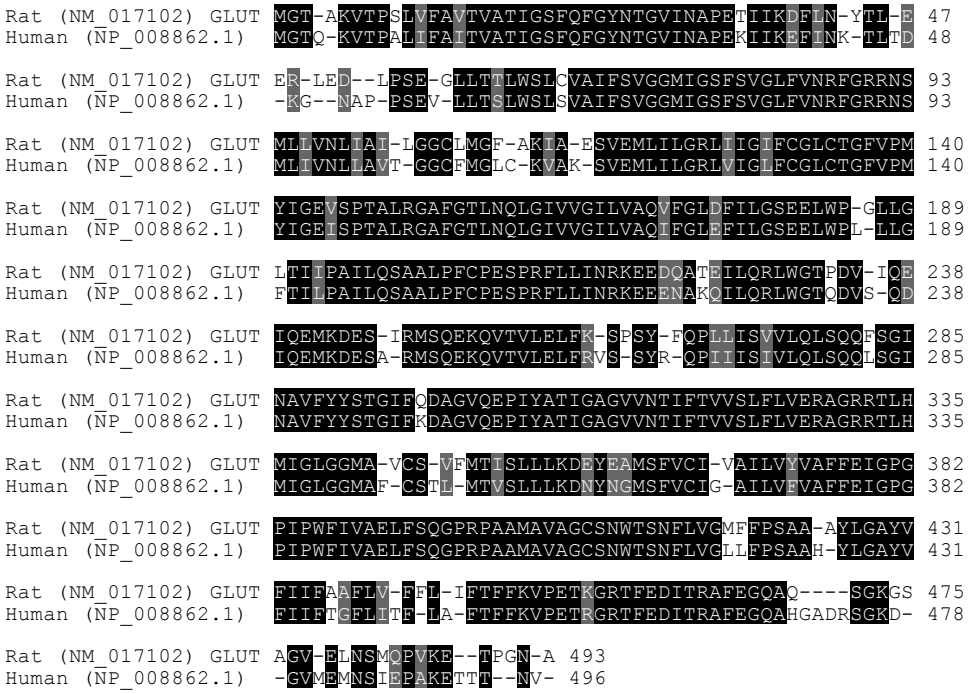


Fig. 6: Color Conserved alignment comparing Human and Rat GLUT3 protein sequences. Identical sequences are given a black background, similar sequences are given a gray background. The remaining residues receive a white background

Alignment Length	**522**
Identical Residues	**413**
Similar Residues	**37**
Percent Identity (%)	**79.12**
Percent Similarity (%)	**86.21**

Table 2: Identity and Similarity Results for Human versus Rat GLUT3 protein sequence alignment.

The sequence alignments show that Human and Rat GLUT3 have a significant similarity in sequence which points to similar protein folding (structure) and function. The Identity and Similarity Results show that both GLUT3s have 413 identical residues of 522 amino acid residues, 79.12% identity, and 86.21% similarity. Both proteins possess the same conserved domains, have similar structure and carry out the same function. Thus, rat GLUT3 can be used as an experimental model for human GLUT3 function.

Sequence Analysis of Proposed Construct

We conduct sequence alignment analysis of our proposed GLUT3 construct (pcDNA3_HA_GLUT3_EGFP) with Rat GLUT3. We highlight similarity of our construct to the wild-type GLUT3 cDNA sequence and examine how the added sequences might affect secondary structure of our proposed construct.

GLUT3_WT versus HA_GLUT3

Here, we compile the protein sequences of our GLUT3_WT versus HA_GLUT3, which is the original GLUT3 sequence with the HA (Human influenza hemagglutinin) protein tag, ~9 amino acids added at the first exofacial loop of GLUT3. We perform a sequence alignment of both sequences and examine the identity and similarity results for the alignment.

>GLUT3_WT
KLMGTAKVTPSLVFAVTVATIGSFQFGYNTGVINAPETIIKDFLN---------YTLEER
LEDLPSEGLLTTLWSLCVAIFSVGGMIGSFSVGLFVNRFGRRNSMLLVNLIAILGGCLMG
FAKIAESVEMLILGRLIIGIFCGLCTGFVPMYIGEVSPTALRGAFGTLNQLGIVVGILVA
QVFGLDFILGSEELWPGLLGLTIIPAILQSAALPFCPESPRFLLINRKEEDQATEILQRL
WGTPDVIQEIQEMKDESIRMSQEKQVTVLELFKSPSYFQPLLISVVLQLSQQFSGINAVF
YYSTGIFQDAGVQEPIYATIGAGVVVNTIFTVVSLFLVERAGRRTLHMIGLGGMAVCSVFM
TISLLLKDEYEAMSFVCIVAILVYVAFFEIGPGPIPWFIVAELFSQGPRPAAMAVAGCSN
WTSNFLVGMFFPSAAAYLGAYVFIIFAAFLVFFLIFTFFKVPETKGRTFEDITRAFEGQA
QSGKGSAGVELNSMQPVKETPGNAEF

>HA_GLUT3
KLMGTAKVTPSLVFAVTVATIGSFQFGYNTGVINAPETIIKDFLNSRYPYDVPDYAMEER
LEDLPSEGLLTTLWSLCVAIFSVGGMIGSFSVGLFVNRFGRRNSMLLVNLIAILGGCLMG
FAKIAESVEMLILGRLIIGIFCGLCTGFVPMYIGEVSPTALRGAFGTLNQLGIVVGILVA
QVFGLDFILGSEELWPGLLGLTIIPAILQSAALPFCPESPRFLLINRKEEDQATEILQRL
WGTPDVIQEIQEMKDESIRMSQEKQVTVLELFKSPSYFQPLLISVVLQLSQQFSGINAVF
YYSTGIFQDAGVQEPIYATIGAGVVVNTIFTVVSLFLVERAGRRTLHMIGLGGMAVCSVFM
TISLLLKDEYEAMSFVCIVAILVYVAFFEIGPGPIPWFIVAELFSQGPRPAAMAVAGCSN
WTSNFLVGMFFPSAAAYLGAYVFIIFAAFLVFFLIFTFFKVPETKGRTFEDITRAFEGQA
QSGKGSAGVELNSMQPVKETPGNAEF

The sequences above are of GLUT3_WT and HA_GLUT3 protein: showing the optimal global alignment of both sequences.

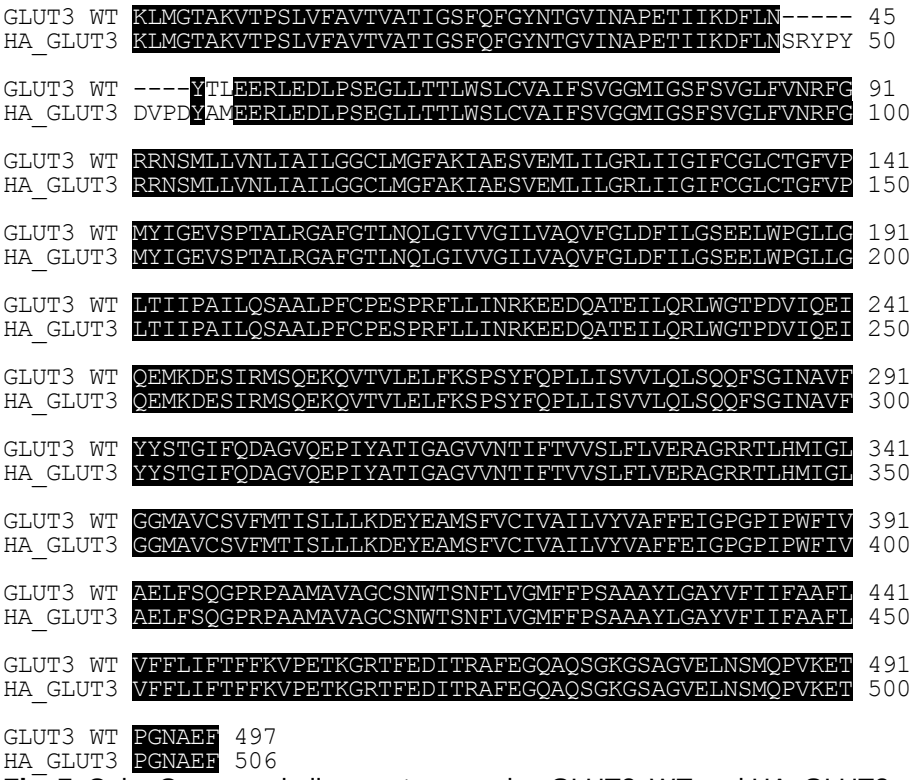


Fig. 7: Color Conserved alignment comparing GLUT3_WT and HA_GLUT3 protein sequences. Identical sequences are given a black background, similar sequences are given a gray background. The remaining residues receive a white background

Alignment Length	**506**
Identical Residues	**495**
Similar Residues	**0**
Percent Identity (%)	**97.83**
Percent Similarity (%)	**97.83**

Table 3: Identity and Similarity Results for Rat GLUT3_WT versus Rat HA_GLUT3 protein sequence

HA_GLUT3 versus HA_GLUT3_EGFP

First, we compile the protein sequences for HA_GLUT3 seen above and HA_GLUT3_EGFP, which is the original GLUT3 sequence with the HA (Human influenza hemagglutinin) protein tag, ~9 amino acids added at the first exofacial loop and an EGFP protein,

~166 amino acids at the C-terminal end of GLUT3_WT. We perform a sequence alignment of both sequences and examine the identity and similarity results for the alignment.

>HA_GLUT3_EGFP
KLMGTAKVTPSLVFAVTVATIGSFQFGYNTGVINAPETIIKDFLNSRYPYDVPDYAMEER
LEDLPSEGLLTTLWSLCVAIFSVGGMIGSFSVGLFVNRFGRRNSMLLVNLIAILGGCLMG
FAKIAESVEMLILGRLIIGIFCGLCTGFVPMYIGEVSPTALRGAFGTLNQLGIVVGILVA
QVFGLDFILGSEELWPGLLGLTIIPAILQSAALPFCPESPRFLLINRKEEDQATEILQRL
WGTPDVIQEIQEMKDESIRMSQEKQVTVLELFKSPSYFQPLLISVVLQLSQQFSGINAVF
YYSTGIFQDAGVQEPIYATIGAGVVNTIFTVVSLFLVERAGRRTLHMIGLGGMAVCSVFM
TISLLLKDEYEAMSFVCIVAILVYVAFFEIGPGPIPWFIVAELFSQGPRPAAMAVAGCSN
WTSNFLVGMFFPSAAAYLGAYVFIIFAAFLVFFLIFTFFKVPETKGRTFEDITRAFEGQA
QSGKGSAGVELNSMQPVKETPGNAEFMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEG
EGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYV
QERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIM
ADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNE
KRDHMVLLEFVTAAGITLGMDELYK

>HA_GLUT3
KLMGTAKVTPSLVFAVTVATIGSFQFGYNTGVINAPETIIKDFLNSRYPYDVPDYAMEER
LEDLPSEGLLTTLWSLCVAIFSVGGMIGSFSVGLFVNRFGRRNSMLLVNLIAILGGCLMG
FAKIAESVEMLILGRLIIGIFCGLCTGFVPMYIGEVSPTALRGAFGTLNQLGIVVGILVA
QVFGLDFILGSEELWPGLLGLTIIPAILQSAALPFCPESPRFLLINRKEEDQATEILQRL
WGTPDVIQEIQEMKDESIRMSQEKQVTVLELFKSPSYFQPLLISVVLQLSQQFSGINAVF
YYSTGIFQDAGVQEPIYATIGAGVVNTIFTVVSLFLVERAGRRTLHMIGLGGMAVCSVFM
TISLLLKDEYEAMSFVCIVAILVYVAFFEIGPGPIPWFIVAELFSQGPRPAAMAVAGCSN
WTSNFLVGMFFPSAAAYLGAYVFIIFAAFLVFFLIFTFFKVPETKGRTFEDITRAFEGQA
QSGKGSAGVELNSMQPVKETPGNAEF----------------------------------

The sequences above are of HA_GLUT3 and HA_GLUT3_EGFP protein: showing the optimal global alignment of both sequences.

Fig. 8: Color Conserved alignment comparing HA_GLUT3 and HA_GLUT3_EGFP protein sequences. Identical sequences are given a black background, similar sequences are given a gray background. The remaining residues receive a white background

Alignment Length	**745**
Identical Residues	**506**
Similar Residues	**0**
Percent Identity (%)	**67.92**
Percent Similarity (%)	**67.92**

Table 4: Identity and Similarity Results for Rat HA_GLUT3 versus Rat HA_GLUT3_EGFP protein sequence

Predicted Solvent Accessibility Composition (core/surface ratio) for Protein

GLUT3 Protein Seq.	**%b**	**%e**
GLUT3 Wild Type	71.83	28.17
HA_GLUT3	71.54	28.46
HA_GLUT3_EGFP	61.21	38.79
Standard Deviation	6.05	6.05
Mean	68.19	31.80

Key

e: residues exposed with more than 16% of their surface

b: all other residues.

Table 5: Predicted Solvent accessibility for GLUT3_WT, HA_GLUT3 and HA_GLUT3_EGFP.

The percent solvent accessibility prediction for the protein sequences examined shows similar parameters between GLUT3_WT and HA_GLUT3 featuring only a 9 amino acid difference in sequence. However, the solvent accessibility of HA_GLUT3_EGFP compared to HA_GLUT3 and GLUT3_WT shows that more of the HA_GLUT3_EGFP protein sequence is exposed and accessible to solvent. The percent difference can be attributed to the ~173 amino acids added with the HA and EGFP components of the construct.

Residue Composition of Protein (%)

Amino Acid Residue	***GLUT3 WT***	***HA_GLUT3***	***HA_GLUT3_EGFP***
A	*7.80*	*7.90*	*6.40*
C	*1.60*	*1.60*	*1.30*
D	*1.80*	*2.20*	*3.90*
E	*6.20*	*6.10*	*6.30*
F	*8.70*	*8.50*	*7.40*
G	*9.70*	*9.50*	*9.40*
H	*0.20*	*0.20*	*1.30*
I	*8.40*	*8.30*	*7.20*
K	*2.60*	*2.60*	*4.40*

L	*11.30*	*10.90*	*10.20*
M	*3.00*	*3.20*	*3.00*
N	*2.80*	*2.80*	*3.60*
P	*4.60*	*4.90*	*4.70*
Q	*4.00*	*4.00*	*3.80*
R	*3.20*	*3.40*	*3.10*
S	*6.40*	*6.50*	*5.80*
T	*5.60*	*5.30*	*5.80*
V	*8.70*	*8.70*	*8.30*
W	*1.00*	*1.00*	*0.80*
Y	*2.20*	*2.60*	*3.20*

Table 6: Residue composition of GLUT3_WT, HA_GLUT3 and HA_GLUT3_EGFP

The residue composition of the protein sequences examined show minor differences in percentage while keeping a constant amino acid residue profile. The residues that are present in low percentages in the GLUT3_WT stay consistent for HA_GLUT3 and HA_GLUT3_EGFP. This shows that the protein tags do not change secondary structure in major ways that would affect protein folding.

Predicted Secondary Structure Composition (% in protein)

Protein	***H***	***E***	***L***
GLUT3_WT	*53.12*	*16.90*	*29.98*
HA_GLUT3	*54.94*	*13.04*	*32.02*
HA_GLUT3_EGFP	*41.21*	*17.58*	*41.21*

Key: H - Helix; E - Beta; L- Loop

Table 7: Percentage of Predicted Secondary Structure of protein

The percentage of predicted secondary structures fluctuate for the protein sequences examined. The percentage of loop structures increase in both HA_GLUT3 and HA_GLUT3_EGFP. The increase in unstructured loop sequences is expected and can be attributed to the added amino acid residues. On the other hand, the percentage of helix structures reduce significantly in

HA_GLUT3_EGFP and increases slightly in HA_GLUT3. The percentage of beta structures reduces slightly in HA_GLUT3 but stays reasonably consistent for all the sequences. These changes are being attributed to the increased number of amino acid residues in HA_GLUT3_EGFP and the increase in loop regions of HA_GLUT3 with respect to other regions respectively.

Secondary Structure Analysis

Comparison of the secondary structure of GLUT3_WT, HA_GLUT3, and HA_GLUT3_EGFP shows that the topological arrangement of the wildtype protein is preserved with the addition of the HA protein tag and the EGFP. The protein tags added constitute the unstructured loop regions of the proposed construct as seen in Table 8 [See Appendix I]. We examine the amino Acid sequence, Predicted transmembrane helices (H- Helical membrane; L- no helical membrane), and Predicted topology of Transmembrane loops (i= inside loop; T= Transmembrane; o= outside loop). Based on the analysis, the mutations are not expected to cause significant changes in proteins secondary structure [See Appendix I].

Restriction Site Analysis

We use the Sequence manipulation Suite to constitute a restriction summary of the DNA sequences: GLUT3-WT, HA_GLUT3, and HA_GLUT3_EGFP. Examination of this summary shows that the HA tag does not introduce any new or unique restriction sites however, the EGFP sequence introduces 2 new restriction sites which do not interfere with the restriction sites present in wild type GLUT3 or the sites to be used in plasmid construction [See Appendix II].

STRING Analysis Results

We perform a STRING analysis to explore whether proteins that co-expressed or in some ways associated proteins of GLUT3 may be involved in its trafficking. The STRING database contains ~5,090 organisms and ~24.6 million proteins whose connection with GLUT3 could be examined

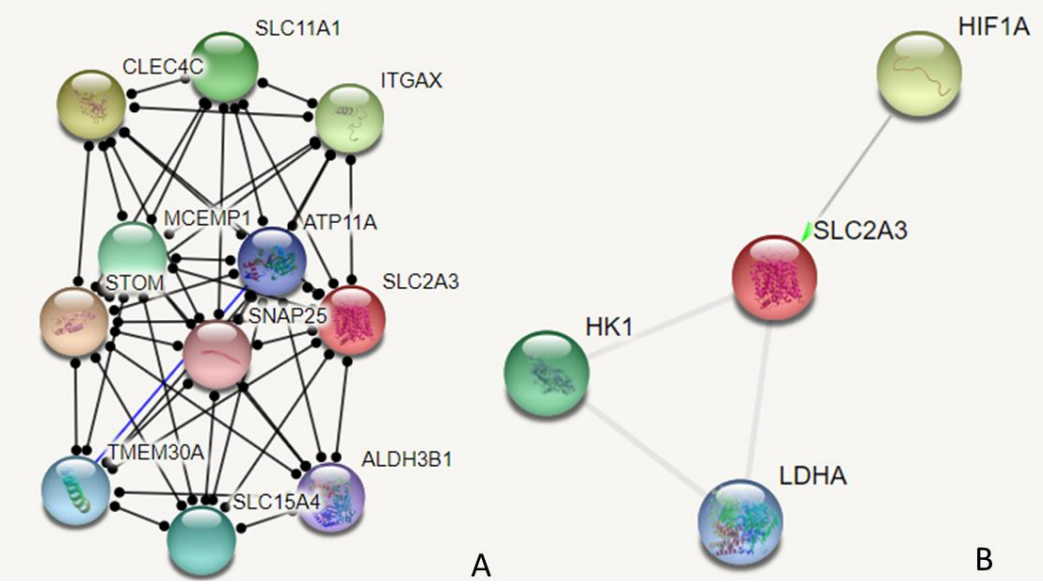


Fig. 9 A&B: STRING ANALYSIS of H. sapiens GLUT3 highlighting interaction with proteins derived from text mining, curated databases, and co-expression. Blue line indicates binding between proteins, TMEM30A and ATP11A which form a P4-ATPase flippase complex. The green arrow indicates positive interaction between proteins, HIF1A and SLC2A3 (GLUT3) (Szklarczyk et al., 2015).

In the STRING diagram, (Fig. 9A), several proteins involved with vesicle formation (ATP11A, TMEM30A), docking and membrane fusion proteins (SNAP25) were present. ATP11A and TMEM30A function as part of a P4-ATPase flippase complex which catalyzes ATP hydrolysis coupled to aminophospholipid transport across membranes (UniProt Consortium, 2019). These proteins could be part of the cellular process for GLUT3 transport to cell surface. The ATP consumption of these proteins could also contribute to increase AMP in the cell which would cause AMPK activation. SNAP25, a member of the SNARE complex regulates vesicles fusion with plasma membrane at nerve terminal. This process controls neurotransmitter release (stimulus driven neurotransmission) and plays a role in synaptic function of neuronal systems (Tafoya et al., 2006; UniProt Consortium, 2019). SNAP25 may be a good candidate involved in GLUT3 transport to cell surface.

STRING diagram (Fig. 9B) shows GLUT3's association with HIF1A (Hypoxia inducible factor 1 alpha) and HK1 (hexokinase 1) based on scientific journals explored by the software.

HIF1A regulates the transcriptional response of cells under conditions of hypoxia (oxygen deficiency) (UniProt Consortium, 2019). One of the responses of cerebral hypoxia is an upregulation of GLUT3 as seen in a study by Vannuci et al. (1996). HK1 is a pertinent protein in glucose metabolism pathway – converting glucose to glucose-6-phosphate in an ATP dependent manner (UniProt Consortium, 2019). This protein interaction with GLUT3 is expected.

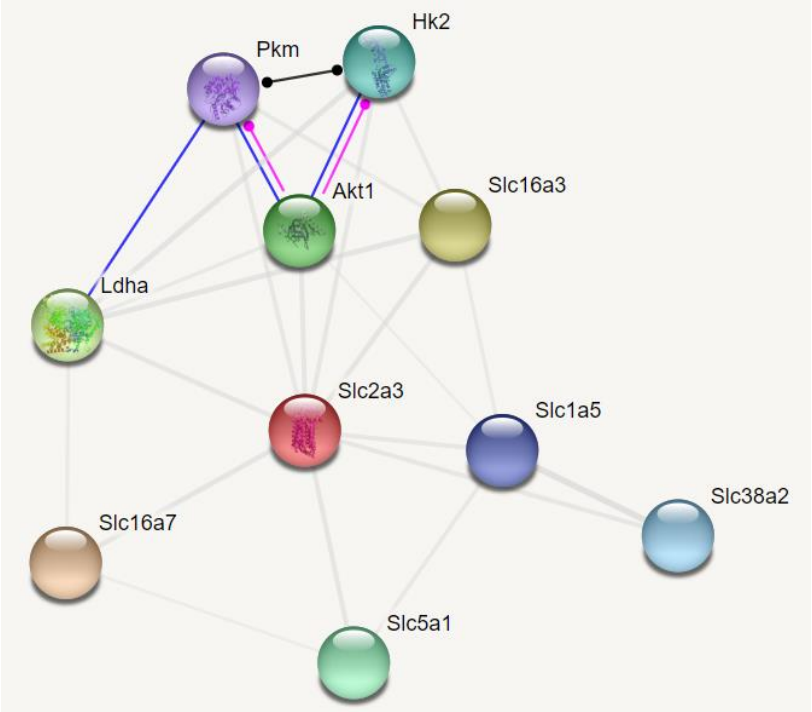


Fig. 10: STRING ANALYSIS of R. norvegicus GLUT3 highlighting interaction with proteins derived from text mining, experiments, databases, and co-expression. Blue lines indicate binding between proteins: Ldha and Pkm, Akt1 and Pkm, Akt1 and Hk2. Pink lines indicate post translational modifications carried out by Akt1 on Pkm and Hk2 (Szklarczyk et al., 2015).

Figure 10 shows R. norvegicus GLUT3 interaction with other proteins in the solute carrier family as well as proteins such as HK2 (hexokinase 2) and Pkm (pyruvate kinase) which are involved in glucose metabolism and are expected interactions with GLUT3. Of specific interest is the interaction between GLUT3 (Slc2a3) and Akt1, a protein which is responsible for the regulation of glucose uptake via insulin induced translocation of GLUT4 to cell surface in adipocytes (Hernandez, Teruel, & Lorenzo, 2001). Studies explored in this paper also implicate Akt in GLUT3 surface expression (Khatri & Man, 2013; Rameau et al., 2007).

Gene	Location	Protein Name	STRING	Function
TMEM30A	cell membrane/golgi	Cell cycle control protein 50A	H. Sapiens GLUT3	Catalytic component of a P4-ATPase flippase complex; vessicle formation via phospholipid translocation
ATP11A	membrane/ ER	ATPase phospholipid transporting 11A	H. Sapiens GLUT3	Catalytic component of a P4-ATPase flippase complex; vessicle formation via phospholipid translocation
HIF1A	cytoplasm	Hypoxia-inducible factor 1-alpha	H. Sapiens GLUT3	regulates the adaptive response to hypoxia
HKI	cytosol/mitochondria	Hexokinase-1	H. Sapiens GLUT3	Catalyzes the phosphorylation of various hexoses
SNAP25	cell membrane	Synaptosomal-associated protein 25	H. Sapiens GLUT3	Associates with proteins involved in vesicle docking and membrane fusion
HK2	mitochondria	Hexokinase-2	R. norvegicus GLUT3	Catalyzes the phosphorylation of various hexoses - glycolytic

PKM	cytoplasm	Pyruvate kinase PKM	R. norvegicus GLUT3	catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, generating ATP - glycolytic
AKT1		RAC-alpha serine/threonine-protein kinase	R. norvegicus GLUT3	AKT is responsible of the regulation of glucose uptake by mediating translocation of glucose transporter to the cell surface

Table 10: Summary/Classification of Protein - Protein Interactions of GLUT3

Expression Plasmid Construction

Recombinant plasmid constructs are a strategic molecular biology tool used to study genes and gene products. Ashrafi et al. (2017), Burchfield et al. (2013), and Dawson et al. (2001) have used molecular biology techniques to successfully create traceable glucose transporter proteins which possess specific protein tags at ideal sites for imaging and quantitation. Based on the structure of our protein of interest (GLUT3), its similarity with GLUT4, the protein tags in the studies mentioned earlier and previous work done, we decided to pursue a similar approach to tagging our GLUT3 cDNA.

We set out to install a Myc tag or a Human Influenza Hemagglutinin (HA) tag (9 amino acids - YPYDVPDYA) at the first extracellular loop of GLUT3 ("HA-tag Protein Expression, Production, and Purification Basics," n.d.). Previous works done on GLUT4 and GLUT3 show that the proteins retain their original function after insertion of protein tags at this loop site (Ashrafi, Wu, Farrell, & Ryan, 2017; Burchfield et al., 2013; Dawson, Aviles-Hernandez, Cushman, & Malide, 2001; Mahon, 2011). Also, the structural plasticity of protein loop regions and the loop's ability to

function as a tool for functional modification and evolution of proteins make this region ideal for our construct (Guo, Choe, & Loeb, 2004; Hayes, Hallet, & Cao, 1997; Heinis & Johnsson, 2010).

Using similar molecular biology techniques employed in these papers, we proceeded to tag our GLUT3 cDNA with the HA tag at the first extracellular loop and create a GFP fusion at its C-terminal end. This construct will be used for experiments to identify and examine GLUT3 surface expression. The construct would also be used as the springboard to make specific constructs for further experiments which include and are not limited to Imaging, truncation, and mutation of GLUT3 to examine its functional mechanism and expression.

Strategy 1

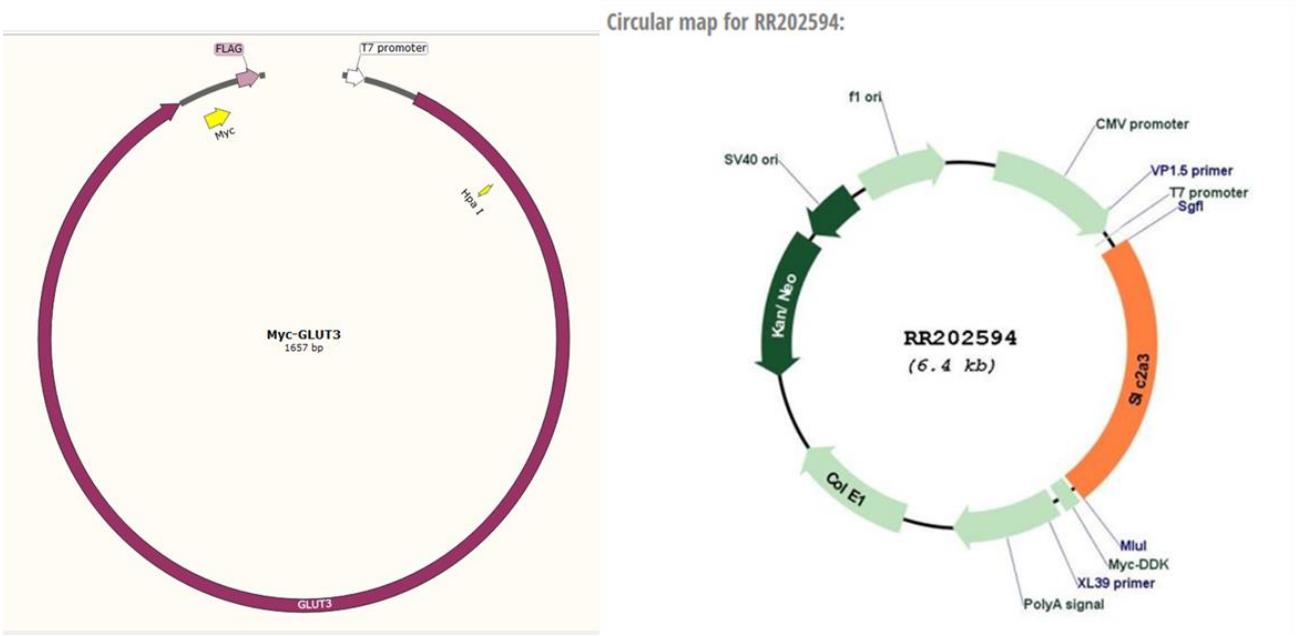


Fig. 11: Hpa1 site installation in RR202594. Note: pCMV6 vector not included in illustration to the left.

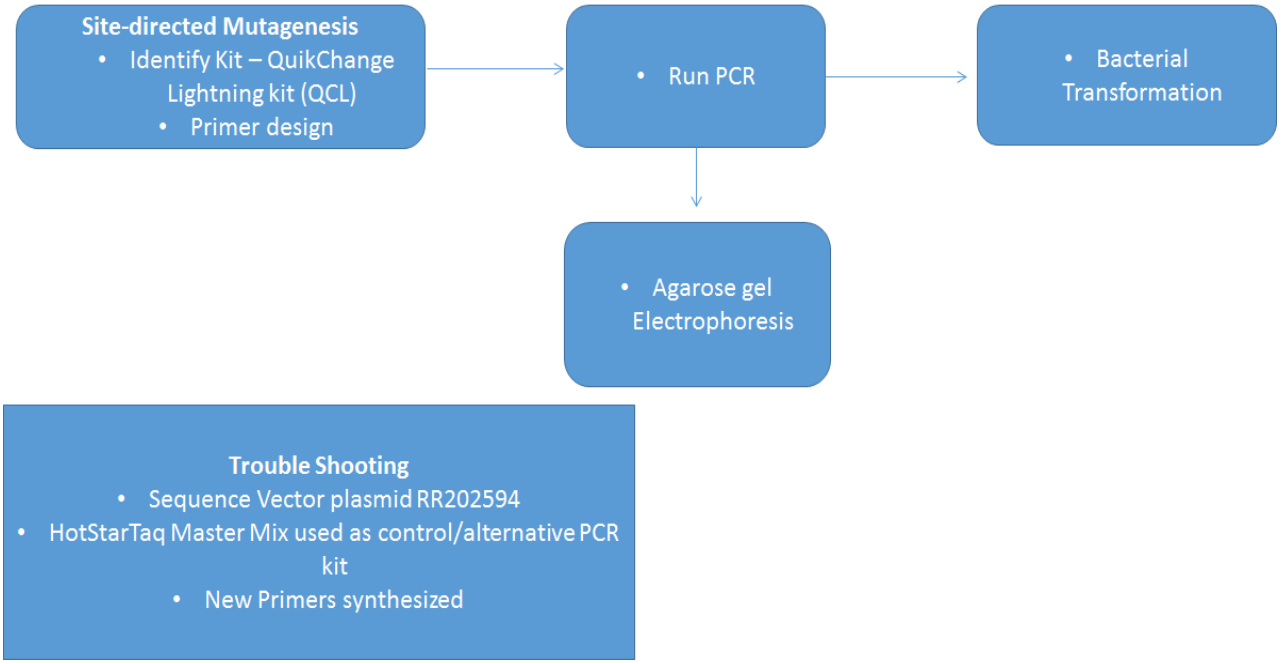


Fig. 12: flow chart of strategy 1 to install a unique Hpa1 site at the first exofacial loop of GLUT3

Results

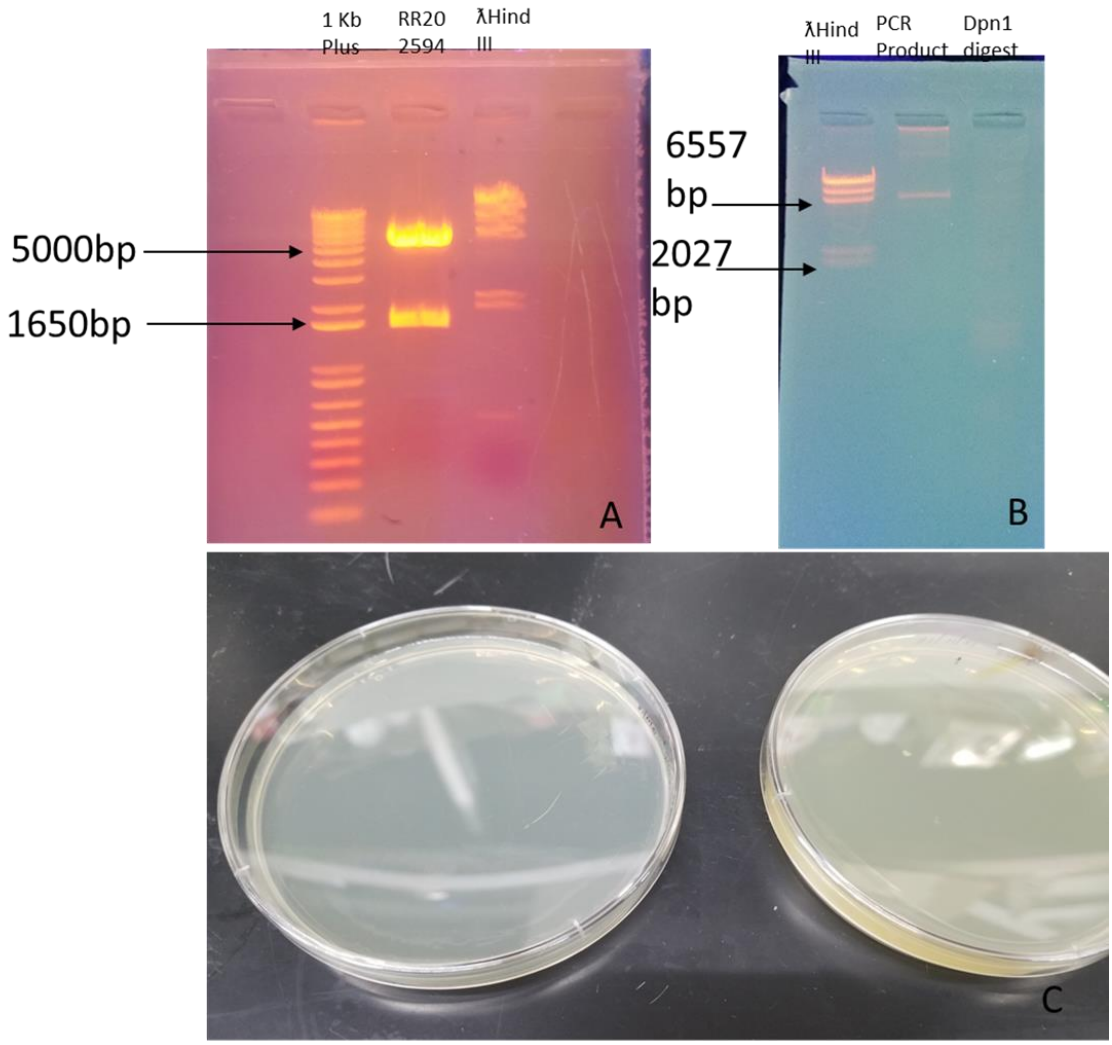


Fig. 13: Agarose gel electrophoresis showing (a) double restriction enzyme digest diagnostic of RR202594 using enzymes Mlu1 and Sgf1 **: ~5Kb (pCMV6 vector), ~1.5Kb (GLUT3 insert)** (b) PCR products after Site directed mutagenesis: before and after Dpn1 digest as directed by the kit (c) PCR products of Site directed mutagenesis after bacterial transformation in XL-10 gold competent cells.

The results showed successful site directed mutagenesis PCR of the control from the kit. However, we did not see consistent results in agarose gel electrophoresis conducted after site directed mutagenesis PCR of our cloning vector with GLUT3 insert (RR202594). We also did not see colony growth after transformation of our PCR product in the XL-10 competent cells provided with the kit supplied by the company.

Strategy 2

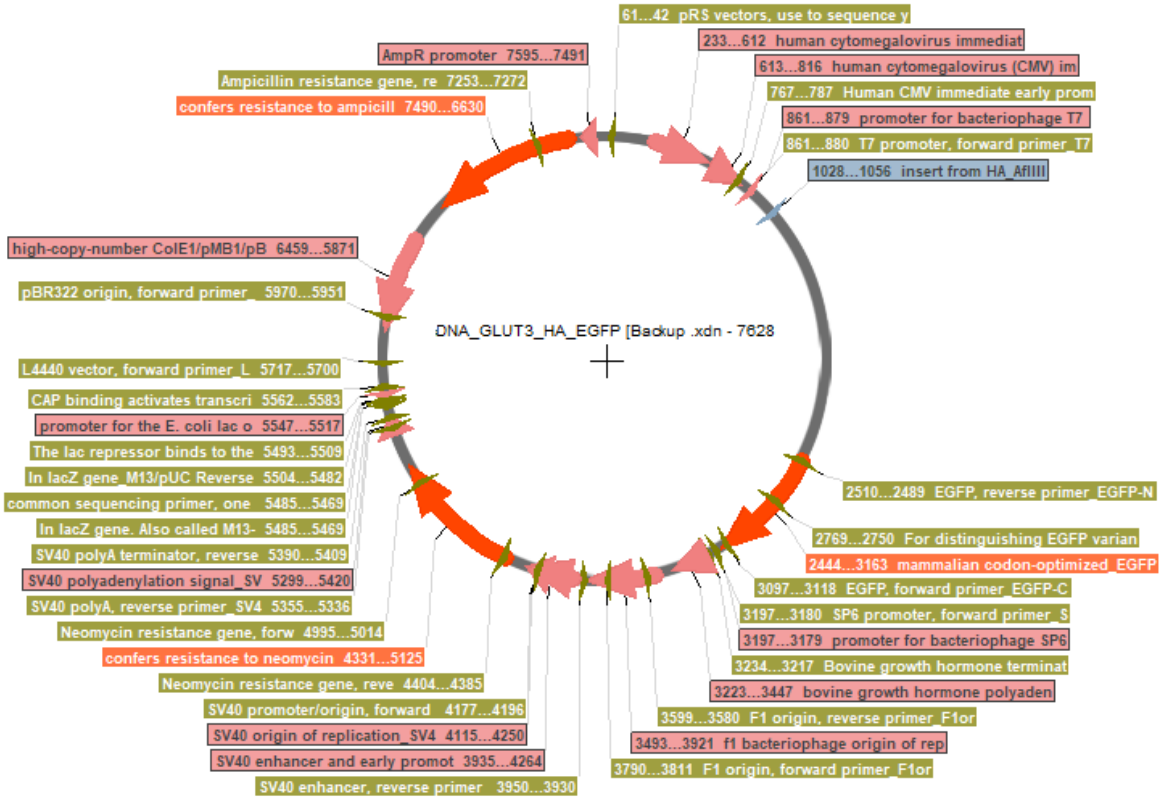


Fig. 14: pcDNA3_HA_GLUT3_EGFP construction using Serial Cloner software

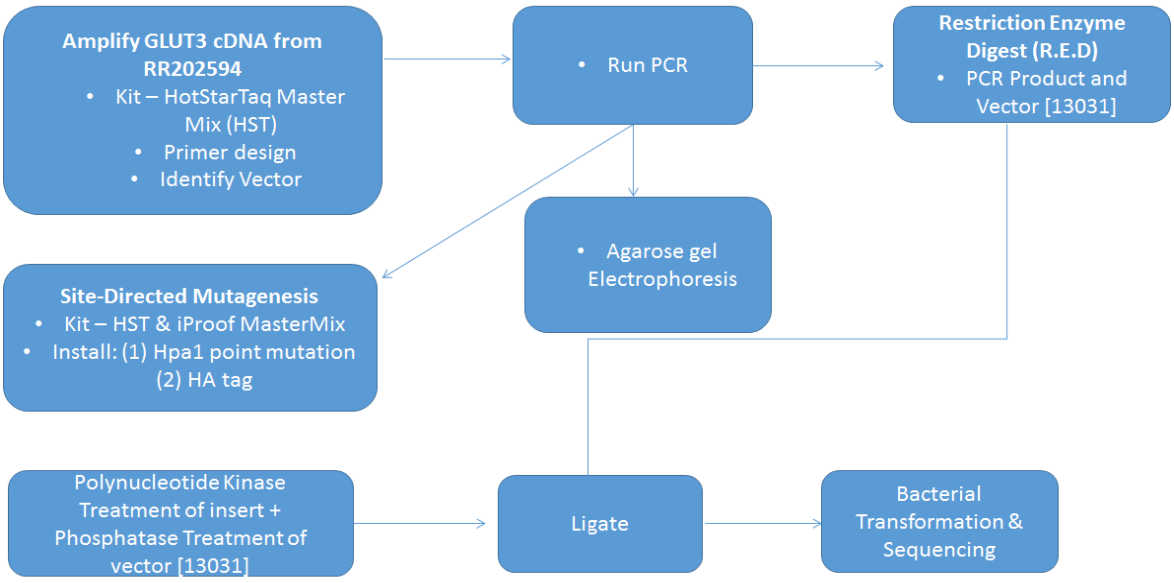


Fig. 15: flowchart of Strategy 2 steps to create pcDNA_HA_GLUT3_GFP

Results

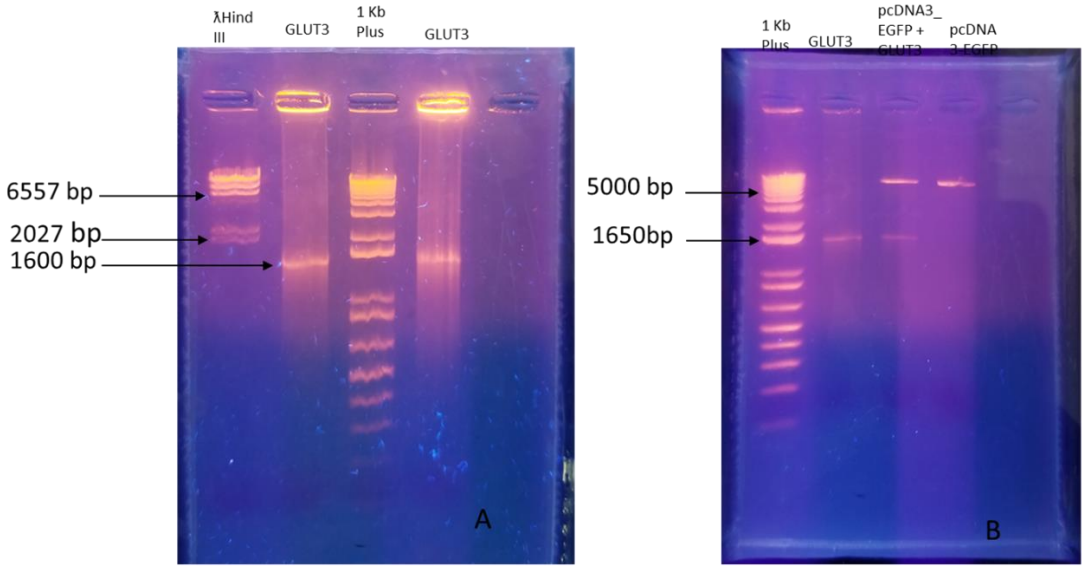


Fig. 16: Agarose gel electrophoresis showing (a) PCR amplification of GLUT3 (~1.5Kb) (b) Ligation product pcDNA3-EGFP + GLUT3

The results showed successful PCR amplification of GLUT3 cDNA clone. However, we did not see successful pcDNA3-EGFP ligation with GLUT3 insert in agarose gel electrophoresis conducted after ligation reaction.

Chapter 4: Discussion

Alignments, Physicochemical and Secondary Properties

The sequence alignment score is a numerical value that tells us the extent to which two sequences have the same residues at the same positions in the alignment sequence. The PAM30 matrix particularly highlights similarities between two sequences. The high percent similarity and sequence conservation between human and rat GLUT3 affirms that the use of the RR202594 GLUT3 mRNA would produce similar function as the human GLUT3 mRNA. Also, experiments conducted using the RR202594 plasmid or the GLUT3 mRNA derived from this plasmid would produce results that could be attributed to human GLUT3 functioning and expression mechanism in a cell. The rat GLUT3 mRNA is also ideal for use in our lab experiments because we use rat neuronal cultures.

Sequence alignments used to compare GLUT3_WT, HA_GLUT3, and HA_GLUT3_EGFP coupled with the results from the query for physicochemical properties of the sequences of interest show minor to no changes in predicted solvent accessibility and percent residue composition. The secondary structure analysis shows that the added components for the proposed construct constitute predicted unstructured regions in the protein, leading us to conclude that the protein tags will not have major effects on regular protein folding of GLUT3. Therefore, the construct would produce functional GLUT3 proteins after successful construction and expression in cell culture.

STRING Analysis

Known and predicted protein-protein associations with GLUT3 were examined using the STRING software to shed more light on the possible NO→AMPK→PI3K/Akt pathway to GLUT3 surface expression. STRING analysis of H. sapiens GLUT3 and R. norvegicus GLUT3 suggested possible proteins that may interact with GLUT3, and that in some ways may influence GLUT3 trafficking or surface expression.

H. sapiens GLUT3 is associated with proteins such as STOM: Stomatin which regulates ion channel activity and transmembrane ion transport. Also, SNAP25: Synaptosomal-associated

protein 25 which is involved in the molecular regulation of neurotransmitter release, synaptic function of neural systems and is associated closely with GLUT3 expression (Szklarczyk et al., 2015; Tafoya et al., 2006; UniProt Consortium, 2019; Vannucci et al., 1998). Also, the STRING diagram in Fig. 9a suggested interactions derived from curated databases between GLUT3 and ATP11A: a phospholipid-transporting ATPase IH; ATP11A is a component of a P4-ATPase flippase complex which catalyzes the hydrolysis of ATP coupled to the transport of aminophospholipids from the outer to the inner leaflet of various membranes. ATP11A drives the transport of ions such as calcium across membranes and ensures the maintenance of asymmetric distribution of phospholipids ("ATP11A ATPase phospholipid transporting 11A [Homo sapiens (human)]—Gene—NCBI," n.d.; Szklarczyk et al., 2015). There is also a protein interaction between GLUT3 and HIF1A (Hypoxia-inducible factor 1-alpha) which is known to regulate GLUT3 expression under certain conditions in neurons via PI3k/Akt/mTOR pathway (Yu et al., 2012). HIF1A is also shown to be repressed as a result of the effects of NO on the mitochondria (Erusalimsky & Moncada, 2007). HK1 (hexokinase 1), glycolytic enzyme that breaks down glucose also interacts with GLUT3.

R. norvegicus GLUT3 has interactions with other proteins of the major facilitator superfamily in the STRING database. Fig.10 shows GLUT3 interaction with other major facilitator proteins (SLC16a7 and SLC16a3) which are expressed at cell membrane. There is also an interaction with Akt1: a serine/threonine-protein kinase which regulates processes such as metabolism, growth, and cell survival. This protein is known to regulate glucose uptake by mediating translocation of glucose transporter in GLUT1 (Barthel et al., 1999). This is mediated through serine and/or threonine phosphorylation of a range of downstream substrates. Khatri and Man (2013) states that PI3K/AKT pathway activation due to upstream AMPK action, leads to increased glucose uptake by GLUT3 in neurons (Khatri & Man, 2013). These interactions support the function of GLUT3 in neurons and are advantageous in studying transport and expression of GLUT3. The proteins from this STRING analysis provide some support for GLUT3 expression via AMPK → Akt pathway.

Plasmid Construction

Constructs previously done (Ashrafi et al., 2017; Burchfield et al., 2013; Dawson et al., 2001) along with our bioinformatics analysis give enough evidence that making a construct of GLUT3 with a protein tag on the first exofacial loop does not adversely impact the function of the glucose transporter. The strategies employed to create the proposed GLUT3 construct were used and proven effective by the previous works done by these scientists.

Our first strategy was simple with minimum but effective steps. However, we did not see the expected results with the prescribed steps in the protocol. We performed troubleshooting procedures with no successful outcomes.

Troubleshooting Strategy 1

After several trials using the protocol from the **site directed mutagenesis** kit (210518), we had the following issues after PCR:

- No bands in trial wells
- Bright bands in trial wells only after PCR
- Bright bands in trial wells and bright bands at ~200bp of trial wells after PCR
- No colonies after bacterial transformation

To address these issues, we carried out these troubleshooting procedures:

- *Sequence Vector DNA*: we sequenced RR202594 using the provided sequencing primers provided by OriGene technologies. The sequencing results show the presence of the correct cDNA sequence.
- *Re-ordering oligos*: we ordered the same Agilent designed primer oligos from a different facility (Johns Hopkins University Sequencing and Synthesis Facility). We ran the oligos in agarose gel electrophoresis and confirmed presence of DNA at the expected size range.
- *Using different PCR machines*: we ran a trial using two different thermal cyclers (
- *Checking for presence of DNA in template and primers*: we ran agarose gel electrophoresis of Vector template and all primers used prior to conducting the PCR reaction.

- *Using a control DNA polymerase*: As a control for the polymerase, we used HotStarTaq master mix (Qiagen: 203643) to run the same reactions
- *DMSO as positive control*: DMSO (dimethyl sulfoxide) has been identified as a binding agent for GC rich regions to enhance amplification by reducing the annealing temperature. We added DMSO as a variable agent in our troubleshooting process for the SDM strategy.

Our second strategy involved successful amplification of GLUT3 insert from RR202594 using HotStarTaq Master Mix (Qiagen: 203643). We then attempt to perform PCR to (1) install our unique Hpa1 site and (2) install our HA tag at the first exofacial loop of GLUT3 using primers: A, B, C, D, E & F [See Appendix V]. This experiment did not produce the expected bands in the agarose gel electrophoresis performed. Therefore, we moved on to ligation of GLUT3 with the pcDNA3-EGFP vector (addgene: 13031). We then perform restriction digest cloning of HA tag into the first exofacial loop of GLUT3 using AflIII restriction enzyme (NEB R0541S). This approach did not change the outcome of our experiments.

Chapter 5: Conclusion and Future Direction

The STRING analyses did not show direct interactions between GLUT3 and AMPK or nNOS, however, experiments by Ferreira et al. (2011) and Weisova et al. (2009) show an AMPK and nNOS link to GLUT3 surface expression. Results from experiments by Ferreira et al. (2011) show that blocking NO production prevents increase surface GLUT3 suggesting a role for NO in the mechanism involved in GLUT3 expression at cell surface in neurons. Supporting information on the activity of NO with AMP is seen in some of the foundational literature featured in this thesis (Almeida, Moncada, & Bolaños, 2004; Cidad et al., 2004; Moncada et al., 1989). Also, the AKT interaction highlighted in the STRING analyses provide some support of our stated hypothesis of a NO/AMPK/PI3K/Akt pathway to increased GLUT3 expression at neuronal cell surface.

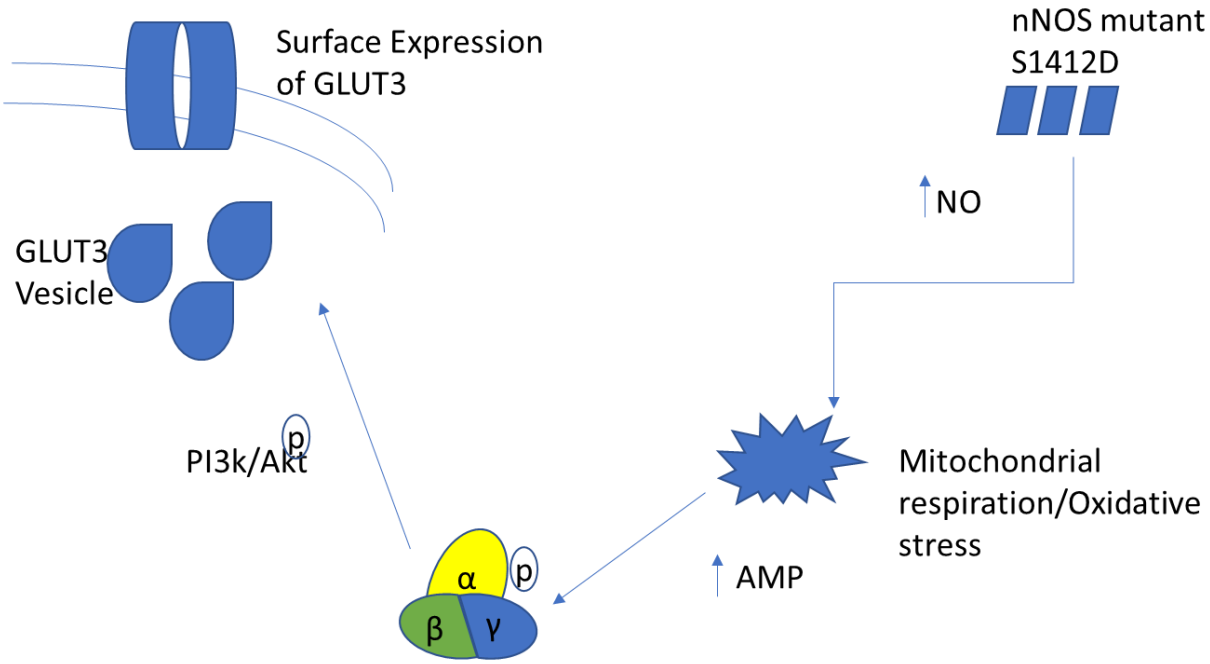


Fig. 17: Proposed model of our NO → AMPK → PI3K/Akt pathway to increase surface GLUT3 expression in neurons

The bioinformatics analyses performed shows that the construct (HA_GLUT3_EGFP) design is sound. This supports the previous works done in this direction (Ashrafi et al., 2017; Burchfield et al., 2013; Dawson et al., 2001). Our next steps for the plasmid construction are to complete the construction process, amplify the product, and confirm the presence of the intended

construct by sequencing and expression of the plasmid in cultured cells *in vitro*. In the future, we would like to carry out experiments that would address the mechanism of the pathway (NO → AMPK → (PI3K/AKT) → GLUT3). Also, we would like to construct mutants that could be used to elucidate specific domain function of GLUT3 in neurons. Studies by (Inukai et al., 2004) have shown that creating a chimera of a GLUT can direct its position of expression in a cell (apical or basal). We would like to explore and identify possible direction-determining sequences in GLUT3 structure.

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Appendices

Appendix I Secondary Structure comparison of Wild Type and Mutant GLUT3

GLUT3_WT				**HA_GLUT3**			**HA_GLUT3_EGFP**		
No	**AA**	**PHL**	**PiTo**	**AA**	**PHL**	**PiTo**	**AA**	**PHL**	**PiTo**
1	K	L	i	K	L	o	K	L	o
2	L	L	i	L	L	o	L	L	o
3	M	L	i	M	L	o	M	L	o
4	G	L	i	G	L	o	G	L	o
5	T	L	i	T	L	o	T	L	o
6	A	L	i	A	L	o	A	L	o
7	K	L	i	K	L	o	K	L	o
8	V	L	i	V	L	o	V	L	o
9	T	L	i	T	L	o	T	L	o
10	P	L	i	P	L	o	P	L	o
11	S	L	i	S	L	o	S	L	o
12	L	L	i	L	L	o	L	L	o
13	V	L	i	V	L	o	V	L	o
14	F	L	i	F	L	o	F	L	o
15	A	H	T	A	L	o	A	L	o
16	V	H	T	V	L	o	V	L	o
17	T	H	T	T	L	o	T	L	o
18	V	H	T	V	L	o	V	H	o
19	A	H	T	A	H	o	A	H	o
20	T	H	T	T	H	o	T	H	o
21	I	H	T	I	H	o	I	H	o
22	G	H	T	G	H	o	G	H	o

23	S	H	T	S	H	o	S	H	o
24	F	H	T	F	H	o	F	H	o
25	Q	H	T	Q	H	o	Q	H	o
26	F	H	T	F	L	o	F	L	o
27	G	H	T	G	L	o	G	L	o
28	Y	H	T	Y	L	o	Y	L	o
29	N	H	T	N	L	o	N	L	o
30	T	H	T	T	L	o	T	L	o
31	G	H	T	G	L	o	G	L	o
32	V	H	T	V	L	o	V	L	o
33	I	H	o	I	L	o	I	L	o
34	N	H	o	N	L	o	N	L	o
35	A	L	o	A	L	o	A	L	o
36	P	L	o	P	L	o	P	L	o
37	E	L	o	E	L	o	E	L	o
38	T	L	o	T	L	o	T	L	o
39	I	L	o	I	L	o	I	L	o
40	I	L	o	I	L	o	I	L	o
41	K	L	o	K	L	o	K	L	o
42	D	L	o	D	L	o	D	L	o
43	F	L	o	F	L	o	F	L	o
44	L	L	o	L	L	o	L	L	o
45	N	L	o	N	L	o	N	L	o
46	Y	L	o	S	L	o	S	L	o
47	T	L	o	R	L	o	R	L	o
48	L	L	o	Y	L	o	Y	L	o
49	E	L	o	P	L	o	P	L	o

50	E	L	o	Y	L	o	Y	L	o
51	R	L	o	D	L	o	D	L	o
52	L	L	o	V	L	o	V	L	o
53	E	L	o	P	L	o	P	L	o
54	D	L	o	D	L	o	D	L	o
55	L	L	o	Y	L	o	Y	L	o
56	P	L	o	A	L	o	A	L	o
57	S	L	o	M	L	o	M	L	o
58	E	L	o	E	L	o	E	L	o
59	G	L	o	E	L	o	E	L	o
60	L	L	o	R	L	o	R	L	o
61	L	L	o	L	L	o	L	L	o
62	T	L	o	E	L	o	E	L	o
63	T	L	o	D	L	o	D	L	o
64	L	H	o	L	L	o	L	L	o
65	W	H	o	P	L	o	P	L	o
66	S	H	T	S	L	o	S	L	o
67	L	H	T	E	L	o	E	L	o
68	C	H	T	G	L	o	G	L	o
69	V	H	T	L	L	o	L	L	o
70	A	H	T	L	L	o	L	L	o
71	I	H	T	T	L	o	T	L	o
72	F	H	T	T	L	o	T	L	o
73	S	H	T	L	L	o	L	L	o
74	V	H	T	W	L	o	W	L	o
75	G	H	T	S	L	T	S	L	o
76	G	H	T	L	H	T	L	L	T

77	M	H	T	C	H	T	C	H	T
78	I	H	T	V	H	T	V	H	T
79	G	H	T	A	H	T	A	H	T
80	S	H	T	I	H	T	I	H	T
81	F	H	T	F	H	T	F	H	T
82	S	H	T	S	H	T	S	H	T
83	V	H	T	V	H	T	V	H	T
84	G	H	T	G	H	T	G	H	T
85	L	H	i	G	H	T	G	H	T
86	F	H	i	M	H	T	M	H	T
87	V	L	i	I	H	T	I	H	T
88	N	L	i	G	H	T	G	H	T
89	R	L	i	S	H	T	S	H	T
90	F	L	i	F	H	T	F	H	T
91	G	L	i	S	H	T	S	H	T
92	R	L	i	V	L	T	V	L	T
93	R	L	i	G	L	i	G	L	T
94	N	L	i	L	L	i	L	L	i
95	S	H	i	F	L	i	F	L	i
96	M	H	i	V	L	i	V	L	i
97	L	H	T	N	L	i	N	L	i
98	L	H	T	R	L	i	R	L	i
99	V	H	T	F	L	i	F	L	i
100	N	H	T	G	L	i	G	L	i
101	L	H	T	R	L	i	R	L	i
102	I	H	T	R	L	i	R	L	i
103	A	H	T	N	L	i	N	L	i

104	I	H	T	S	L	i	S	L	i
105	L	H	T	M	L	i	M	L	T
106	G	H	T	L	H	T	L	H	T
107	G	H	T	L	H	T	L	H	T
108	C	H	T	V	H	T	V	H	T
109	L	H	T	N	H	T	N	H	T
110	M	H	T	L	H	T	L	H	T
111	G	H	T	I	H	T	I	H	T
112	F	H	T	A	H	T	A	H	T
113	A	H	T	I	H	T	I	H	T
114	K	H	T	L	H	T	L	H	T
115	I	H	o	G	H	T	G	H	T
116	A	H	o	G	H	T	G	H	T
117	E	H	o	C	H	T	C	H	T
118	S	H	o	L	H	T	L	H	T
119	V	H	o	M	H	T	M	H	T
120	E	H	o	G	H	T	G	H	T
121	M	H	o	F	H	T	F	L	T
122	L	H	T	A	L	T	A	L	T
123	I	H	T	K	L	T	K	L	o
124	L	H	T	I	L	o	I	L	o
125	G	H	T	A	L	o	A	L	o
126	R	H	T	E	L	o	E	L	o
127	L	H	T	S	H	o	S	H	o
128	I	H	T	V	H	o	V	H	o
129	I	H	T	E	H	T	E	H	o
130	G	H	T	M	H	T	M	H	T

131	I	H	T	L	H	T	L	H	T
132	F	H	T	I	H	T	I	H	T
133	C	H	T	L	H	T	L	H	T
134	G	H	T	G	H	T	G	H	T
135	L	H	T	R	H	T	R	H	T
136	C	H	T	L	H	T	L	H	T
137	T	H	T	I	H	T	I	H	T
138	G	H	T	I	H	T	I	H	T
139	F	H	T	G	H	T	G	H	T
140	V	H	i	I	H	T	I	H	T
141	P	H	i	F	H	T	F	H	T
142	M	H	i	C	H	T	C	H	T
143	Y	H	i	G	H	T	G	H	T
144	I	H	i	L	H	T	L	H	T
145	G	H	i	C	H	T	C	H	T
146	E	L	i	T	H	T	T	H	T
147	V	L	i	G	H	T	G	H	T
148	S	L	i	F	H	i	F	H	i
149	P	L	i	V	H	i	V	H	i
150	T	L	i	P	H	i	P	H	i
151	A	L	i	M	H	i	M	H	i
152	L	L	i	Y	L	i	Y	L	i
153	R	L	i	I	L	i	I	L	i
154	G	L	i	G	L	i	G	L	i
155	A	L	i	E	L	i	E	L	i
156	F	L	i	V	L	i	V	L	i
157	G	H	i	S	L	i	S	L	i

158	T	H	i	P	L	i	P	L	i
159	L	H	T	T	L	i	T	L	i
160	N	H	T	A	L	i	A	L	i
161	Q	H	T	L	L	i	L	L	i
162	L	H	T	R	L	i	R	L	i
163	G	H	T	G	L	i	G	L	i
164	I	H	T	A	L	i	A	L	i
165	V	H	T	F	L	i	F	L	i
166	V	H	T	G	L	i	G	L	i
167	G	H	T	T	L	i	T	L	i
168	I	H	T	L	L	T	L	L	i
169	L	H	T	N	L	T	N	L	T
170	V	H	T	Q	H	T	Q	H	T
171	A	H	T	L	H	T	L	H	T
172	Q	H	T	G	H	T	G	H	T
173	V	H	T	I	H	T	I	H	T
174	F	H	T	V	H	T	V	H	T
175	G	H	T	V	H	T	V	H	T
176	L	H	T	G	H	T	G	H	T
177	D	H	o	I	H	T	I	H	T
178	F	H	o	L	H	T	L	H	T
179	I	H	o	V	H	T	V	H	T
180	L	L	o	A	H	T	A	H	T
181	G	L	o	Q	H	T	Q	H	T
182	S	L	o	V	H	T	V	H	T
183	E	L	o	F	H	T	F	H	T
184	E	L	o	G	H	T	G	H	T

185	L	L	o	L	L	T	L	L	T
186	W	L	o	D	L	o	D	L	T
187	P	H	o	F	L	o	F	L	o
188	G	H	o	I	L	o	I	L	o
189	L	H	T	L	L	o	L	L	o
190	L	H	T	G	L	o	G	L	o
191	G	H	T	S	L	o	S	L	o
192	L	H	T	E	L	o	E	L	o
193	T	H	T	E	L	o	E	L	o
194	I	H	T	L	L	o	L	L	o
195	I	H	T	W	L	o	W	L	o
196	P	H	T	P	L	o	P	L	o
197	A	H	T	G	L	o	G	L	o
198	I	H	T	L	L	T	L	L	T
199	L	H	T	L	H	T	L	H	T
200	Q	H	T	G	H	T	G	H	T
201	S	H	T	L	H	T	L	H	T
202	A	H	T	T	H	T	T	H	T
203	A	H	T	I	H	T	I	H	T
204	L	H	T	I	H	T	I	H	T
205	P	H	T	P	H	T	P	H	T
206	F	H	T	A	H	T	A	H	T
207	C	H	i	I	H	T	I	H	T
208	P	H	i	L	H	T	L	H	T
209	E	L	i	Q	H	T	Q	H	T
210	S	L	i	S	H	T	S	H	T
211	P	L	i	A	H	T	A	H	T

212	R	L	i	A	H	T	A	H	T
213	F	L	i	L	H	T	L	H	T
214	L	L	i	P	H	T	P	H	T
215	L	L	i	F	H	T	F	H	T
216	I	L	i	C	L	i	C	L	i
217	N	L	i	P	L	i	P	L	i
218	R	L	i	E	L	i	E	L	i
219	K	L	i	S	L	i	S	L	i
220	E	L	i	P	L	i	P	L	i
221	E	L	i	R	L	i	R	L	i
222	D	L	i	F	L	i	F	L	i
223	Q	L	i	L	L	i	L	L	i
224	A	L	i	L	L	i	L	L	i
225	T	L	i	I	L	i	I	L	i
226	E	L	i	N	L	i	N	L	i
227	I	L	i	R	L	i	R	L	i
228	L	L	i	K	L	i	K	L	i
229	Q	L	i	E	L	i	E	L	i
230	R	L	i	E	L	i	E	L	i
231	L	L	i	D	L	i	D	L	i
232	W	L	i	Q	L	i	Q	L	i
233	G	L	i	A	L	i	A	L	i
234	T	L	i	T	L	i	T	L	i
235	P	L	i	E	L	i	E	L	i
236	D	L	i	I	L	i	I	L	i
237	V	L	i	L	L	i	L	L	i
238	I	L	i	Q	L	i	Q	L	i

239	Q	L	i	R	L	i	R	L	i
240	E	L	i	L	L	i	L	L	i
241	I	L	i	W	L	i	W	L	i
242	Q	L	i	G	L	i	G	L	i
243	E	L	i	T	L	i	T	L	i
244	M	L	i	P	L	i	P	L	i
245	K	L	i	D	L	i	D	L	i
246	D	L	i	V	L	i	V	L	i
247	E	L	i	I	L	i	I	L	i
248	S	L	i	Q	L	i	Q	L	i
249	I	L	i	E	L	i	E	L	i
250	R	L	i	I	L	i	I	L	i
251	M	L	i	Q	L	i	Q	L	i
252	S	L	i	E	L	i	E	L	i
253	Q	L	i	M	L	i	M	L	i
254	E	L	i	K	L	i	K	L	i
255	K	L	i	D	L	i	D	L	i
256	Q	L	i	E	L	i	E	L	i
257	V	L	i	S	L	i	S	L	i
258	T	L	i	I	L	i	I	L	i
259	V	L	i	R	L	i	R	L	i
260	L	L	i	M	L	i	M	L	i
261	E	L	i	S	L	i	S	L	i
262	L	L	i	Q	L	i	Q	L	i
263	F	L	i	E	L	i	E	L	i
264	K	L	i	K	L	i	K	L	i
265	S	L	i	Q	L	i	Q	L	i

266	P	L	i	V	L	i	V	L	i
267	S	L	i	T	L	i	T	L	i
268	Y	L	i	V	L	i	V	L	i
269	F	L	i	L	L	i	L	L	i
270	Q	H	i	E	L	i	E	L	i
271	P	H	T	L	L	i	L	L	i
272	L	H	T	F	L	i	F	L	i
273	L	H	T	K	L	i	K	L	i
274	I	H	T	S	L	i	S	L	i
275	S	H	T	P	L	i	P	L	i
276	V	H	T	S	L	i	S	L	i
277	V	H	T	Y	L	i	Y	L	i
278	L	H	T	F	L	i	F	L	i
279	Q	H	T	Q	L	i	Q	L	i
280	L	H	T	P	L	i	P	L	i
281	S	H	T	L	H	i	L	H	T
282	Q	H	T	L	H	T	L	H	T
283	Q	H	T	I	H	T	I	H	T
284	F	H	T	S	H	T	S	H	T
285	S	H	T	V	H	T	V	H	T
286	G	H	T	V	H	T	V	H	T
287	I	H	T	L	H	T	L	H	T
288	N	H	T	Q	H	T	Q	H	T
289	A	H	T	L	H	T	L	H	T
290	V	H	T	S	H	T	S	H	T
291	F	H	T	Q	H	T	Q	H	T
292	Y	H	T	Q	H	T	Q	H	T

293	Y	H	T	F	H	T	F	H	T
294	S	H	T	S	H	T	S	H	T
295	T	H	T	G	L	T	G	H	T
296	G	H	o	I	H	T	I	H	T
297	I	H	o	N	H	T	N	H	T
298	F	H	o	A	H	T	A	H	T
299	Q	H	o	V	H	T	V	H	T
300	D	H	o	F	H	o	F	H	T
301	A	H	o	Y	H	o	Y	H	o
302	G	L	o	Y	H	o	Y	H	o
303	V	L	o	S	H	o	S	H	o
304	Q	L	o	T	L	o	T	L	o
305	E	H	o	G	L	o	G	L	o
306	P	H	o	I	L	o	I	L	o
307	I	H	o	F	L	o	F	L	o
308	Y	H	T	Q	L	o	Q	L	o
309	A	H	T	D	L	o	D	L	o
310	T	H	T	A	L	o	A	L	o
311	I	H	T	G	L	o	G	L	o
312	G	H	T	V	L	o	V	L	o
313	A	H	T	Q	L	o	Q	L	o
314	G	H	T	E	L	o	E	L	o
315	V	H	T	P	L	o	P	L	o
316	V	H	T	I	L	o	I	L	o
317	N	H	T	Y	H	o	Y	H	o
318	T	H	T	A	H	T	A	H	T
319	I	H	T	T	H	T	T	H	T

320	F	H	T	I	H	T	I	H	T
321	T	H	T	G	H	T	G	H	T
322	V	H	T	A	H	T	A	H	T
323	V	H	T	G	H	T	G	H	T
324	S	H	T	V	H	T	V	H	T
325	L	H	T	V	H	T	V	H	T
326	F	H	T	N	H	T	N	H	T
327	L	H	T	T	H	T	T	H	T
328	V	H	i	I	H	T	I	H	T
329	E	H	i	F	H	T	F	H	T
330	R	L	i	T	H	T	T	H	T
331	A	L	i	V	H	T	V	H	T
332	G	L	i	V	H	T	V	H	T
333	R	L	i	S	H	T	S	H	T
334	R	L	i	L	H	T	L	H	T
335	T	L	i	F	H	T	F	H	T
336	L	L	i	L	H	i	L	H	i
337	H	L	i	V	H	i	V	L	i
338	M	H	i	E	L	i	E	L	i
339	I	H	i	R	L	i	R	L	i
340	G	H	T	A	L	i	A	L	i
341	L	H	T	G	L	i	G	L	i
342	G	H	T	R	L	i	R	L	i
343	G	H	T	R	L	i	R	L	i
344	M	H	T	T	L	i	T	L	i
345	A	H	T	L	L	i	L	L	i
346	V	H	T	H	L	i	H	L	i

347	C	H	T	M	L	i	M	L	i
348	S	H	T	I	L	i	I	L	i
349	V	H	T	G	H	T	G	H	T
350	F	H	T	L	H	T	L	H	T
351	M	H	T	G	H	T	G	H	T
352	T	H	T	G	H	T	G	H	T
353	I	H	T	M	H	T	M	H	T
354	S	H	T	A	H	T	A	H	T
355	L	H	T	V	H	T	V	H	T
356	L	H	T	C	H	T	C	H	T
357	L	H	T	S	H	T	S	H	T
358	K	H	T	V	H	T	V	H	T
359	D	H	o	F	H	T	F	H	T
360	E	H	o	M	H	T	M	H	T
361	Y	H	o	T	H	T	T	H	T
362	E	H	o	I	H	T	I	H	T
363	A	H	T	S	H	T	S	H	T
364	M	H	T	L	H	T	L	H	T
365	S	H	T	L	H	T	L	H	T
366	F	H	T	L	H	T	L	H	T
367	V	H	T	K	L	o	K	L	o
368	C	H	T	D	L	o	D	L	o
369	I	H	T	E	L	o	E	L	o
370	V	H	T	Y	L	o	Y	L	o
371	A	H	T	E	H	o	E	L	o
372	I	H	T	A	H	o	A	H	o
373	L	H	T	M	H	o	M	H	o

374	V	H	T	S	H	o	S	H	o
375	Y	H	T	F	H	T	F	H	T
376	V	H	T	V	H	T	V	H	T
377	A	H	T	C	H	T	C	H	T
378	F	H	T	I	H	T	I	H	T
379	F	H	T	V	H	T	V	H	T
380	E	H	T	A	H	T	A	H	T
381	I	H	T	I	H	T	I	H	T
382	G	H	T	L	H	T	L	H	T
383	P	H	T	V	H	T	V	H	T
384	G	H	T	Y	H	T	Y	H	T
385	P	H	T	V	H	T	V	H	T
386	I	H	T	A	H	T	A	H	T
387	P	H	T	F	H	T	F	H	T
388	W	H	i	F	H	T	F	H	T
389	F	H	i	E	H	T	E	H	T
390	I	H	i	I	H	T	I	H	T
391	V	H	i	G	H	T	G	H	T
392	A	H	i	P	H	T	P	H	T
393	E	H	i	G	H	T	G	H	T
394	L	H	i	P	H	T	P	H	T
395	F	H	i	I	H	T	I	H	T
396	S	H	i	P	H	i	P	H	T
397	Q	H	i	W	H	i	W	H	i
398	G	L	i	F	H	i	F	H	i
399	P	L	i	I	H	i	I	H	i
400	R	L	i	V	H	i	V	H	i

401	P	L	i	A	L	i	A	L	i
402	A	L	i	E	L	i	E	L	i
403	A	H	i	L	L	i	L	L	i
404	M	H	i	F	L	i	F	L	i
405	A	H	i	S	L	i	S	L	i
406	V	H	i	Q	L	i	Q	L	i
407	A	H	T	G	L	i	G	L	i
408	G	H	T	P	L	i	P	L	i
409	C	H	T	R	L	i	R	L	i
410	S	H	T	P	L	i	P	L	i
411	N	H	T	A	L	i	A	L	i
412	W	H	T	A	L	i	A	L	i
413	T	H	T	M	L	i	M	L	i
414	S	H	T	A	L	i	A	L	i
415	N	H	T	V	L	i	V	L	i
416	F	H	T	A	H	i	A	H	T
417	L	H	T	G	H	T	G	H	T
418	V	H	T	C	H	T	C	H	T
419	G	H	T	S	H	T	S	H	T
420	M	H	T	N	H	T	N	H	T
421	F	H	T	W	H	T	W	H	T
422	F	H	T	T	H	T	T	H	T
423	P	H	T	S	H	T	S	H	T
424	S	H	T	N	H	T	N	H	T
425	A	H	T	F	H	T	F	H	T
426	A	H	o	L	H	T	L	H	T
427	A	H	o	V	H	T	V	H	T

428	Y	H	o	G	H	T	G	H	T
429	L	H	o	M	H	T	M	H	T
430	G	H	T	F	H	T	F	H	T
431	A	H	T	F	H	T	F	H	T
432	Y	H	T	P	H	T	P	H	T
433	V	H	T	S	H	T	S	H	T
434	F	H	T	A	H	T	A	H	o
435	I	H	T	A	H	o	A	H	o
436	I	H	T	A	H	o	A	H	o
437	F	H	T	Y	H	o	Y	H	o
438	A	H	T	L	H	o	L	H	T
439	A	H	T	G	H	T	G	H	T
440	F	H	T	A	H	T	A	H	T
441	L	H	T	Y	H	T	Y	H	T
442	V	H	T	V	H	T	V	H	T
443	F	H	T	F	H	T	F	H	T
444	F	H	T	I	H	T	I	H	T
445	L	H	T	I	H	T	I	H	T
446	I	H	T	F	H	T	F	H	T
447	F	H	T	A	H	T	A	H	T
448	T	H	i	A	H	T	A	H	T
449	F	H	i	F	H	T	F	H	T
450	F	H	i	L	H	T	L	H	T
451	K	H	i	V	H	T	V	H	T
452	V	L	i	F	H	T	F	H	T
453	P	L	i	F	H	T	F	H	T
454	E	L	i	L	H	T	L	H	T

455	T	L	i	I	H	T	I	H	T
456	K	L	i	F	H	T	F	H	T
457	G	L	i	T	H	i	T	H	i
458	R	L	i	F	H	i	F	H	i
459	T	L	i	F	H	i	F	L	i
460	F	L	i	K	L	i	K	L	i
461	E	L	i	V	L	i	V	L	i
462	D	L	i	P	L	i	P	L	i
463	I	L	i	E	L	i	E	L	i
464	T	L	i	T	L	i	T	L	i
465	R	L	i	K	L	i	K	L	i
466	A	L	i	G	L	i	G	L	i
467	F	L	i	R	L	i	R	L	i
468	E	L	i	T	L	i	T	L	i
469	G	L	i	F	L	i	F	L	i
470	Q	L	i	E	L	i	E	L	i
471	A	L	i	D	L	i	D	L	i
472	Q	L	i	I	L	i	I	L	i
473	S	L	i	T	L	i	T	L	i
474	G	L	i	R	L	i	R	L	i
475	K	L	i	A	L	i	A	L	i
476	G	L	i	F	L	i	F	L	i
477	S	L	i	E	L	i	E	L	i
478	A	L	i	G	L	i	G	L	i
479	G	L	i	Q	L	i	Q	L	i
480	V	L	i	A	L	i	A	L	i
481	E	L	i	Q	L	i	Q	L	i

482	L	L	i	S	L	i	S	L	i
483	N	L	i	G	L	i	G	L	i
484	S	L	i	K	L	i	K	L	i
485	M	L	i	G	L	i	G	L	i
486	Q	L	i	S	L	i	S	L	i
487	P	L	i	A	L	i	A	L	i
488	V	L	i	G	L	i	G	L	i
489	K	L	i	V	L	i	V	L	i
490	E	L	i	E	L	i	E	L	i
491	T	L	i	L	L	i	L	L	i
492	P	L	i	N	L	i	N	L	i
493	G	L	i	S	L	i	S	L	i
494	N	L	i	M	L	i	M	L	i
495	A	L	i	Q	L	i	Q	L	i
496	E	L	i	P	L	i	P	L	i
497	F	L	i	V	L	i	V	L	i
				K	L	i	K	L	i
				E	L	i	E	L	i
				T	L	i	T	L	i
				P	L	i	P	L	i
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				N	L	i	N	L	i
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Table 8: Secondary Structure comparison (Yachdav et al., 2014).

Key: AA - Amino Acid, PHL - Predicted transmembrane helices (H- Helical membrane; L- no helical membrane), PiTo - Predicted topology of Transmembrane loops (i= inside loop; T= Transmembrane; o= outside loop)

Appendix II Restriction Summary of GLUT3_WT, HA_GLUT3 and HA_GLUT3_EGFP

	GLUT3_WT	HA_GLUT3	HA_GLUT3_EGFP
Site:	**Positions:**	**Positions:**	**Positions:**
AatI agg|cct	1020	1047	1047
AccII cg|cg	none	none	1848, 2166
AfaI gt|ac	428	455	455, 1949, 2231
Alw441 g|tgcac	406	433	433
AluI ag|ct	4, 484, 553, 590, 784, 801, 1095, 1205	4, 511, 580, 617, 811, 828, 1122, 1232	4, 511, 580, 617, 811, 828, 1122, 1232, 1540, 1573, 1645, 1678, 1894, 1942, 2053, 2227
ApaI gggcc|c	1196	1223	1223
ApaLI g|tgcac	406	433	433
AvaI c|ycgrg	623, 1392	650, 1419	650, 1419
BalI tgg|cca	209	236	236
BamHI g|gatcc	501	528	528
BbuI gcatg|c	1456	1483	1483
BfaI c|tag	293, 798	320, 825	320, 825
Bsp1407I t|gtaca	426	453	453, 2229
Bsp19I c|catgg	1209	1236	1236
BsrGI t|gtaca	426	453	453, 2229
BstUI cg|cg	none	none	1848, 2166
DpnII |gatc	366, 501, 678, 711, 720, 858	393, 528, 705, 738, 747, 885	393, 528, 705, 738, 747, 885, 2019, 2167, 2205
EcoRI g|aattc	1487	1514	1514
HaeIII gg|cc	209, 375, 559, 1020, 1130, 1146, 1152, 1194, 1280, 1397	236, 402, 586, 1047, 1157, 1173, 1179, 1221, 1307, 1424	236, 402, 586, 1047, 1157, 1173, 1179, 1221, 1307, 1424, 1593, 1693, 1982, 2094
HincII gty|rac	262, 298, 949	289, 325, 976	289, 325, 976
HindIII a|agctt	2	2	2
HinfI g|antc	93, 252, 350, 628, 888	93, 279, 377, 655, 915	93, 279, 377, 655, 915
HpaI gtt|aac	none	none	none

HpaII c|cgg	90, 704, 1393	90, 731, 1420	90, 731, 1420, 1548, 1611, 1671, 2202
MboI |gatc	366, 501, 678, 711, 720, 858	393, 528, 705, 738, 747, 885	393, 528, 705, 738, 747, 885, 2019, 2167, 2205
MscI tgg|cca	209	236	236
MseI t|taa	120, 132, 575, 648	120, 132, 602, 675	120, 132, 602, 675
MspI c|cgg	90, 704, 1393	90, 731, 1420	90, 731, 1420, 1548, 1611, 1671, 2202
NcoI c|catgg	1209	1236	1236
NdeII |gatc	366, 501, 678, 711, 720, 858	393, 528, 705, 738, 747, 885	393, 528, 705, 738, 747, 885, 2019, 2167, 2205
NlaIII catg|	232, 289, 1015, 1033, 1054, 1093, 1213, 1456	259, 316, 1042, 1060, 1081, 1120, 1240, 1483	259, 316, 1042, 1060, 1081, 1120, 1240, 1483, 1522, 1756, 1786, 1981, 2176, 2221
PhoI gg|cc	209, 375, 559, 1020, 1130, 1146, 1152, 1194, 1280, 1397	236, 402, 586, 1047, 1157, 1173, 1179, 1221, 1307, 1424	236, 402, 586, 1047, 1157, 1173, 1179, 1221, 1307, 1424, 1593, 1693, 1982, 2094
PvuII cag|ctg	484, 1205	511, 1232	511, 1232
RsaI gt|ac	428	455	455, 1949, 2231
SacI gagct|c	786	813	813
SmaI ccc|ggg	1394	1421	1421
SphI gcatg|c	1456	1483	1483
SstI gagct|c	786	813	813
StuI agg|cct	1020	1047	1047
TaqI t|cga	881, 1380, 1401	908, 1407, 1428	908, 1407, 1428, 1569, 1863, 1890, 1905, 2034
XmaI c|ccggg	1392	1419	1419

Table 9: Restriction Summary of GLUT3_WT, HA_GLUT3 and HA_GLUT3_EGFP (Stothard, 2000)

Key: Green: cuts once (unique) Yellow: cuts twice

Appendix III List of Reagents

Reagent or Resource	**Source**	**Identifier**
Critical commercial Assays		
QuikChange Lightning Site-Directed Mutagenesis Kit, 10 Rxn	Agilent Technologies	210518
QIAquick Gel Extraction Kit (50)		28704
iProof		
HotStarTaq Master Mix	Qiagen Inc	203643
Restriction Enzymes		
SgfI, GQ, 250u	Promega Corp	R7103
HindIII-HF	New England BioLabs	R3104S
HpaI	New England BioLabs	R0105S
BamHI	New England BioLabs	R0136S
MLU1: 1,000 units	New England BioLabs	R0198S
AflIII	New England BioLabs	R0541S
Sequence-Based Reagents		
Slc2a3 (NM_017102) Rat Tagged ORF Clone	OriGene Technologies	RR202594
GLUT3_Hpa1		

pcDNA3-EGFP	Addgene	13031
Other Reagents		
1 Liter Nuclease Free water	Integrated DNA Technologies (IDT)	11-05-01-04
T4 DNA Ligase	New England BioLabs	M0202T
Software and Algorithms		
SnapGene Free Trial & Viewer	SnapGene	https://www.snapgene.com/try-snapgene/
Agilent Technologies Primer Design Tool	Agilent Technologies	https://www.chem.agilent.com/store/primerDesignProgram.jsp
STRING Database		https://string-db.org/
CLUSTAL W		https://www.genome.jp/tools-bin/clustalw
Serial Cloner		http://serialbasics.free.fr/Serial_Cloner.html
Bioinformatics: Sequence manipulation Suite		https://www.bioinformatics.org/sms2/pairwise_align_dna.html
PredictProtein		https://www.predictprotein.org/
Equipment		
T100 Thermal Cycler	Bio Rad	1861096

Appendix IV SEQUENCES

>NP_008862.1 solute carrier family 2, facilitated glucose transporter member 3 [Homo sapiens]

```
      1 MGTQKVTPAL IFAITVATIG SFQFGYNTGV INAPEKIIKE FINKTLTDKG
     51 NAPPSEVLLT SLWSLSVAIF SVGGMIGSFS VGLFVNRFGR RNSMLIVNLL
    101 AVTGGCFMGL CKVAKSVEML ILGRLVIGLF CGLCTGFVPM YIGEISPTAL
    151 RGAFGTLNQL GIVVGILVAQ IFGLEFILGS EELWPLLLGF TILPAILQSA
    201 ALPFCPESPR FLLINRKEEE NAKQILQRLW GTQDVSQDIQ EMKDESARMS
    251 QEKQVTVLEL FRVSSYRQPI IISIVLQLSQ QLSGINAVFY YSTGIFKDAG
    301 VQEPIYATIG AGVVNTIFTV VSLFLVERAG RRTLHMIGLG GMAFCSTLMT
    351 VSLLLKDNYN GMSFVCIGAI LVFVAFFEIG PGPIPWFIVA ELFSQGPRPA
    401 AMAVAGCSNW TSNFLVGLLF PSAAHYLGAY VFIIFTGFLI TFLAFTFFKV
    451 PETRGRTFED ITRAFEGQAH GADRSGKDGV MEMNSIEPAK ETTTNV
```

>Rat GLUT3 from NM_017102

```
      1 MGTAKVTPSL VFAVTVATIG SFQFGYNTGV INAPETIIKD FLNYTLEERL
     51 EDLPSEGLLT TLWSLCVAIF SVGGMIGSFS VGLFVNRFGR RNSMLLVNLI
    101 AILGGCLMGF AKIAESVEML ILGRLIIGIF CGLCTGFVPM YIGEVSPTAL
    151 RGAFGTLNQL GIVVGILVAQ VFGLDFILGS EELWPGLLGL TIIPAILQSA
    201 ALPFCPESPR FLLINRKEED QATEILQRLW GTPDVIQEIQ EMKDESIRMS
    251 QEKQVTVLEL FKSPSYFQPL LISVVLQLSQ QFSGINAVFY YSTGIFQDAG
    301 VQEPIYATIG AGVVNTIFTV VSLFLVERAG RRTLHMIGLG GMAVCSVFMT
    351 ISLLLKDEYE AMSFVCIVAI LVYVAFFEIG PGPIPWFIVA ELFSQGPRPA
    401 AMAVAGCSNW TSNFLVGMFF PSAAAYLGAY VFIIFAAFLV FFLIFTFFKV
    451 PETKGRTFED ITRAFEGQAQ SGKGSAGVEL NSMQPVKETP GNA
```

>Rat GLUT3

```
      1 KLMGTAKVTP SLVFAVTVAT IGSFQFGYNT GVINAPETII KDFLNYTLEE
     51 RLEDLPSEGL LTTLWSLCVA IFSVGGMIGS FSVGLFVNRF GRRNSMLLVN
    101 LIAILGGCLM GFAKIAESVE MLILGRLIIG IFCGLCTGFV PMYIGEVSPT
    151 ALRGAFGTLN QLGIVVGILV AQVFGLDFIL GSEELWPGLL GLTIIPAILQ
    201 SAALPFCPES PRFLLINRKE EDQATEILQR LWGTPDVIQE IQEMKDESIR
    251 MSQEKQVTVL ELFKSPSYFQ PLLISVVLQL SQQFSGINAV FYYSTGIFQD
    301 AGVQEPIYAT IGAGVVNTIF TVVSLFLVER AGRRTLHMIG LGGMAVCSVF
    351 MTISLLLKDE YEAMSFVCIV AILVYVAFFE IGPGPIPWFI VAELFSQGPR
    401 PAAMAVAGCS NWTSNFLVGM FFPSAAAYLG AYVFIIFAAF LVFFLIFTFF
    451 KVPETKGRTF EDITRAFEGQ AQSGKGSAGV ELNSMQPVKE TPGNAEF
```

> NM_017102.2 GLUT3_HA Rat Sequence

```
      1 KLMGTAKVTP SLVFAVTVAT IGSFQFGYNT GVINAPETII KDFLNSRYPY
     51 DVPDYAMEER LEDLPSEGLL TTLWSLCVAI FSVGGMIGSF SVGLFVNRFG
    101 RRNSMLLVNL IAILGGCLMG FAKIAESVEM LILGRLIIGI FCGLCTGFVP
    151 MYIGEVSPTA LRGAFGTLNQ LGIVVGILVA QVFGLDFILG SEELWPGLLG
    201 LTIIPAILQS AALPFCPESP RFLLINRKEE DQATEILQRL WGTPDVIQEI
    251 QEMKDESIRM SQEKQVTVLE LFKSPSYFQP LLISVVLQLS QQFSGINAVF
    301 YYSTGIFQDA GVQEPIYATI GAGVVNTIFT VVSLFLVERA GRRTLHMIGL
    351 GGMAVCSVFM TISLLLKDEY EAMSFVCIVA ILVYVAFFEI GPGPIPWFIV
    401 AELFSQGPRP AAMAVAGCSN WTSNFLVGMF FPSAAAYLGA YVFIIFAAFL
    451 VFFLIFTFFK VPETKGRTFE DITRAFEGQA QSGKGSAGVE LNSMQPVKET
    501 PGNAEF
```

>rf 1 NM_017102.2 HA_GLUT3_EGFP Rat Sequence

```
      1 KLMGTAKVTP SLVFAVTVAT IGSFQFGYNT GVINAPETII KDFLNSRYPY
     51 DVPDYAMEER LEDLPSEGLL TTLWSLCVAI FSVGGMIGSF SVGLFVNRFG
    101 RRNSMLLVNL IAILGGCLMG FAKIAESVEM LILGRLIIGI FCGLCTGFVP
    151 MYIGEVSPTA LRGAFGTLNQ LGIVVGILVA QVFGLDFILG SEELWPGLLG
    201 LTIIPAILQS AALPFCPESP RFLLINRKEE DQATEILQRL WGTPDVIQEI
```

```
251 QEMKDESIRM SQEKQVTVLE LFKSPSYFQP LLISVVLQLS QQFSGINAVF
301 YYSTGIFQDA GVQEPIYATI GAGVVNTIFT VVSLFLVERA GRRTLHMIGL
351 GGMAVCSVFM TISLLLKDEY EAMSFVCIVA ILVYVAFFEI GPGPIPWFIV
401 AELFSQGPRP AAMAVAGCSN WTSNFLVGMF FPSAAAYLGA YVFIIFAAFL
451 VFFLIFTFFK VPETKGRTFE DITRAFEGQA QSGKGSAGVE LNSMQPVKET
501 PGNAEFMVSK GEELFTGVVP ILVELDGDVN GHKFSVSGEG EGDATYGKLT
551 LKFICTTGKL PVPWPTLVTT LTYGVQCFSR YPDHMKQHDF FKSAMPEGYV
601 QERTIFFKDD GNYKTRAEVK FEGDTLVNRI ELKGIDFKED GNILGHKLEY
651 NYNSHNVYIM ADKQKNGIKV NFKIRHNIED GSVQLADHYQ QNTPIGDGPV
701 LLPDNHYLST QSALSKDPNE KRDHMVLLEF VTAAGITLGM DELYK
```

Appendix V Primers

A - GLUT3 F

5’ – AAGCTTATGGGGACAGCGAAG – 3’

B - GLUT3 R

5’ – GAATTCGGCATTGCCAGGGGT – 3’

C - Hpa1 F

5’ – GCTCTTCCAACGTGTAGTTAACAAAGTCCTTAATGATTGTCTC – 3’

D - Hpa1 R

5’ – GAGACAATCATTAAGGACTTTGTTAACTACACGTTGGAAGAGC – 3’

E- HA_GLUT3 F

5’ – TACACGTTACCCATACGATGTTCCAGATTACGCTA – 3’

F - HA_GLUT3 R

5’ – GTAGCGTAATCTGGAACATCGTATGGGTAACGTGTA – 3’

G - Myc_Hpa1 F

5’ – TACACGGAACAAAAACTCATCTCAGAAGAGGATCTGA – 3’

H - Myc_Hpa1 R

5’ – GCTTGTTTTTGAGTAGAGTCTTCTCCTAGACTACC – 3’