The functional characterization of the gene coding for *O*-linked β-*N*-acetylglucosaminidase (OGA) in *Drosophila melanogaster*

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Abstract

O-linked β-*N*-acetylglucosaminidase (OGA) is the enzyme responsible for removing the *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification from serine and threonine residues of a variety of proteins, while its addition to protein targets is catalyzed by *O*-linked β-*N*-acetylglucosamine transferase (OGT). *sxc/Ogt* is essential in *Drosophila melanogaster*; however, it is unknown whether *Oga* is also essential in flies. I found that, in flies, a significant decrease in *Oga* transcript induced by RNAi knockdown is not lethal and that a nonsense mutation that putatively results in the translation of a C-terminally truncated version of OGA is viable when crossed to a deficiency known to span the *Oga* locus in the genome; however, reduced viability was observed when ubiquitously overexpressing two copies of *Oga* cDNA. Reduced expression of *Oga* and *Ogt* in *Drosophila* insulin-producing cells, via targeted RNAi expression with a *dILP2-GAL4* driver, results in a slight increase and decrease, respectively, in male body weight.

Keywords: *O*-linked β-*N*-acetylglucosaminidase (OGA); *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc); *O*-linked β-*N*-acetylglucosamine transferase (OGT); *Drosophila melanogaster*

For my father, mother and sister,
the best family a girl could ask for.

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List of Acronyms

4MU-GlcNAc	4-methylumbelliferyl N-acetyl-β-D-glucosaminide
4-MU	4-methylumbelliferone (also known as 7-hydroxy-4-methylcoumarin)
CD-Search	Conserved Domain-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)
CDD	Conserved Domain Database (from NBCI, used for CD-Search to look for conserved domains)
EBI	European Bioinformatics Institute (http://www.ebi.ac.uk)
EMBL	European Molecular Biology Laboratory (http://www.ebi.ac.uk)
FHCRC	Fred Hutchinson Cancer Research Center
HBP	Hexosamine biosynthetic pathway
JCVI	J. Craig Venter Institute (http://sift.jcvi.org)
NCBI	National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)
NAPS	Nucleic Acid Protein Service
NMD	Nonsense-mediated mRNA decay
O-GlcNAc	*O*-linked β-*N*-acetylglucosamine
OGA	*O*-linked β-*N*-acetylglucosaminidase (*O*-GlcNAcase)
OGT	*O*-linked β-*N*-acetylglucosaminyl transferase (*O*-GlcNAc transferase)
ORF	Open reading frame
PTC	Premature translation termination codon
PTM	Posttranslational modification
RFU	Relative fluorescence unit
SIFT	Sorting Intolerant from Tolerant
SNP	Single nucleotide polymorphism
SDTP	The Seattle *Drosophila* TILLING Project (http://tilling.fhcrc.org)
TPR	Tetratricopeptide repeat
UDP-GlcNAc	Uridine diphosphate *N*-acetylglucosamine
VDRC	Vienna Drosophila Resource Centre (http://stockcenter.vdrc.at/control/main)

1. General Introduction

1.1. What is *O*-GlcNAc?

O-linked β-*N*-acetylglucosamine (O-GlcNAc) is a posttranslational modification (PTM) that has been studied extensively since its identification on lymphocyte proteins in 1984 (Torres & Hart, 1984); the modification consists of the addition/removal of a single GlcNAc moiety on serine or threonine residues of the over one-thousand nuclear and/or cytoplasmic protein targets (Bullen et al., 2014; Gao, Wells, Comer, Parker, & Hart, 2001; Holt & Hart, 1986; Wells, Vosseller, & Hart, 2001; Zeidan & Hart, 2010). *O*-GlcNAc is considered a signal transduction modification because it is constantly being added and removed in response to certain stimuli (Wells et al., 2001). *O*-linked β-*N*-acetylglucosamine transferase (OGT) catalyzes the addition of at least one *O*-GlcNAc group to protein targets, whereas *O*-linked β-*N*-acetylglucosaminidase (OGA) catalyzes the removal of the GlcNAc moieties (Hart, Housley, & Slawson, 2007). This process, termed *O*-GlcNAc cycling, is found mostly within the cytoplasm or nucleoplasm (Hart et al., 2007). *O*-GlcNAcylation is one of the most abundant PTMs and it is thought to fine tune various cellular processes in response to nutrient levels and stress cues within the cell (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011).

Perturbed *O*-GlcNAc signalling has been putatively linked to many diseases, including Alzheimer's disease, cardiovascular disease, diabetes and cancer (Slawson, Copeland, & Hart, 2010). The large number of functionally diverse OGT/OGA protein targets include components of many crucial cellular processes; a few noteworthy examples include regulation of transcription, translation, various signalling pathways and the stress response (Hart et al., 2007; Slawson et al., 2010). Clearly, the vast array of protein targets suggests that many pathways are reliant on particular substrates being *O*-GlcNAc modified to provide appropriate downstream cellular responses; and, that *O*-

GlcNAc cycling is an important regulator of protein activity (Hanover, 2001; Love & Hanover, 2005; Sekine, Love, Rubenstein, & Hanover, 2010).

Interestingly, OGT and OGA are the sole enzymes responsible for *O*-GlcNAcylation and de-*O*-GlcNAcylation, respectively; this is in distinct contrast with the large number of protein kinases and phosphatases responsible for phosphorylation/dephosphorylation of specific targets (Lewis, 2013; Ozcan, Andrali, & Cantrell, 2010; Slawson & Hart, 2011). Thus, it is likely that OGT, and possibly OGA, rely on the help of other protein partners for recruitment to appropriate substrates in many cases (Cheung & Hart, 2008; Ozcan et al., 2010; X. Yang et al., 2008) . For example, Yang, Zhang, & Kudlow (2002) found that mSin3A recruits OGT to gene promoters via OGTs tetratricopeptide repeat (TPR) domain in order to promote gene silencing via *O*-GlcNAc modification, which is proposed to inactivate transcription factors and RNA polymerase II. Since like phosphorylation, *O*-GlcNAcylation is a dynamic process, it is not surprising to find that OGT is an essential enzyme in many eukaryotes, including *Drosophila melanogaster* (Ingham, 1984), mammals (O'Donnell, Zachara, Hart, Marth, & Donnell, 2004; Shafi et al., 2000) and various other vertebrates (Kenwrick, Amaya, & Papalopulu, 2004; Love, Krause, & Hanover, 2010; Webster et al., 2009). Unfortunately, there is not as much information available as to organisms that require properly regulated and functioning OGA for viability.

The main focus of my work centered on experiments designed to investigate the functional importance of OGA in *Drosophila*. My most basic goal was to determine whether, like OGT, OGA is essential for fly survival. In addition, I wanted to provide as much insight as possible into other aspects of the fly's requirements for OGA.

1.2. Enzymes involved

1.2.1. Mammalian OGT

The gene that encodes OGT is located on the X chromosome in mammals and the protein has high sequence conservation across all tested eukaryotes (Nolte & Müller, 2002; Shafi et al., 2000). A tetratricopeptide repeat (TPR) domain is found in the N-terminus of OGT and the catalytic domain is located in the C-terminus (Kreppel,

Blomberg, & Hart, 1997; Kreppel & Hart, 1999). Three splice variant mRNAs encoding three polypeptides, which differ in terms of their N-terminal TPR domain lengths and respective localizations, have been identified to date: ncOGT (nucleocytoplasmic OGT), mOGT (mitochondrial OGT), and sOGT (short isoform of OGT) (Hanover et al., 2003; Vaidyanathan, Durning, & Wells, 2014). The ncOGT isoform is the longest of the three at 116 kDa in size containing 12.5 TPR motifs and mOGT is only slightly smaller at 103 kDa containing 9.5 TPR motifs (Hanover et al., 2003; reviewed in Harwood & Hanover, 2014); these two isoforms are aptly named for their localization in the nucleus/cytoplasm and in the mitochondria, respectively. mOGT contains a mitochondrial-targeting sequence upstream of its TPR domain (Love, Kochran, Cathey, Shin, & Hanover, 2003). sOGT is the smallest isoform at 78 kDa with 2.5 TPRs and has been shown to localize to the nucleocytoplasm (Hanover et al., 2003; Nolte & Müller, 2002; Shafi et al., 2000). These TPRs are thought to facilitate protein-protein interactions and may be involved in substrate recognition and multimerization of OGT (Hanover et al., 2003; Kreppel & Hart, 1999; Lubas & Hanover, 2000; Lubas, Smith, Starr, & Hanover, 1995). Various factors are thought to impact OGT activation including protein-protein interaction, localization, substrate accessibility and post-translational modification (PTM) (Whelan, Lane, & Hart, 2008; X. Yang et al., 2008).

1.2.2. Mammalian OGA

OGA was originally identified as an autoantigen associated with meningioma (meningioma-expressed antigen 5; MGEA5) (Hanover, 2001; Heckel et al., 1998). The mammalian gene encodes two splice variants resulting in two isoforms: the long isoform, OGA-L, which is 916 amino acids in length and 102 kDa in weight, and the short isoform, OGA-S, which is 677 amino acids in length and 76 kDa in weight (reviewed in Harwood & Hanover, 2014). OGA-L, localized primarily in the cytoplasm, contains a sequence similar to a histone acetyl transferase (HAT) domain at its C-terminus in addition to its N-terminal catalytic domain (Hanover, 2001; Heckel et al., 1998); however, whether OGA-L has HAT activity *in vivo* remains unclear (Butkinaree, Park, & Hart, 2010; Harwood & Hanover, 2014; Rao et al., 2013; Toleman, Paterson, Whisenhunt, & Kudlow, 2004). It has been suggested that the HAT domain may function to help facilitate OGA's interactions with transcription machinery (Hart et al., 2011). OGA-L has a linker region

between the N and C termini that includes a cleavage site for caspase 3 and thus the protein is cleaved during apoptosis (Butkinaree et al., 2010); however, this cleavage has not been reported to abolish OGA activity (Hart et al., 2011). OGA-L has been demonstrated to be *O*-GlcNAc modified by OGT (B. D. Lazarus, Love, & Hanover, 2006; Whisenhunt et al., 2006).

OGA-S, has been shown to have nuclear localization by Comtesse, Maldener, and Meese (2001) as well as lipid-droplet associated localization by Keembiyehetty, Krzeslak, Love, and Hanover (2011); this shorter form has an identical N-terminal hyaluronidase domain to OGA-L, but no C-terminal HAT-like domain (Harwood & Hanover, 2014). OGA-S has been demonstrated to target to the surface of nascent lipid droplets, where colocalization with a key player in the formation of lipid droplets, perilipin-2, can be observed (Bickel, Tansey, & Welte, 2009; Brasaemle, 2007; Londos, Brasaemle, Schultz, Segrest, & Kimmel, 1999). Genetic variations in perilipin have been correlated to various metabolic phenotypes such as type 2 diabetes mellitus (Bickel et al., 2009). Duggirala et al. (1999) demonstrated that human *MGEA5* is a diabetes susceptibility locus in people of Mexican American heritage. This was later confirmed by Lehman et al. (2005) in a study that showed an association of a particular single nucleotide polymorphism (SNP), located in intron 10 of the human MGEA5 gene, with diabetes traits in a Mexican American population. The crystal structure of human OGA has not been solved, but catalytic mechanisms have been proposed based on the structure of two bacterial homologues (Dennis et al., 2006; Rao et al., 2006).

1.3. The relationship between *O*-GlcNAcylation and phosphorylation

As mentioned above, OGT modifies serine/threonine residues of protein targets and it has been well established that serine/threonine residues can also be phosphorylated by various kinases; the two modifications, *O*-GlcNAc and phosphate, have a complex relationship, termed cross talk, that aids in the regulation of target protein function based on which modification is present on a particular residue at any given time (Hart et al., 2011). Both *O*-GlcNAc and phosphate are dynamic modifications that are added and removed in response to various stimuli. The relationship between

these two PTMs appears to change depending on the protein target in question. In some cases proteins can have concurrent *O*-GlcNAc and phosphate modifications, such as insulin receptor substrate-1 (IRS-1) (Ball, Berkaw, & Buse, 2006; Hart et al., 2011), and in other cases the addition of one modification may regulate the addition of adjacent modifications (Dias, Cheung, Wang, & Hart, 2009). In some proteins, the two modifications are mutually exclusive, meaning that they can both modify the same site but at different times and/or one modification may prevent the other from being added too close in proximity (Comer & Hart, 2001). For example, the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II) can be modified by both OGT and CTD kinases on the same amino acid residues; however, *O*-GlcNAc inhibits phosphorylation of adjacent sites and vice versa (Comer & Hart, 2001). There are also various protein substrates in which the two modifications compete for nearby sites, such as calcium/calmodulin-dependent kinase IV (CaMKIV), and the combination of modifications present is associated with changes in protein activity (Dias et al., 2009; Hart et al., 2011). This competitive relationship may be due to the large size of the *O*-GlcNAc sugar modification, the phosphate group having a negative charge, or because protein conformations may change with the addition/removal of each modification (Hart et al., 2011). Cross talk between phosphorylation and *O*-GlcNAcylation is obviously very important for the regulation of protein activity and thus, will continue to be explored.

1.4. The hexosamine biosynthetic pathway (HBP) and its link to *O*-GlcNAcylation

O-GlcNAc metabolism is becoming more widely accepted as part of the nutrient-sensing hexosamine biosynthetic pathway (HBP), because *O*-GlcNAc modifications can be partially representative of the cell's nutritional state (Hanover, Krause, & Love, 2012; Harwood & Hanover, 2014). The hexosamine biosynthetic pathway (HBP) is a metabolically driven pathway regulated in part by cellular nutrient levels and the availability of nutrient processing enzymes (Rossetti, 2000). Only ~2-5% of cellular glucose is funnelled into the HBP (Vaidyanathan et al., 2014). The rate-limiting step of the HBP is the conversion of fructose-6-phosphate to glucose-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT) (Kornfeld, Kornfeld, Neufeld, & O'Brien, 1964; Marshall, Bacote, & Traxinger, 1991); thus, GFAT is a crucial enzyme in the HBP

and therefore in *O*-GlcNAcylation also, because the HBP culminates in the production of UDP-GlcNAc, the donor sugar nucleotide for *O*-GlcNAcylation by OGT (Haltiwanger, Holt, & Hart, 1990).

Various studies have emerged outlining both protective and damaging roles for the HBP in several model organisms. For example, increased hexosamine biosynthetic pathway activity in flies was shown to cause heart damage while a decrease in pathway activity had a protective effect on the hearts of flies raised on a high sucrose diet (Na et al., 2013). A more recent study, conducted by Denzel et al. (2014), describes novel *Caenorhabditis elegans* gain of function (gof) mutations in *gfat-1,* which was found to be a novel longevity gene, that result in increased lifespan of the nematode by up to 42%; the authors determined that the levels of cellular UDP-HexNAc, a measure of combined UDP-GlcNAc and UDP-N-acetylgalactosamine (UDP-GalNAc), were elevated by up to 10 fold in *gfat-1* gof mutants. Denzel et al. (2014) also found that supplementing wild-type nematodes with GlcNAc, up to a concentration of 10 mM, resulted in an extended lifespan. Together these data suggest that increased cellular UDP-HexNAc, either through supplementation or through the novel gof *gfat-1* mutations, result in an extended *C. elegans* lifespan (Denzel et al., 2014). Another previous study reported that *oga-1* deletion mutants may also help to extend the lifespan of nematodes, presumably by persisting *O*-GlcNAc modifications on target proteins (Rahman et al., 2010); this study also reported that *ogt-1* deletion mutants showed a decrease in median lifespan, perhaps from the lack of *O*-GlcNAc modifications. Although, the median lifespan may be affected in the *ogt-1* and *oga-1* mutants, it is still interesting to note that they are viable and fertile and that they have an effect on insulin-like signalling, dauer entry and macronutrient storage in either case (Forsythe et al., 2006; Hanover et al., 2005).

1.5. *O*-GlcNAcylation in *Drosophila melanogaster*

In *Drosophila,* the essential gene *sxc* (*super sex combs*)/*Ogt* is located in the right arm of chromosome 2 (2R) heterochromatin and encodes OGT, which is critical for proper embryonic development (Gambetta, Oktaba, & Müller, 2009; Ingham, 1984; Sinclair et al., 2009). *sxc* is a member of the Polycomb group (PcG) of genes, which encode conserved proteins that function as transcriptional repressors to regulate

development (Gambetta et al., 2009). *sxc* loss of function alleles display homeotic defects due to the misregulation of Hox genes (Gambetta et al., 2009; Sinclair et al., 2009). Currently, there is a large body of information available in terms of the putative functions and regulation of OGT, but investigations are ongoing with the intention of elucidating the exact functions of OGT in flies and other eukaryotes. However, the functional role of OGA in flies is largely undefined and there is a scarcity of information regarding the function of OGA in eukaryotes in general. The suitability of *D. melanogaster* as an appropriate model organism to study several human gene orthologues and the disorders associated with their respective dysfunctions, is well established; thus, the wealth of genetic tools available in flies should be useful for defining the basic functions of OGA. At this point, there are studies that suggest a role for OGA in insulin signalling in *Drosophila* and claim that the disruption of OGA activity results in significant changes to fly body weight (Park et al., 2011; Sekine et al., 2010).

1.6. The genetics of the *Oga* gene in flies and TILLING for *Oga* mutants

In *Drosophila,* the *Oga* gene, CG5871, is located at the cytological position, **93C6-93C7**, in the right arm of chromosome 3 (3R) (http://flybase.org). Although some chromosomal deficiencies lacking the gene are available, none exist that are deficient for *Oga* exclusively and, until very recently, no *Oga* allele has been discovered (FlyBase; Radermacher et al., 2014); the discovery, characterization and confirmation of a bona fide null, or even a hypomorphic *Oga* mutant in which gene function is reduced, would help to elucidate the function of OGA in flies. This information would be useful for defining the role of the protein in eukaryotes in general. Importantly, one must always consider the possibility that the *Oga* gene is nonessential in flies; this may not be surprising, since it is estimated that an approximate 75% of *Drosophila* genes are nonessential (Koundakjian, Cowan, Hardy, & Becker, 2004). Even if *Oga* proves to be nonessential in flies, the isolation and characterization of appropriate *Oga* mutants would be important steps in characterizing the function and regulation of the gene. *Oga* has been shown to be essential during development in mice as gene knockout results in embryonic lethality (Y. R. Yang et al., 2012). Perhaps this is indicative of a functional

trend across species and *Oga* will prove to be essential during fly development as studies continue to progress and the importance of OGA becomes increasingly clear.

There are many transposon inserts that map near the 5' and 3' ends of *Oga* (http://flybase.org). Radermacher et al. (2014) recently reported an allele, Oga^{P}, in which a transposon (P{GSV6}) had inserted into exon 1 of *Oga* introducing a premature stop codon; flies homozygous for Oga^{P} were viable and fertile. The homozygotes were analyzed via western blot and Oga^{P} appeared to be protein-null (Radermacher et al., 2014). Further investigation into these findings will be required to confirm whether this allele encodes an amorphic *Oga* mutant. The Honda laboratory has previously attempted to generate and isolate *Oga* mutants via the imprecise excision of a transposon (P{Supor-P}) from its insertion site near the 5' end of *Oga* in flies with the $Oga^{KG04950}$ allele (unpublished). However, all recessive lethal derivatives of this experiment were associated with deletions of both *Oga* and the adjacent gene(s) (D. Sinclair, unpublished). A useful reverse-genetics approach is the method of mutant identification within an assemblage of mutant fly lines, such as the Zuker collection, entitled, TILLING or Targeting Induced Local Lesions IN Genomes. TILLING exploits the high-point mutational densities found within flies treated with the chemical mutagen ethyl methanesulfonate (EMS) (Winkler et al., 2005).

The Zuker collection comprises mutant fly lines resulting from a collaborative project designed with the objective of using EMS mutagenesis to generate at least one novel mutant allele for every nonessential autosomal gene (Koundakjian et al., 2004); this collection has provided a continuous, forward-genetic resource of balanced second and third chromosome mutations for phenotypic screening (Cooper, Greene, et al., 2008). Interestingly, even though only stocks of flies homozygous for mutagenized second or third chromosomes were established, Koundakjian et al. (2004) report that approximately one third of these lines became homozygous lethal; the authors attribute this to the EMS induction of mosaic mutations. The lethal third chromosome lines from this collection have been extremely valuable for the ongoing functional genetic analysis of third chromosome heterochromatin in the Honda laboratory (Syrzycka, 2009).

Cooper et al. (2008) have made use of the Zuker collection to provide a reverse-genetic resource for TILLING in the *D. melanogaster* genome (Fly-TILL,

http://tilling.fhcrc.org/fly/). TILLING begins with the use of a web-based program, CODDLE (for Codons Optimized to Detect Deleterious Lesions, http://proweb.org/coddle) to select an appropriate primer pair in order to amplify a region within which, based on sequence conservation, a missense mutation is likely to disrupt the protein product of the gene of interest (Henikoff et al., 2004). The primers are used in PCR reactions with pooled genomic DNA as the template and the products are subsequently denatured; slow re-annealing of the products facilitates the generation of heteroduplexes at sites with mismatched base pairs where point mutations altering the sequence from wild-type are located (Cooper, Till, & Henikoff, 2008). Next, the mutations are identified via digestion with *Cel*I, a single-strand-specific endonuclease, and electrophoretic separation; then all of the DNA samples from within the pooled genomic DNA that produced heteroduplexes are tested individually to determine which samples contained the mutations of interest (Cooper, Till, et al., 2008). Finally, the mutations are sequenced and this information is made accessible to the research community (Cooper, Till, et al., 2008). Winkler et al. (2005), using their own collection of mutagenized chromosome lines, tested the TILLING process to identify mutations for a known essential *Drosophila* gene and found that five out of twenty-five total mutations resulted in lethality, thereby indicating that *Cel*I-mediated TILLING of EMS generated mutants is a rapid and efficient method of finding mutations in a gene of interest in flies (Winkler et al., 2005).

1.7. Disruption of *Oga* regulation via targeted transgene expression using UAS/GAL4 system

Historically, genetic analysis has largely involved the forward genetics approach of generating random mutations, which are then screened for desired phenotypes displayed by mutant lines. Next, the genes involved are mapped and characterized genetically and, where possible, molecularly. However, currently, the existence of annotated sequence data for model organisms has provided a wealth of powerful reverse genetics tools, including the identification of mutations via TILLING-based approaches. Site-directed mutagenesis is another useful reverse genetics method; it can be used for generating customized mutations, such as protein null mutations, affecting the protein product of a gene of interest. One can, of course, link this to

transgenic analysis in order to test the biological effects of the mutations. However, in the absence of suitable biochemical reagents, such as an antibody for the product, the confirmation of protein null mutations may be problematic. Fortunately, there are also other reverse genetics methods that can enable one to investigate the effects of altered expression of a gene of interest. The most popular and useful of these methods rely on targeted expression of gene-specific transgenes using the UAS/GAL4 system; this system allows one to have spatial and/or temporal control of UAS-transgene expression (Brand & Perrimon, 1993; Duffy, 2002). It is based on the fact that the Upstream Activation Sequences (UAS) that regulate expression of the target transgene recruit the yeast-specific transcription factor, GAL4, whose expression itself is controlled in the desired pattern in the same fly (Duffy, 2002). This system is extremely versatile since it allows one to induce targeted misexpression or downregulation of the gene of interest, depending on the type and position of the transgene. Another alternative reverse genetics approach to generate gene specific mutations is to use the clustered regularly interspersed short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, through the injection of RNA into *D. melanogaster* embryos to generate germline transmittable mutations (Bassett, Tibbit, Ponting, & Liu, 2013) or through the use of guide RNA expression plasmids and transgenic Cas9 lines to knockout specific genes using transgenic animals (Port, Chen, Lee, & Bullock, 2014).

1.8. Project aims

There are numerous studies investigating the function and regulation of OGT in *D. melanogaster*, but not nearly as many investigating OGA. It has been well established that *Ogt* is an essential gene in flies, but whether the same is true for *Oga* is still unknown; therefore, my work largely involved determining the causative effect of disrupting *Oga* or its regulation in the fly in an attempt to elucidate the function of the gene and the respective encoded protein. To this end, I analyzed various *Oga* mutants obtained from the Seattle *Drosophila* TILLING Project. Each allele was crossed to a known third chromosome deficiency to assess the viability of flies hemizygous for their respective mutations. I also wanted to determine where the putative active site is in *Drosophila* OGA and predict whether the amino acid substitutions in the mutants may affect the function of this well-conserved protein.

While characterizing the available mutant lines, I was also exploring other methods known to alter gene expression in *Drosophila*. I used the UAS/GAL4 system and transgenic animals to reduce or increase the level of *Oga* transcript ubiquitously within the fly, using RNAi or cDNA transgenic constructs, respectively, to determine if an effect could be observed. I determined the effectiveness of the knockdown or overexpression experiments using quantitative PCR (qPCR) with three reference genes to measure the level of *Oga* transcript relative to control flies.

Previous research has been conducted that associates disruption of *O*-GlcNAc cycling in the *Drosophila* insulin producing cells (IPCs) with a change in body weight. I wanted to determine if I could repeat these findings and extend the experiment to include our most promising mutant, Oga^{415}, which putatively encodes a C-terminally truncated OGA protein, as well as flies with *UAS-Oga cDNA* transgenes to overexpress *Oga*. I used a GAL4 driver, *Drosophila insulin-like peptide 2-GAL4* (*dILP2-GAL4*), to target transgene expression exclusively to the ICPs and measured the body weight of affected flies.

As previously mentioned, the importance of OGT function in *Drosophila* has been clearly established. Thus, strong *sxc*/*Ogt* alleles kill the fly at the pharate adult stage (Gambetta et al., 2009; Ingham, 1984; Sinclair et al., 2009). The fact that *sxc*-null mutants survive to this stage suggests that the maternal contribution of *Ogt* mRNA and protein is sufficient to sustain development as far as this advanced stage; however, the resulting flies do not eclose and other studies in our laboratory show that there is a continuous requirement for OGT for adult survival (J. Radke, personal communication). In addition to mutant analysis, down-regulation of *Ogt* using GAL4-induced, ubiquitous expression of RNAi transgenes causes embryonic death (Sinclair et al., 2009). The latter result is consistent with the previous data of Ingham (1984) in which pole-cell transplantation was used to demonstrate that SXC/OGT is required for embryonic development. Nevertheless, many questions remain about the exact nature of the fly's requirements for OGT. One interesting question is whether the catalytic function of OGT is essential or if other critical non-catalytic roles exist. Therefore, in collaboration with researchers in the Vocadlo laboratory, I participated in an experiment to test the ability of a putative, catalytic-site defective version of human OGT to rescue combinations of *sxc*/*Ogt* alleles in the fly.

2. Materials and Methods

2.1. *Drosophila* stocks and crosses

Fly stocks and crosses were maintained on standard yeast-cornmeal-molasses media; crosses using the UAS-GAL4 system for targeted gene expression were incubated at 29°C for optimal transgene expression (Duffy, 2002). All other crosses were raised at 25°C unless stated otherwise. Crosses combined ten virgin females and six males of the desired genotypes, unless otherwise stated. Cross progeny were genotypically distinguished on the basis of the presence/absence of markers on the relevant balancer chromosomes. The term "ubiquitous" shall be used to describe expression patterns when they follow those of genes expressed during every stage of life and in every cell type (e.g. *tubulin*).

The following stocks were obtained from the Bloomington Stock Centre and are listed with their respective stock numbers in parentheses: [*w*; P{Ilp2-GAL4.R}2*](#37516) (Rulifson, Kim, & Nusse, 2002) will henceforth be referred to as the *dILP2-GAL4* driver (*Drosophila* insulin-like peptide 2); [y^1*w*; P{tubP-GAL4}LL7/TM3* Sb^1](#5138) (Lee & Luo, 1999), will henceforth be referred to as the *tub-GAL4* driver; [w^{1118}*; Df(3R)ED10845, P{3'.RS5+3.3'}ED10845/TM6C,* cu^1 Sb^1](#9487) (Ryder et al., 2007), will henceforth be referred to as *Df(3R)ED10845*; [*UAS-Dcr-2*](#24646) (Dietzl et al., 2007), will henceforth be referred to as *UAS-Dcr-2*; and [w^{1118}] was the wild-type control strain used in this study. The following stocks were obtained from the Vienna Drosophila Resource Centre (VDRC) and are listed with their respective stock numbers in parentheses: [w^{1118}*; UAS-Oga RNAi*](#41822); [w^{1118}*; UAS-Oga RNAi*](#41823); and [w^{1118}*; UAS-Oga RNAi*](#106670). More specific information about the VDRC RNAi lines can be found on the FlyBase site (http://www.flybase.organd/orhttp://stockcenter.vdrc.at) and other relevant information is given in appendix A.

The Seattle *Drosophila* TILLING Project (STDP) at the Fred Hutchinson Cancer Research Center (FHCRC) provided putative *Oga* mutants; these will henceforth be referred to as *Oga* alleles (Cooper, Till, et al., 2008). The origins of the *sxc* alleles used in this study are as follows: sxc^{1}, sxc^{3}, sxc^{4}, and sxc^{5} were generated and characterized by Ingham (1984) by; sxc^{NC130} was generated by Myster & Peifer (2003) and shown to be an sxc allele by Gambetta et al. (2009) and Sinclair et al. (2009); sxc^{2637} was generated by Spradling et al. (1999) and shown to be an sxc allele by Sinclair et al. (2009). The w^{1118}; *UAS-Ogt cDNA 3632-2-2M* and w^{1118}; *UAS-Ogt RNAi 2824-1-4M* lines were generated as described in Sinclair et al. (2009) and the w^{1118}; *UAS-Gfat1 RNAi 2664-1-1M* and w^{1118}; *UAS-Gfat1 cDNA 4145-1-3M* lines were generated as described by Jackson (2007). *UAS-Oga cDNA* lines 8302-3-1M to 8302-4-10F were generated via plasmid injection into w^{1118}embryos by BestGene, following the cloning of the appropriate plasmid constructs by standard cloning procedures detailed in section 2.6 of this study. Eleven *Oga* RNAi lines, provided by Kristina Pohl (unpublished), were made following procedures summarized in section 2.4 of this study; two of the lines had transgene insertion sites on the second chromosome (23-9 and 23-10) and nine had transgene insertion sites on the third chromosome (23-1, 23-2, 23-4, 23-5, 23-6, 23-7, 23-8, 23-11, and 23-12).

2.2. Hemizygous viability of *Oga* alleles

All but one of the *Oga* alleles were maintained in combination with the *TM3Sb Ser* balancer. Oga^{89} was maintained as a homozygote. Males from each of the nine *Oga* mutant lines were crossed separately to *Df(3R)ED10845/TM6CSb* virgin females. F_1 offspring (a minimum of 114) were examined for the presence/absence of *Sb and Ser,* markers indicative of the *TM3* balancer, within the respective crosses. The relative viability of each *Oga* allele when hemizygous, was calculated as a ratio of the number of flies with the genotype Oga^{-}/*Df(3R)ED10845* to the number of internal control flies with the genotype Oga^{-}/*TM6CSb.*

2.3. Online analysis of wild-type and mutant *Oga* protein sequences

The wild-type OGA protein sequence (appendix B), was obtained from the FlyBase Website (http://www.flybase.org) (St. Pierre, Ponting, Stefancsik, McQuilton, & the FlyBase Consortium, 2014). Two web-based tools were used to analyze the sequence for potential conserved structural and functional domains: the National Center for Biotechnology's (NCBI's) CD-Search (Conserved Domain-Search) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the InterPro Protein Sequence Analysis and Classification tool hosted by EMBL-EBI (European Molecular Biology Laboratory- European Bioinformatics Institute) (https://www.ebi.ac.uk/interpro/). The CD-Search searches for conserved domains within a sequence query based on homology to previously annotated protein domains from various databases, including the Conserved Domain Database (CDD), in order to allow users to predict possible functions of their protein of interest; the search methodology is based on an algorithm called Reverse Position-Specific BLAST (RPS-BLAST); it relies on the assumption that important amino acid positions or critical stretches of amino acids representing functional domains will be well conserved within protein families and across species (Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2009, 2011, 2013).

The CD-Search was conducted with the default settings against each of the applicable databases: CDD v3.11 – 45746 PSSMs, Pfam v27.0 – 14831 PSSMs, SMART v6.0 – 1013 PSSMs, KOG v1.0 – 4825 PSSMs, and TIGR v13.0 – 4284 PSSMs. CD-Search uses an algorithm called Reverse Position-Specific BLAST (RPS-BLAST) (Marchler-Bauer et al., 2002, 2011). RPS-BLAST is an adaptation of Position-Specific Iterated BLAST (PSI-BLAST) (Altschul et al., 1997).

InterPro is a web-based tool that analyzes protein sequences and classifies them to generate a comprehensive report with information about respective protein families, conserved domains and predicted functional sites (Hunter et al., 2012; Jones et al., 2014). This search tool compares the query protein sequence to information housed in some of the same databases used with the CD-Search tool, as well as those of a few other resources (Hunter et al., 2012). The InterPro search was conducted using default settings.

Missense *Oga* alleles were analyzed using SIFT (Sorting Intolerant from Tolerant) to predict which, if any, of the amino acid substitutions may be deleterious to the subsequently translated mutant *Oga* proteins. SIFT assesses the frequency and type of tolerated amino acids within a particular position of homologous protein sequences and uses this information to determine the probability that the substitution in question will have an adverse affect on the resulting protein or be tolerated within the protein structure (Ng & Henikoff, 2001, 2002, 2003). SIFT analysis was completed by the SDTP and the report made available on their website. A SIFT search can also be completed on the JCVI website (http://sift.jcvi.org) using the amino acid sequence of the *Oga* protein translation from FlyBase (http://www.flybase.org), shown in appendix B, and the amino acid substitutions present in the *Oga* mutants (Table 2).

2.4. Transposition of an *Oga* RNAi transgene to obtain enhanced expression (Kristina Pohl, unpublished)

Our initial *Oga* knockdown experiments using RNAi lines from VDRC (41822 and 41823) did not show an effect on viability; to determine if the targeted transgene expression was being affected by the loci in which they were inserted we attempted to move the P element, from stock number 41823, to loci that facilitated better transgene expression. Males bearing the transgenic X-chromosome RNAi line from VDRC (stock number 41823) were mated with females carrying $\Delta 2.3^{HH}$ ("hi-hop"), a transposase source; this cross tentatively allowed for the transposition of the P element to other loci. The progeny with enhanced w^+ reporter gene expression, observed as an increase in red eye pigment in a w^- background, were selected to generate new RNAi stocks that presumably contained the RNAi transgene in a more permissive chromatin environment.

2.5. *Oga* RNAi crosses and calculation of relative viability

UAS-Dcr-2/UAS-Dcr-2; *tub-GAL4/TM3Sb* virgin females were crossed separately to males from each of the fourteen available *Oga* RNAi stocks listed in appendix A. For line 23-10, the RNAi transgene was heterozygous balanced over *CyO,* as the insert was recessive lethal, and in line 41823 the transgene was hemizygous, as the insert was on

the X chromosome; however, all of the other crosses consisted of males homozygous for the RNAi transgenes. F_1 progeny (a minimum of 159) were genotypically distinguished on the basis of the presence/absence of the *Sb* marker carried by the balancer. Relative viability was calculated as the ratio of the number of flies with *tub-GAL4* driven RNAi transgenes to the number of internal control flies with the *TM3* balancer. *Oga* RNAi line 106670 from the VDRC phage site-specific insertion library was the last to become available (see 3.5) and it was predicted by VDRC to have one off-target effect that may affect the expression of gene CG10574 (*Inhibitor-2*) alongside CG5871 (*Oga*) (Appendix A).

2.6. Molecular cloning of *Oga* cDNA transgene constructs

Drosophila melanogaster (Dm) Oga cDNA was cloned into the multiple cloning site of pUAST, a GAL4-regulated *Drosophila* gene expression vector originally developed by Brand & Perrimon (1993); the desired construct was used to express *Oga* cDNA ubiquitously *in vivo*. The P{pUAST-*Dm Oga*} cDNA construct was cloned as follows: the cDNA was subcloned from P{pFLC1-*Dm Oga*} cDNA clones (kindly provided by Chandra Lebovitz) into the *Not*I and *Kpn*I sites of the pUAST polylinker, in order to allow directional cloning of the insert to ensure the desired orientation. The insert and vector ends were prepared by conducting separate double digest reactions with *Not*I and *Kpn*I restriction enzymes (Fermentas). The *Oga* cDNA insert was then ligated into the compatible restriction sites in the vector.

The P{pUAST 2xflag-*Dm Oga*} cDNA construct was made as follows: *Oga* cDNA was PCR amplified from a pFLC1 clone housing *Drosophila Oga* cDNA (kindly provided by Chandra Lebovitz). *Not*I restriction sites were added to the 5' and 3' ends of the *Oga* cDNA fragment by incorporating the NotI recognition site into the forward (5'-TTT TGC GGC CGC CAC AAG GTG CAA TTG TCC GAA CC-3') and reverse (5'-TTT GCG GCC GCC GAA ACG GCG ACC CAT GTA AAT AC-3') PCR primers designed to amplify the entire *Oga* cDNA sequence. The *Oga* cDNA template was PCR amplified using High Fidelity PCR Enzyme Mix (Thermo Scientific) and the following PCR program: 94°C 5 min., 33 cycles of [94°C 30 sec., 67.5°C 30 sec., 72°C 3 min. 30 sec.], 70°C 10 min., hold at 4°C). The resulting PCR product was purified (QIAquick PCR Purification Kit,

Quiagen) and ligated into a pJET1.2 shuttle vector following standard procedure for the CloneJET PCR Cloning Kit (Thermo Scientific). The appropriate shuttle vector clone, containing the desired insert, and the empty pUAST 2x FLAG vector (see below) were separately digested with *Not*I restriction enzyme (Fermentas) to generate the appropriate sticky ends for the subsequent ligation reaction. The *Not*I cut vector was treated with Shrimp Alkaline Phosphatase (SAP; Fermentas) following standard procedures, prior to ligation with the insert. The pUAST 2xflag vector is a modified version of pUAST containing a 3' 2x flag tag that was generated and kindly supplied by Graham Hallson (Hallson et al., 2012).

The pUAST/pUAST2xFLAG constructs containing *Drosophila Oga* cDNA were isolated, sent to the NAPS (Nucleic Acid Protein Service) unit at UBC and I subsequently confirmed that the sequence generated matched the sequence listed on FlyBase (appendix C); a sample was then sent to BestGene for microinjection into w^{1118} embryos to generate transgenic *D. melanogaster* stocks using standard procedures (Rubin & Spradling, 1982).

2.7. Overexpression of *Oga* and calculation of relative viability

y^1w^*; *tub-GAL4/TM3Sb*^{1} virgin females were crossed separately to males that were either heterozygous or homozygous for each of the P{pUAST-*Dm Oga*} or the P{pUAST 2xflag-*Dm Oga*} constructs. F_1 progeny (a minimum of 133) were distinguished on the basis of presence/absence of the *Sb* marker carried by the *TM3* balancer. The relative viability was measured as the ratio of the number of flies with a *tub-GAL4* driven *Oga* cDNA transgene to the number of internal control flies containing the cDNA transgene over the *TM3* balancer.

I also used standard genetic methods to carry out an experiment in which Oga^+ transgenes representing five different second chromosome transgenic lines (8302-3-1M, 8302-3-2M, 8302-4-3M, 8302-4-5M and 8302- 4-8M)) were separately rendered homozygous in the presence of the *tub-GAL4* driver transgene on the third chromosome. To accomplish this, virgin females from each of the five stocks were mated separately to

males of the same genotype. The resulting F_1 progeny (a minimum of 49) were scored by the presence/absence of the *Sb* marker on the *TM3* balancer chromosome. The relative viability for each cross was calculated as the ratio of the number of progeny containing two copies of the *Oga* cDNA transgene under control of a *tub-GAL4* driver to the number of flies containing only one copy of the transgene driven with the *tub-GAL4* driver.

2.8. Transheterozygous *sxc* mutant rescue experiment

An important question is whether the essential functions of OGT stem from its catalytic capability or if they are related to other cellular roles of the protein. I collaborated with Matthew Macauley and David Shen from the Vocadlo laboratory, with a view to investigate this question. Based on existing research information, Matthew Macauley constructed a mutated version of *Homo sapiens (Hs) Ogt* cDNA that was intended to code for the wild-type protein with the exception of a single amino acid substitution; the location of this residue change was thought to be a critical histidine (H) residue within the OGT active site, H558 (Martinez-Fleites et al., 2008). Thus the altered gene in which histidine was substituted by alanine (A) at amino acid position 558 and where no discernable enzymatic activity exists was designated as "catalytic-site-dead" (CSD) (Martinez-Fleites et al., 2008). Next, he cloned H558A mutant *Hs Ogt* cDNA, the *Ogt* cDNA CSD, into a targeted fly gene expression vector and sent the purified plasmid to BestGene for injection into w^{1118} embryos; this process resulted in four fly lines, each containing the *UAS-Hs Ogt cDNA CSD* transgene in a different insertion site within the genome (1CSD, 2CSD, 3CSD, 4CSD).

My role in the collaboration was to test whether expression of the *UAS-Hs Ogt cDNA CSD* transgene was able to rescue transheterozygous combinations of different *sxc* alleles. In this experiment, nine different combinations involving the *sxc* alleles 1, 3, 4, 5, NC130 and 2637 were used and each allele combination was repeated with the four CSD fly lines. In each case, the third chromosome *tub-GAL4* driver was used to express the CSD construct constituitively. The entire set of crosses was repeated with *Drosophila melanogaster (Dm) Ogt* cDNA transgenes in place of the CSD to serve as a positive control. The F_1 offspring (minimum of 124) were distinguished via the

presence/absence of appropriate dominant markers known to be on the balancer chromosomes used. Don Sinclair prepared the following stocks by standard genetic methods: *sxc^{2637}/CyRoi; UAS-Hs Ogt cDNA CSD/TM3Sb* (separate stocks for 1CSD, 2CSD, 3CSD, and 4 CSD), *sxc^{2637}/CyRoi; UAS-Dm Ogt cDNA/TM3Sb* and *sxc^{3}/CyRoi; tub-GAL4/TM3Ser*. I generated the remaining genotypes using standard genetic methods: *sxc^{1}/CyRoi; UAS-Hs Ogt cDNA CSD/TM3Ser* (separate stocks for 1CSD, 2CSD, 3CSD, and 4 CSD)*, sxc^{1}/CyRoi; UAS-DmeI Ogt cDNA/TM3Ser, sxc^{1}/CyRoi; tub-GAL4/TM3Sb, sxc^{4}/CyRoi; tub-GAL4/TM3Sb, sxc^{5}/CyRoi; tub-GAL4/TM3Sb,* and *sxc^{NC130}/CyRoi; tub-GAL4/TM3Sb.*

David Shen conducted OGT activity assays for rescued flies, using radiolabeled [^{3}H]UDP-GlcNAc (American Radiolabel) as the substrate donor and followed protocols adapted from his published work (see Shen, Gloster, Yuzwa, & Vocadlo, 2012). For each of these assays, one-day-old, adult, female flies of the genotype *sxc^{2637}/sxc^{3}; UAS-Hs Ogt cDNA 1CSD/tub-GAL4,* were used to assay the OGT activity relative to activity in wild-type, *w^{1118},* flies.

2.9. Quantitative PCR (qPCR)

RNA was isolated from one-day-old adult female flies of the following genotypes by TRIzol (Invitrogen) and chloroform extraction followed by precipitation in isopropanol using standard protocols: *UAS-Oga* RNAi transgenes ubiquitously expressed with *tub-GAL4* and *UAS-Dcr-2*, *UAS-Oga* cDNA transgenes ubiquitously expressed with *tub-GAL4*, *Oga^{415}/Df(3R)ED10845*) and *w^{1118}* control flies. RNA from each genotype was treated with DNase I (Invitrogen) for 1 hour at 37°C and then heated in a 65°C water bath for 15 minutes. The RNA was then used as template for reverse transcriptase to generate cDNA via the iScript cDNA synthesis kit (Bio-Rad). Using the available *Oga* cDNA sequence (FlyBase; Appendix C), Kristina Pohl designed qPCR primers to amplify a region of *Oga* cDNA spanning the boundary between exons 6 and 7 (Table 1). Graham Hallson designed primer sets to amplify three reference genes: *RP49, Khc Set4,* and *Tub 56D* (Table 1).

Each 10 μl qPCR reaction was prepared in triplicate and contained the following: 1 μl cDNA, 0.2 μl forward primer, 0.2 μl reverse primer, 5 μl KAPA SYBR FAST master mix (KAPABIOSYSTEMS), and 3.6 μl nuclease free water. The qPCR program used was as follows: 20 sec at 95 °C, 40 cycles of [3 sec at 95 °C, 30 sec at 60 °C], 15 sec at 95 °C and then 60 sec at 80 °C. The quantity of *Oga* transcript, as well as the quantity of the three reference gene transcripts, in each cDNA sample relative to the w^{1118} control was measured using the 48 well StepOne™ Real-Time PCR System (Applied Biosystems®).

Table 1. Primers used to determine Oga expression in samples relative to wild-type control via quantitative PCR (qPCR)

Gene Target	Primer Sequence
Oga	5'- GCA GCT ACA CAT GGT TTA GCA AGG -3' 5'- CAC AAA TCG TTG ACT TGC TGT TCG -3'
RP49	5'- CATGTGGCGGGTGCGCTTGTTC -3' 5'- TGCTAAGCTGTCGCACAAATGGCG -3'
Khc Set4	5'- GATCTCCTGGGATTTCTGGT -3' 5'- AGG AGG AAC TCA TCG CTA AC -3'
Tub 56D	5'- TCA GTG CTC GAT GTT GTC C -3' 5'- CTT GGA AAT CAG CAG GGT TC -3'

Kristina Pohl designed the primers used to amplify a portion of *Oga* cDNA and Graham Hallson designed the primers used to amplify part of the cDNA of reference genes *RP49*, *Khc Set4* and *Tub56D*.

2.10. Weight assay

dILP2-GAL4/CyRoi females were crossed to w^{1118} to generate the control class, *dILP2-GAL4/+,* and also crossed separately to males from each of the following transgenic lines to produce the desired *dILP2-GAL4; UAS-cDNA transgene* flies for weight analysis: *UAS-Oga cDNA 8302-3-1M, UAS-Ogt cDNA 3632-2-2M*, and *UAS-Gfat1 cDNA 4145-1-3M*. *dILP2-GAL4/CyRoi; UAS-Dcr-2/UAS-Dcr-2* females were crossed separately to males from each of the following RNAi lines to generate *dILP2-GAL4/UAS RNAi transgene; UAS-Dcr-2/+* flies: *UAS-Oga RNAi 23-5, UAS-Ogt RNAi 2824-1-1M,* and *UAS-Gfat1 RNAi 2664-1-1M*. *Df(3R)ED10845/TM6* females were

crossed separately to *Oga*^{415}*/TM3Sb Ser* and *w*^{1118} males to provide *Df(3R)ED10845/Oga*^{415} and *Df(3R)ED10845/+* flies, respectively. All of the crosses were performed in bottles to avoid crowding and the parents were removed after one day. Once the adults eclosed, the desired genotypes were selected, aged for four days at 29°C and stored at -80°C. Fifty flies from each genotype were thawed quickly and weighed individually three times using an OHAUS analytical plus balance. The mean weights were compared using Student's *t* test.

3. Results

3.1. TILLING elicited misense and nonsense point mutations within *Oga*

As mentioned previously, *sxc/Ogt* is an essential gene in *Drosophila melanogaster* (Gambetta et al., 2009; Sinclair et al., 2009). At the outset of my work, it was not clear whether this was also true for the *Oga* gene in flies. In order to investigate this possibility further and in parallel with my RNAi experiments, our laboratory sought EMS-induced *Oga* mutants from the Seattle *Drosophila* TILLING Project (SDTP). To this end, primers were designed using the web-based program, CODDLe to amplify a 1.5 kb region of *Oga* predicted to be the most deleterious to the protein upon point mutation (Cooper, Till, et al., 2008). The SDTP used these primers to screen pooled genomic DNA from mutant flies to look for point mutations in the desired region of *Oga* as per standard TILLING protocols (Cooper, Till, et al., 2008). The SDTP screen located fifteen mutations from the CODDLe selected region of *Oga* including one nonsense mutation (Oga^{415}) that putatively codes for a C-terminally truncated protein only 761 amino acid residues long rather than the 1019 amino acid wild-type OGA protein, eight missense mutations, four silent mutations, and two mutations within intronic DNA regions. The missense and nonsense mutations are listed in Table 2 with their respective amino acid substitutions.

Table 2. Amino acid substitutions of SDTP Oga mutants

Mutant	Effect
Oga ^{415}	W761*
Oga ^{292}	V762I
Oga ^{773}	G780S
Oga ^{1179}	G823R
Oga ^{1021}	G823E
Oga ^{520}	P834S
Oga ^{89}	S899F
Oga ^{255}	E920K
Oga ^{526}	G960E

Missense and nonsense *Oga* mutants, provided by the Seattle *Drosophila* TILLING Project (SDTP), listed with their respective amino acid substitutions in single-letter code at the amino acid position within the 1019 amino acid *Oga* protein.
*Indicates a premature stop codon

3.2. Relevant domain search and protein sequence analysis of *Drosophila Oga* revealed a conserved N-terminal domain

In order to evaluate the potential of the aforementioned mutant *Oga* alleles for disruption of OGA protein function, I decided first to use the wild-type protein sequence to look for conserved domains, which represent putative functional domains, particularly the presumed active site of the enzyme. Once the location of the active site has been pinpointed, the relative positions of the missense amino acid substitutions associated with the *Oga* mutants may become more meaningful. The CD-Search results (Appendix E) showed a conserved region near the N-terminus of the *D. melanogaster* OGA sequence between amino acid residues 17 and 324 of the 1019 amino acid protein; this conserved sequence reportedly belongs to the NAGidase superfamily (accession pfam07555), which has been shown to have β-N-acetylglucosaminidase activity (Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2009, 2011). The superfamily includes conserved regions from: *D. melanogaster* CG5871/OGA, *Homo sapiens*

MGEA5 (bifunctional protein NCOAT isoforms a and b), *Caenorhabditis elegans* OGA-1d, a putative hyaluronidase within *Enterococcus faecium* and several other proteins within various prokaryotic and eukaryotic species (Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2009, 2011). To determine the validity of the CD-Search output, I supplemented the data with a search using the protein analysis and classification tool, InterPro.

InterPro identified a conserved region of the query protein sequence between residues 17 and 313 belonging to the protein family, β-N-acetylglucosaminidase (Appendix E); the program also identified two conserved domain types within the sequence. The first domain type, extending from residues 15 through 324, belongs to the glycoside hydrolase superfamily; the second type includes two regions extending from residue 799 to 906 and from residue 936 to 1015, which are Acyl-CoA N-acyltransferase domains (Appendix E).

The two searches, CD-Search and InterPro, were congruent as they both identified an N-terminal domain with proposed β-N-acetylglucosaminidase activity; however, only InterPro identified putative C-terminal Acyl-CoA N-acyltransferase domains.

3.3. SIFT analysis predicts three of eight missense *Oga* mutants may be damaging to protein function

In order to investigate the biological usefulness of the *Oga* alleles further, I next used the SIFT algorithm, which searches for well-conserved amino acid residues within protein families and predicts whether the substitution of a particular residue to another will be deleterious to the protein function (Ng & Henikoff, 2003). Three of the missense *Oga* mutants obtained had damaging SIFT scores, meaning that the respective substitutions were predicted to be deleterious to protein function; these are: *Oga* ^{773}, *Oga* ^{89} and *Oga* ^{526} (Table 3). The SIFT algorithm does not analyze nonsense mutations; however, as *Oga*^{415} putatively encodes a C-terminally truncated protein, it is feasible that the proper function of OGA may be affected in the nonsense mutant.

Table 3. SIFT scores of missense Oga mutants

Mutant	Amino Acid Substitution	SIFT Score	IC
Oga ^{292}	V762I	0.08	3.02
Oga ^{773}	G780S	0.04	3.02
Oga ^{1179}	G823R	0.05	4.32
Oga ^{1021}	G823E	0.08	4.32
Oga ^{520}	P834S	0.08	3.40
Oga ^{89}	S899F	0.01	3.02
Oga ^{255}	E920K	0.93	3.02
Oga ^{526}	G960E	0.02	3.02

Data provided by the Seattle *Drosophila* TILLING Project. Mutants with a SIFT score of less than 0.05 are predicted to have deleterious effects to the protein. IC is a measure of confidence in the predictions and a value less than 3.25 is desired. *Oga* ^{773}, *Oga* ^{89} and *Oga* ^{526} mutants meet the SIFT score and IC requirements, thus they are predicted to be deleterious to protein function.

3.4. Flies hemizygous for various EMS-induced *Oga* alleles are viable and fertile

If *Oga* is essential, then one would expect putative deleterious gene lesions (i.e. hypomorphic or amorphic mutants) to be lethal/semi-lethal and/or sterile when hemizygous with a suitable *Oga*$^{-}$ deletion. However, the results of my experiment, which tested all of the *Oga* alleles (Table 4), clearly show that none of the alleles show lethality or sterility when hemizygous. Note that the relative viability, defined as the ratio of *Oga***/Df(3R)ED10845* flies to the number of *Oga***/TM6CSb* internal control flies (where * represents an EMS allele), ranged from 0.78 to 1.39 (Figure 1). It is particularly notable that *Oga*^{415} has no effect on viability, since this mutant encodes a truncated protein that lacks 258 residues from the C-terminal end of the OGA protein. Interestingly, qPCR analysis showed that *Oga* transcript levels are greatly reduced in *Oga*^{415}*/Df(3R)ED10845* flies to 2% of those found in *w*^{1118} flies (Appendix F).

Table 4. Mutant Oga alleles placed over a deficiency spanning the Oga locus are viable

Oga mutant	Total flies	*Oga**/ *Df(3R)ED10845* Expected	*Oga**/ *Df(3R)ED10845* Observed	Observed/ expected (%)
Oga^{415}	174	58	55	94.83
Oga^{1021}	163	54	51	93.87
Oga^{89}	162	81	93	114.81
Oga^{773}	169	56	75	133.14
Oga^{520}	183	61	54	88.52
Oga^{526}	194	65	58	89.69
Oga^{255}	126	42	50	119.05
Oga^{292}	223	74	81	108.97
Oga^{1179}	114	38	52	136.84
w^{1118} *control*	588	294	330	112.24

Oga mutants (*) obtained via TILLING by the Seattle *Drosophila* TILLING Project (SDTP) were viable and fertile when crossed to a known deficiency (*Df(3R)ED10845)* spanning the *Oga* locus in the fly genome.

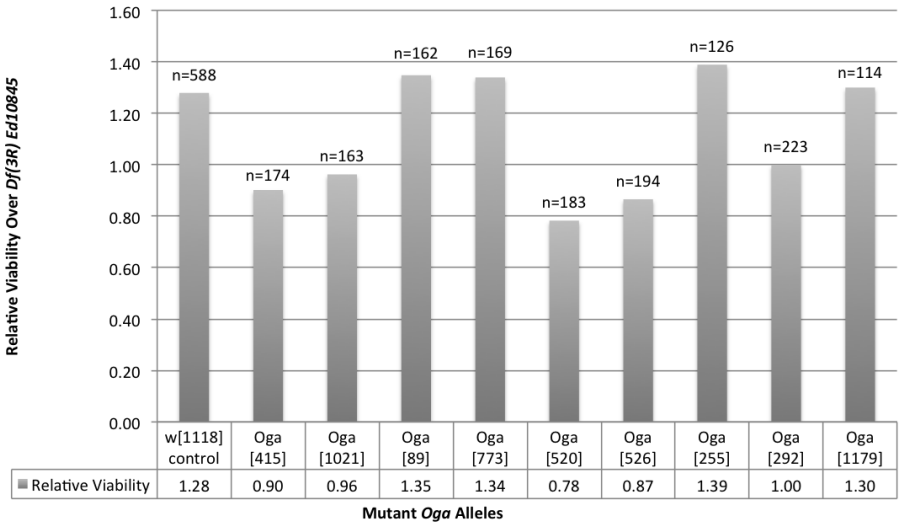

Figure 1. Relative viability of mutant Oga alleles placed over Df(3R)ED10845.

Note. Relative viability was calculated within the F_1 progeny as the ratio of the number of flies that had both a mutant Oga allele and the chromosomal deficiency (Df) mutation to the number of flies that had the mutant Oga allele over the TM6C balancer chromosome. "n" represents the total number of progeny scored in each cross.

3.5. Ubiquitous expression of several different *UAS-Oga RNAi* transgenes results in gene knockdown and moderate semi-lethality

As previously mentioned, the TILLed *Oga* mutant alleles were hemizygous viable and fertile, which indicates that *Oga* is nonessential in flies if the mutant *Oga* protein function was strongly compromised. However, in the absence of an OGA antibody or compelling enzyme assay data, I cannot exclude the possibility that the fly *Oga* gene is essential, but that OGA activity is not sufficiently affected by the mutants available. Fortunately, there were two transgenic *Oga* RNAi lines available (VDRC) to allow me to test whether RNAi-induced down-regulation of *Oga* affects viability; however, no viability effects were observed when each VDRC RNAi line (41822 and 41823) was driven with *tub-GAL4* (Table 5). I speculated that if the RNAi insert was transposed to a more transcriptionally permissive chromatin environment then the RNAi effect might be enhanced; to this end the P element containing *UAS-Oga RNAi* was excised from the X chromosome of 41823 males and progeny in which the insert was relocated to the second or third chromosome were selected (K. Pohl, unpublished). From these progeny she was able to generate several stocks with new autosomal insertion sites for the P element containing the *Oga* RNAi construct; we named the subsequent RNAi lines starting with 23- because they contain the transgene from VDRC line 41823. It was not until after the completion of the transposition experiment that the VDRC RNAi-phiC31 line for *Oga* (106670) became available; the phiC31 RNAi library, called KK, uses phage sites to target RNAi inserts to a known landing site on the second chromosome. This method ensures that inserts will be recombined into loci permissive for transgene expression, rather than into unknown loci in various chromatin environments (Keleman, Micheler, & VDRC project members, 2009).

The surviving male and female flies with driven *UAS-RNAi* constructs, from each of the fourteen available *Oga* RNAi lines, were fertile (data not shown). RNAi lines 23-2, 23-7, 23-9, and 23-11 proved to be moderately semi-lethal upon ubiquitous expression of the transgenes, with only 50% of the expected progeny eclosing (Table 5). RNAi lines 23-8 and 23-5 proved to be even more affective at reducing viability when combined with the *tub-GAL4* driver, with only 35% and 18% of the expected number of progeny eclosing, respectively (Table 5).

Table 5. Knockdown of Oga with several RNAi lines results in moderate semi-lethality in some cases

UAS-Oga RNAi stock number	Total flies	*RNAi + GAL4* flies expected	*RNAi + GAL4* flies observed	Observed/Expected (%)
41822	302	151	189	125
41823	409	205	234	114
23-1	483	242	203	84
23-2	268	134	75	56
23-4	308	154	170	110
23-5	246	82	15	18
23-6	381	191	145	76
23-7	159	80	44	55
23-8	283	142	50	35
23-9	214	107	64	60
23-10	287	72	54	75
23-11	267	134	70	52
23-12	267	134	133	99
106670	387	194	182	94

UAS-Oga RNAi constructs ubiquitously expressed at 29°C using the GAL4/UAS system with a *tub-GAL4* driver and *UAS-Dcr-2* show moderate semi-lethality (<50% of internal control) in six lines: 23-2, 23-5, 23-7, 23-8, 23-9, 23-11. The *RNAi + GAL4* flies were viable and fertile. *Oga* RNAi *23-11 + GAL4* flies showed a blistered wing phenotype.

Relative viability was calculated for each of the RNAi crosses (Figure 2); RNAi line 23-5 proved to have the lowest relative viability, at a ratio of 0.06 versus the internal control class, thus marking it as a candidate worthy of further investigation (Figure 2). Several RNAi lines showed moderate semi-lethality with relative viability ratios lower than 0.5, which could be indicative of reduced *Oga* transcript levels: 23-2, 23-7, 23-8, 23-9, and 23-11 (Figure 2). RNAi line 23-11 showed a blistered wing phenotype upon ubiquitous expression of the transgene (Figure 3).

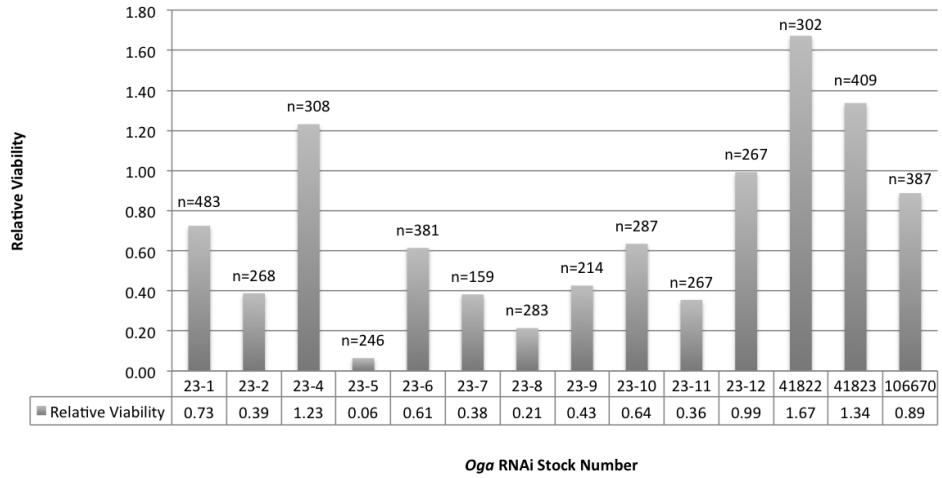

Figure 2. ***Relative viability of ubiquitously expressed Oga RNAi constructs.***

Note. The relative viability was calculated as the ratio of the number F_1 progeny with ubiquitously expressed *UAS-Oga RNAi* transgenes driven with a *tub-GAL4* driver in the presence of *UAS-Dcr-2* to the number of internal control progeny with the *UAS-Oga RNAi* transgene over the *TM3Sb* balancer chromosome. "n" represents the number of flies scored. The surviving *UAS-Dcr-2/+; tub-GAL4/UAS-Oga RNAi* progeny were fertile.

UAS-Dcr-2/+; UAS-Oga RNAi23-11/ tub-GAL4

Wild-type

Figure 3. ***Ubiquitous expression of UAS-Oga RNAi 23-11 with tub-GAL4 results in a blistered wing phenotype.***

Oga mRNA levels in survivors in which the various RNAi transgenes were ubiquitously expressed were determined via qPCR and the data expressed relative to the level of *Oga* mRNA in the control background strain, w^{1118}. The qPCR data show

that, in most cases, the RNAi transgenes caused moderate down-regulation of *Oga* (Figure 4). RNAi lines 23-9 and 41822 reduced *Oga* mRNA levels to 16% of w^{1118} *Oga* transcript (Figure 4). RNAi lines 23-1 and 23-4 showed a reduction in transcript levels to 22% of the control (Figure 4). RNAi lines 23-10 and 23-11 had 26% of *Oga* transcript relative to wild-type, and 23-2, 23-5, and 106670 showed 29% of *Oga* transcript relative to the control (Figure 4). The other lines had 42% of wild-type *Oga* transcript levels and above.

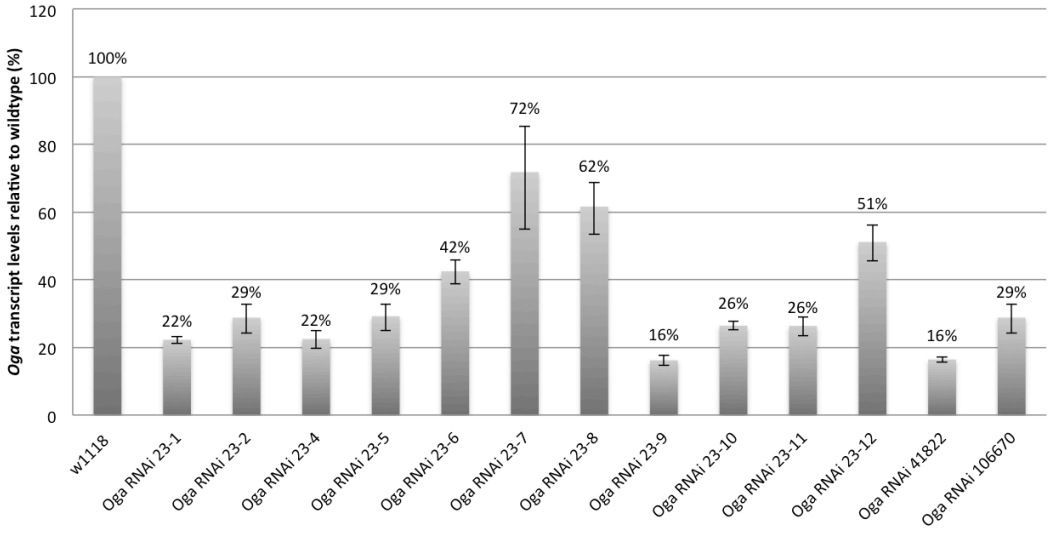

Figure 4. Oga transcript levels, relative to wild-type, in flies with various ubiquitously expressed RNAi transgenes.

Note. *Oga* transcript levels of one-day-old, adult, female flies containing *UAS-Oga RNAi* transgenes driven with *tub-GAL4* were measured relative to w^{1118} control flies using qPCR and three reference genes.

3.6. Ubiquitous overexpression of a single copy of Oga^{+} does not affect viability

Perturbation of *O*-GlcNAc signalling by either decreased *Ogt* function (Gambetta et al., 2009; Sinclair et al., 2009) or ectopic expression of fly or human Ogt^{+} cDNA

causes lethality and semi-lethality, respectively (data not shown). Thus, it was of interest to test whether ectopic expression of *Oga*$^{+}$ disrupts *O*-GlcNAc signalling, presumably via increased removal of GlcNAc from target proteins, sufficiently to affect viability. To accomplish this, *Oga*$^{+}$ was ubiquitously overexpressed using several different *UAS-Oga cDNA* transgenes under the control of a *tub-GAL4* driver. However, in no case was any effect on viability observed (Table 6), and all survivors were male and female fertile (data not shown). In fact, the majority of the transgenic lines had a relative viability ratio over 1 and even the lowest ratio, from line 8302-4-7M, was not overly low at 0.65 (Figure 5).

It is possible that, in the aforementioned experiment, *Oga*$^{+}$ overexpression was insufficient to cause significant depletion of *O*-GlcNAc modifications on protein targets. In an attempt to amplify any possible effects, I used the homozygous viable cDNA lines with insertion sites on the second chromosome to drive two copies of the cDNA transgenes simultaneously within one fly; this experiment resulted in strong semi-lethality in the 8302-3-2M, 8302-4-5M and 8302-4-8M lines (relative viability ratios of 0.01, 0.02, and 0.03, respectively) and moderate semi-lethality in the 8302-3-1M and 8302-4-3M lines (relative viability ratios of 0.30 and 0.33, respectively) (Figure 6).

Table 6. Driving UAS-Oga cDNA transgenes ubiquitously with a tub-GAL4 driver does not result in lethality.

UAS-Oga cDNA	Total flies	*Oga cDNA* + *GAL4* Expected	*Oga cDNA* + *GAL4* Observed	Observed/ Expected (%)
Oga cDNA 8302-3-1M	215	108	96	89
Oga cDNA 8302-3-2M	230	58	62	107
Oga cDNA 8302-3-3M	150	50	53	106
Oga cDNA 8302-4-1M	267	67	71	106
Oga cDNA 8302-4-2M	135	45	57	127
Oga cDNA 8302-4-3M	136	68	93	137
Oga cDNA 8302-4-4M	177	59	70	119
Oga cDNA 8302-4-5M	254	64	77	120
Oga cDNA 8302-4-6M	205	68	79	116
Oga cDNA 8302-4-7M	135	45	33	73
Oga cDNA 8302-4-8M	301	75	82	109

UAS-Oga cDNA	Total flies	*Oga cDNA* + *GAL4* Expected	*Oga cDNA* + *GAL4* Observed	Observed/ Expected (%)
Oga cDNA 8302-4-9M	149	75	82	109
Oga cDNA 8302-4-10F	133	33	52	158

UAS-Oga cDNA (3-) or *UAS-2xflag Oga cDNA* (4-) transgenes were ubiquitously expressed using the UAS/GAL4 system with a *tub-GAL4* driver. *Oga cDNA + GAL4* flies from each cross were fertile.

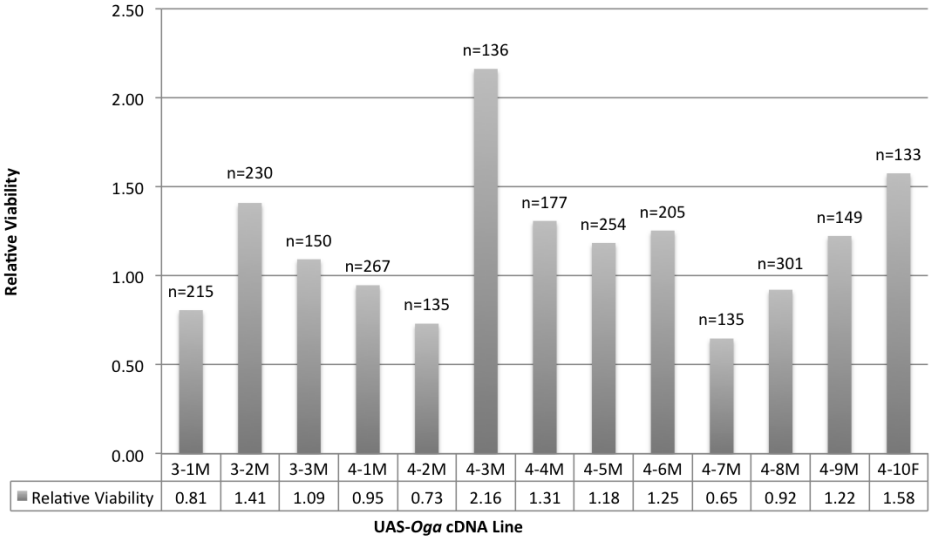

Figure 5. The relative viability of ubiquitously expressed UAS-Oga cDNA transgenes.

Note. *UAS-Oga cDNA* (3-) or *UAS-2xflag Oga cDNA* (4-) transgenes were ubiquitously expressed with a *tub-GAL4* driver and the relative viability was calculated as the ratio of the number of flies with the transgene and driver to the number of flies with the transgene over the *TM3Sb* balancer. Flies with expressed transgenes were viable and fertile. "n" refers to the number of progeny scored.

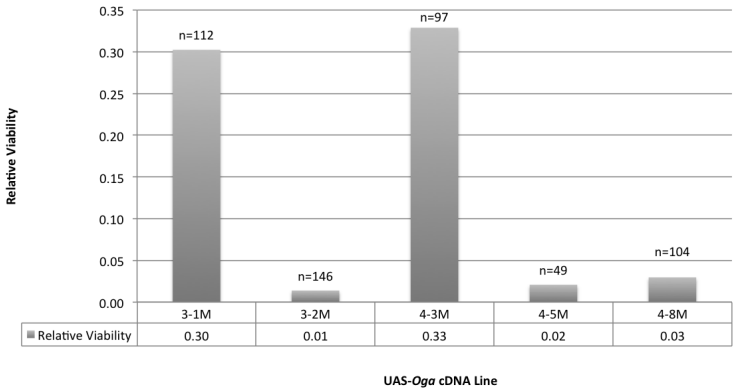

Figure 6. Relative viability of flies with two copies of UAS-Oga cDNA driven ubiquitously with tub-GAL4.

Note. Two copies of *Oga* cDNA (3-) or flag tagged *Oga* cDNA (4-) were ubiquitously expressed with a *tub-GAL4* driver and moderate to strong semi-lethality was observed in all cases.

qPCR analysis was used to measure *Oga* transcript in flies containing single copies of the *UAS-Oga cDNA* transgenes driven ubiquitously with *tub-GAL4* relative to a w^{1118} control sample; *Oga* expression was shown to be increased in every sample by between 5 and 20.5 fold relative to the control sample (Figure 7).

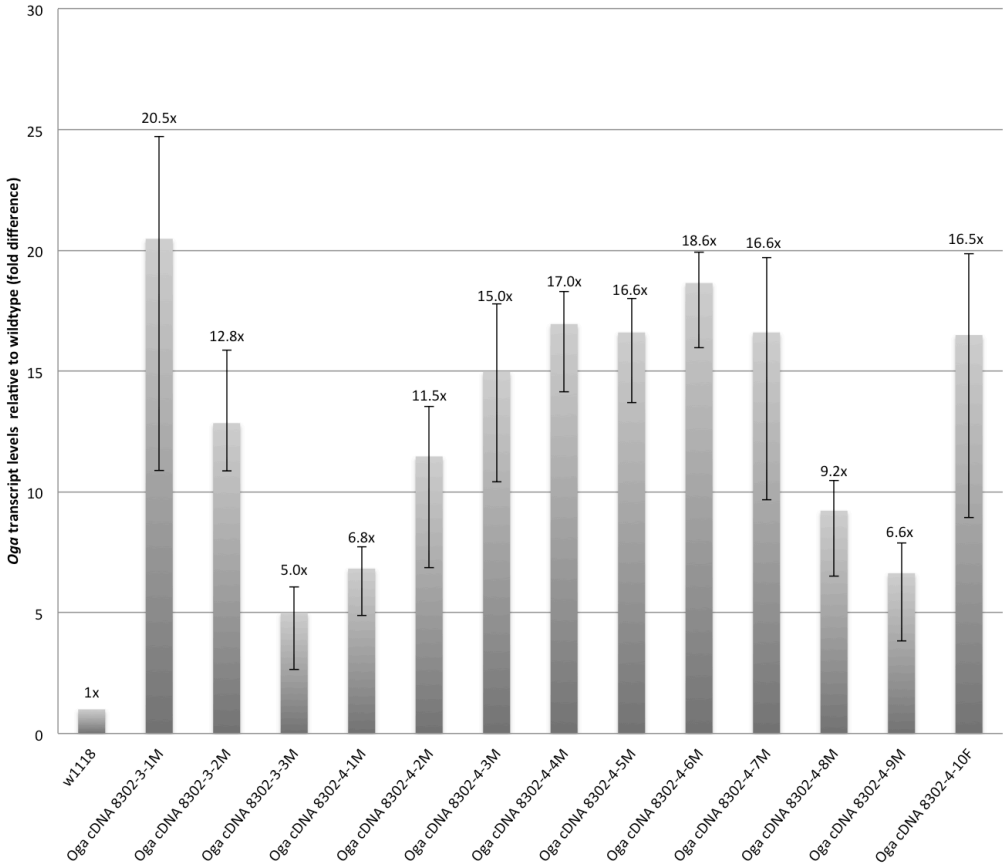

Figure 7. Oga transcript levels in female flies with ubiquitous overexpression of Oga cDNA.

Note. *Oga* cDNA was overexpressed using the UAS/GAL4 targeted gene expression and a *tub-GAL4* driver. The *Oga* transcript levels were measured relative to w^{1118} flies using qPCR with three reference genes.

3.7. Ectopic expression of human *UAS-Hs Ogt cDNA* containing a putative catalytic-site-dead (CSD) mutation can rescue transheterozygous *sxc* mutants

The functional importance of OGT is well-documented (Gambetta et al., 2009; Ingham, 1984; Sinclair et al., 2009). As mentioned previously, it is not completely clear whether the essential functions of OGT are principally due to its catalytic role or to non-catalytic functions of the protein. I participated in a collaborative study to investigate this question *in vivo*. I obtained transgenic fly lines, each containing a *Homo sapiens* (*Hs*) *Ogt* cDNA transgene with a putative "catalytic-site-dead" (CSD) mutation, from

colleagues in the Vocadlo laboratory. Since it has been previously shown that ectopically expressed human *Ogt* cDNA transgenes can rescue the lethality associated with a transheterozygous combination of different *sxc* alleles (Sinclair et al., 2009), it was reasoned that it would be possible to test whether ectopic expression of a catalytically-defective version of the human CSD *Ogt* transgene ubiquitously, would also rescue the *sxc*$^{-}$ flies. Interestingly, the putative CSD transgene did indeed rescue all tested transheterozygous *sxc* mutant combinations (Table 7). The surviving transheterozygotes were fertile and the only two phenotypic differences observed were ectopic wing vein material and a blistered wing phenotype.

As a positive control, I carried out the same set of rescue crosses using the fly *Ogt* cDNA transgene in place of the *UAS-Hs Ogt cDNA CSD*. The rescue crosses with fly cDNA were successful in every transheterozygous *sxc* mutant allele combination tested (Table 8). The rescue flies appeared phenotypically normal and they were fertile. This experiment culminated in the identification of new transheterozygous sxc allele combinations that can be rescued with both human and the fly *sxc/Ogt* transgenes, which extends the previous findings that the combination of *sxc*^{3} and s*xc*^{2637} alleles can be rescued with both human and fly cDNA transgenes under the control of a *tub-GAL4* driver (Sinclair et al., 2009)

However, a key question in terms of the aforementioned hypothesis is whether the putative CSD cDNA actually lacks catalytic function. As part of the collaboration, David Shen, from the Vocadlo laboratory, conducted an assay to determine the enzymatic activity of OGT CSD, relative to wild-type OGT in *sxc*^{3}*/sxc*^{2637}*; UAS-Hs Ogt-CSD/tub-GAL4* rescue flies using the protocols outlined in his thesis (Shen, 2011); he determined that the catalytic activity of OGT in the rescued flies, was at least the equivalent of that of wild-type flies (David Shen, unpublished). This result indicates that the putative CSD mutant substitution did not compromise the catalytic capability of OGT.

Table 7. Rescue of transheterozygous sxc mutants with ubiquitously expressed human Ogt catalytic site dead (CSD) cDNA transgenes

Rescue genotype	Total F1	Rescue expected	Rescue observed	Observed/ expected (%)
sxc^{1}/sxc^{3};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	216	31	47	152.31
sxc^{1}/sxc^{3};UAS-Hs Ogt cDNA -2CSD/tub-GAL4	261	37	33	88.51
sxc^{1}/sxc^{3};UAS-Hs Ogt cDNA-3CSD/tub-GAL4	284	41	43	105.99
sxc^{1}/sxc^{3};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	142	28	39	137.32
sxc^{1}/sxc^{4};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	157	22	17	75.80
sxc^{1}/sxc^{4};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	146	21	7	33.56
sxc^{1}/sxc^{4};UAS-Hs Ogt cDNA-3CSD/tub-GAL4	124	18	2	11.29
sxc^{1}/sxc^{4};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	211	30	39	129.38
sxc^{1}/sxc^{5};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	156	22	33	148.08
sxc^{1}/sxc^{5};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	171	19	4	21.05
sxc^{1}/sxc^{5};UAS-Hs Ogt cDNA-3CSD/tub-GAL4	107	12	19	159.81
sxc^{1}/sxc^{5};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	92	13	21	159.78
sxc^{1}/sxc^{NC130};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	83	12	13	109.64
sxc^{1}/sxc^{NC130};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	145	21	16	77.24
sxc^{1}/sxc^{NC130};UAS-Hs Ogt cDNA 3CSD/tub-GAL4	87	17	13	74.71
sxc^{1}/sxc^{NC130};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	125	14	17	122.40
sxc^{2637}/sxc^{1};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	158	32	41	129.75
sxc^{2637}/sxc^{1};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	99	20	3	15.15
sxc^{2637}/sxc^{1};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	50	7	13	182.00
sxc^{2637}/sxc^{3};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	41	6	7	119.51
sxc^{2637}/sxc^{3};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	83	12	2	16.87
sxc^{2637}/sxc^{3};UAS-Hs Ogt cDNA-3CSD/tub-GAL4	160	23	16	70.00
sxc^{2637}/sxc^{3};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	114	23	24	105.26
sxc^{2637}/sxc^{4};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	55	11	11	100.00
sxc^{2637}/sxc^{4};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	221	44	42	95.02
sxc^{2637}/sxc^{4};UAS-Hs Ogt cDNA-3CSD/tub-GAL4	266	53	56	105.26
sxc^{2637}/sxc^{5};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	174	19	29	150.00

Rescue genotype	Total F1	Rescue expected	Rescue observed	Observed/ expected (%)
sxc^{2637}/sxc^{5};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	90	10	9	90.00
sxc^{2637}/sxc^{5};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	54	6	10	166.67
sxc^{2637}/sxc^{NC130};UAS-Hs Ogt cDNA-1CSD/tub-GAL4	148	30	22	74.32
sxc^{2637}/sxc^{NC130};UAS-Hs Ogt cDNA-2CSD/tub-GAL4	88	13	9	71.59
sxc^{2637}/sxc^{NC130};UAS-Hs Ogt cDNA-3CSD/tub-GAL4	274	55	59	107.66
sxc^{2637}/sxc^{NC130};UAS-Hs Ogt cDNA-4CSD/tub-GAL4	96	11	20	187.50

UAS-Hs Ogt cDNA CSD fly lines provided by M. Macauley. All rescued transheterozygous *sxc* mutant combinations were viable and fertile.

Table 8. Rescue of transheterozygous sxc mutants with fly Ogt cDNA transgene

Rescue genotype	Total F_1	Rescue expected	Rescue observed	Observed/ expected (%)
sxc^{1}/sxc^{3};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	124	18	11	62.10
sxc^{1}/sxc^{4};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	308	44	8	18.18
sxc^{1}/sxc^{5};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	183	37	51	139.34
sxc^{1}/sxc^{NC130};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	137	15	22	144.53
sxc^{2637}/sxc^{1};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	190	38	48	126.32
sxc^{2637}/sxc^{3};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	225	45	44	97.78
sxc^{2637}/sxc^{4};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	263	53	50	95.06
sxc^{2637}/sxc^{5};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	247	49	56	113.36
sxc^{2637}/sxc^{NC130};UAS-Dm Ogt cDNA 3632-2-2M/ tub-GAL4	257	51	53	103.11

UAS-Dm Ogt cDNA was expressed ubiquitously using a *tub-GAL4* driver. The rescued transheterozygous sxc mutants were viable and fertile.

3.8. Effects of perturbations in *O*-GlcNAc signalling on body size

Two previous studies have used global and/or targeted knockdown of *Oga* and *Ogt* via RNAi to show changes in *Drosophila* body size; the RNAi knockdown of *Oga* or *Ogt* result in increased and decreased body size, respectively (Park et al., 2011; Sekine et al., 2010). Furthermore, both studies linked OGT depletion to decreased insulin signalling, while OGA depletion had the opposite effects (Park et al., 2011; Sekine et al., 2010). I decided to use our *Oga* and *Ogt* reagents, including the putative *Oga* mutant, Oga^{415}, in an attempt to confirm their body size findings. In addition, I expanded the analysis by testing the effects of targeted over-expression of *Oga* and *Ogt,* as well as targeted over-expression and knockdown of the *Gfat1* gene; the rationale for the interest in *Gfat1* is as follows. The GFAT enzyme acts at the rate-limiting step of the Hexosamine Biosynthetic Pathway (HBP) (Na et al., 2013). Since the end product of the pathway is UDP-GlcNAc, the substrate required by OGT for target protein modification, it seemed reasonable to speculate that appropriate knockdown of *Gfat1* might mimic that of *Ogt* (i.e. decrease body size), and that perhaps over-expression of *Gfat1* might have the opposite effect. Although, this hypothesis does not take into account that *Gfat1* has a paralogue in *Drosophila*, *Gfat2;* it is feasible that *Gfat2* expression may be partially compensating for the downregulation of *Gfat1*.

For my experiment, I used the available reagents to disrupt *O*-GlcNAc signalling via the UAS/GAL4 system to target misexpression to the *Drosophila* insulin producing cells via the *dILP2-GAL4 driver* (Sekine et al., 2010). I performed various crosses, as mentioned in section 2.10 of this study, that allowed cDNA and RNAi transgenes for *Ogt, Oga* and *Gfat1* to be expressed under the control of the *dILP2-GAL4* driver and measured the body weight of the individual progeny of these crosses. I also included a test of Oga^{415}*/Df(3R)ED10845* in this experiment. The results of this study are shown in Figure 8 and Figure 9.

For the female assay, the average body weight of the *dILP2-GAL4* control, 1.08 mg, was smaller than all of the experimental genotypes (Figure 8). When the *Oga, Ogt* and *Gfat1* RNAi transgenes were driven with *dILP2-GAL4*, with *UAS-Dcr-2* included to enhance the knockdown, I noticed a 16%, 7% and 15% increase in body size,

respectively (Figure 8). When *Oga*, *Ogt*, and *Gfat1* cDNA transgenes were driven with *dILP2-GAL4,* I observed a 13%, 4% and 4% increase in body size, respectively (Figure 8). The hemizygous Oga^{415} mutant showed a 7% increase, while the, *Df(3R)ED10845/+* flies showed a 5% increase in body size relative to the *dILP2-GAL4* control (Figure 8). A Student's t test was conducted and the weight averages for each genotype were deemed to be statistically significant relative to the control ($p<0.05$). The same test was used to compare *Df(3R)ED10845*/Oga^{415} to *Df(3R)ED10845/+* control average and the difference was found to be statistically insignificant ($p>0.05$).

Males from the same above-mentioned crosses were weighed and the averages were less variable than those of the females. The average *dILP2-GAL4/+* male weight was 0.72 mg (Figure 9). When the *Oga, Ogt* and *Gfat1* RNAi transgenes were driven with *dILP2-GAL4*, in combination with *UAS-Dcr-2* included to enhance the knockdown, I noticed a 3% increase, a 4% decrease and 3% increase in body size, respectively (Figure 9). When *Oga*, *Ogt*, and *Gfat1* cDNA transgenes were driven with *dILP2-GAL4,* only *Gfat1* showed a 10% decrease in body size, while both *Oga* and *Ogt* body sizes were consistent with that of the *dILP2-GAL4* control (Figure 9). The hemizygous Oga^{415} mutant and the *Df(3R)ED10845/+* flies had the same average body weight as the *dILP2-GAL4* control flies (Figure 9). The changes in body weight relative to the control class were deemed statistically significant by a Student's t test ($p<0.05$).

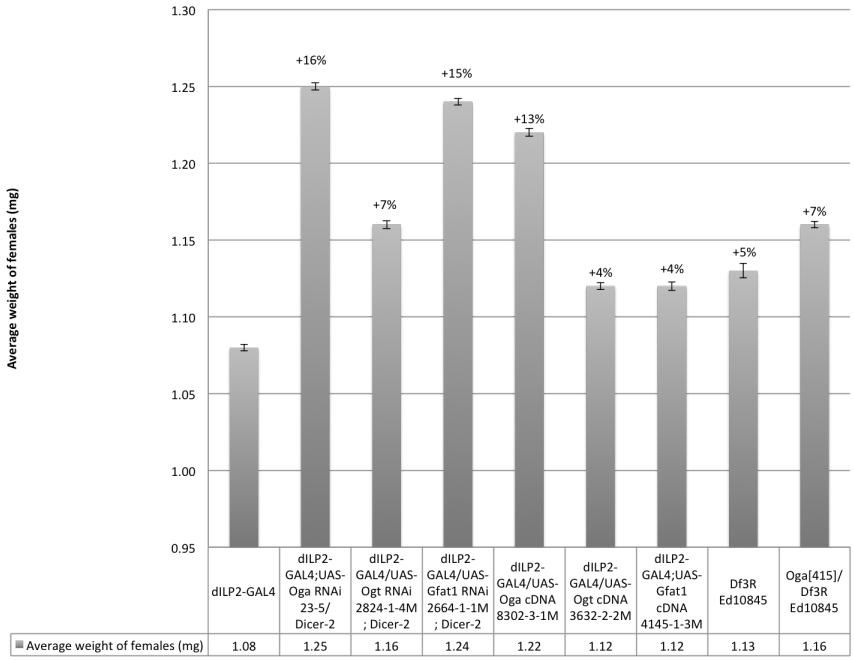

Figure 8. Average female fly weights.

Note. Adult female flies from each genotype, in which various *O*-GlcNAc related proteins were mutated or altered in terms of their respective expression using transgenes and the UAS/GAL4 system. The data labels represent the relative size compared to the *dILP2-GAL4* control weight average shown in the far left column. +/- standard error, all averages are statistically significantly relative to the control as determined by a Student's t test ($p<0.05$). A Student's t test was also used to compare *Df(3R)ED10845/Oga*^{415} to *Df(3R)ED10845/+* control and found the difference to be statistically insignificant ($p>0.05$).

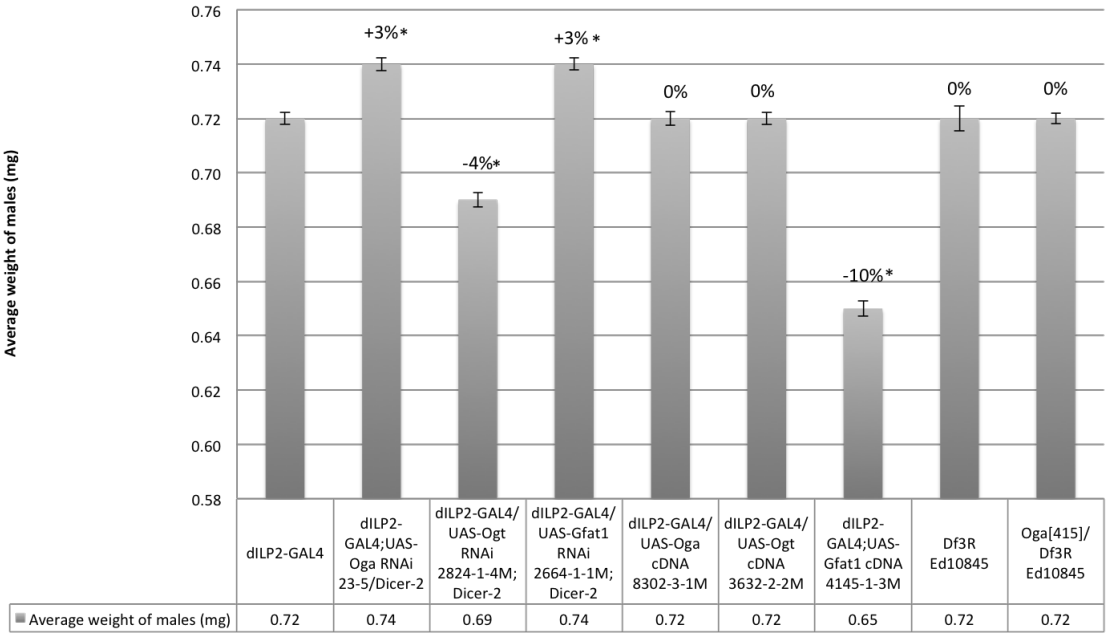

Figure 9. Average male fly weights.

Note. Adult male flies from each genotype with transgenes driven with *dILP2-GAL4* and mutants affecting proteins involved in O-GlcNAc cycling, or within the hexosamine biosynthesis pathway (HBP). The data labels displayed represent the relative size compared to the *dILP2-GAL4* control weight average shown in the leftmost column. Error bars are +/- standard error. The significance of the data was confirmed by a Student's t test relative to the control. *Df(3R)ED10845/Oga*^{415} weights were also compared to *Df(3R)ED10845/+* control weight via Student's t test and the difference was found to be statistically insignificant ($p>0.05$)

* indicates a significant difference from average weight of control ($p<0.05$).

4. Discussion and conclusion

4.1. Analysis of the *Oga* mutants

Protein sequence analysis is a standard bioinformatics procedure that can provide insight into protein structure and function based on conserved amino acid residues and domains. As mentioned in section 3.2, CD-Search and InterPro both identified the N-terminal region of the *D. melanogaster Oga* protein as a conserved domain with β-N-acetylglucosaminidase activity; even though the analyses provided by the two programs differed with respect to the length of the conserved domain, they both positioned the active site of the enzyme at the N-terminus of the protein. Interestingly, the CODDLe program, which pinpoints regions of a protein within which TILLed EMS lesions are most likely to affect the protein's function, identified the C-terminal region of OGA (McCallum, Comai, Greene, & Henikoff, 2000); thus, all of the recovered *Oga* mutants have amino acid substitutions between residues 761 and 960 of the 1019 residue protein (Figure 11). In other words, none of the amino acid substitutions in the *Oga* mutants occur within the conserved active site in the N-terminal domain of the enzyme. However, some or all of these mutations could affect protein function in other ways; for example, amino acid substitutions resulting in improperly folded OGA molecules could lead to the formation of protein aggregates and/or degradation of the misfolded protein. My qPCR analysis indicated low levels of *Oga* transcript in flies hemizygous for the Oga^{415} mutation. As mentioned, Oga^{415} is an allele that contains a premature stop codon, which presumably encodes a C-terminally truncated OGA protein of 761 amino acids (rather than the full-length 1019 amino acid form). The production of polypeptides from mRNAs with premature translation termination codons (PTCs) could result in the aggregation of harmful truncated protein molecules (Nicholson et al., 2010). Therefore, the PTC in Oga^{415} might trigger nonsense-mediated mRNA decay (NMD), which could explain the low levels of *Oga* transcript within the sample. NMD, or RNA surveillance, serves as a quality control mechanism to degrade mRNAs with PTCs that truncate an open reading frame (ORF) (Nicholson et al., 2010). If it is possible to

confirm my findings regarding the transcript levels associated with *Oga*^{415}, then this mutant may prove useful for future studies of OGA function. Another possibility is that *Oga*^{415} is a double mutant containing the aforementioned nonsense mutation, plus a mutation in a regulatory element that reduces *Oga* transcription. An antibody that binds a portion of the N-terminus of fly OGA would also be very useful in the characterization of all of the *Oga* mutants, because it could help to identify which, if any, of the mutants are protein nulls. If the catalytic function of OGA is not affected in *Oga*^{415} but a C-terminally truncated form of OGA is translated, then the mutant line may be useful in studies investigating the function of the C-terminus of OGA.

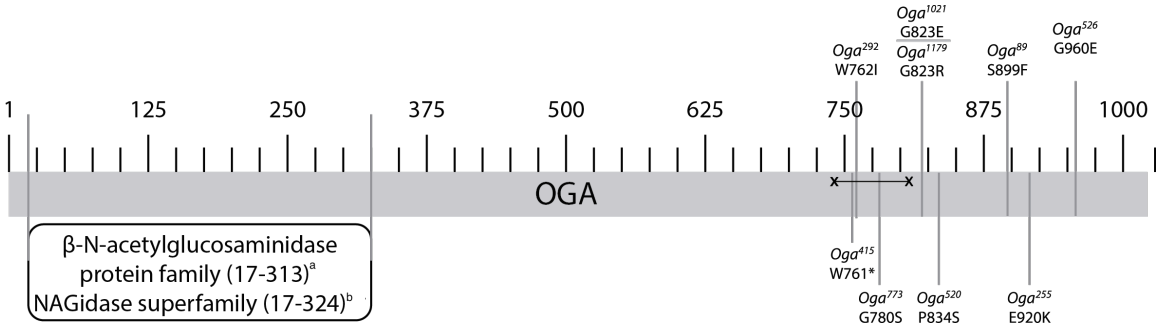

Figure 10. A schematic of the annotated 1019 amino acid Drosophila Oga protein.

Note. The relative position of the conserved active site and the amino acid substitutions of the *Oga* mutants have been marked in this diagram of *Drosophila* OGA protein sequence. The amino acids are listed by their single letter codes, and * is indicative of a premature stop codon.
[a] Identified via InterPro search
[b] Identified via CD-Search
[x-x] Indicates the approximate region of corresponding *Oga* cDNA that would be amplified during the qPCR experiments

The SIFT analysis predicted that the *Oga* ^{773}, *Oga* ^{89} and *Oga* ^{526} mutations would be most likely to affect OGA function (Table 3). However, the SIFT program is known to have both false positive and false negative errors, which are defined by Ng & Henikoff (2002) as predicting a deleterious substitution when the effect is neutral, or predicting a neutral substitution when the effect is deleterious to protein function, respectively. Unfortunately, these errors vary from protein to protein and can only be calculated when a large dataset of tolerated versus intolerated mutations in a particular protein is available (Ng & Henikoff, 2002). The SIFT program is estimated to be approximately

75% accurate at predicting if an amino acid substitution will negatively affect protein function (Ng & Henikoff, 2003). Thus, these mutations may prove useful for future work.

4.2. Is *Oga* an essential gene?

None of the selected TILLed *Oga* alleles are lethal when hemizygous. However, without definitive evidence about the molecular consequences of the alleles, it is difficult to assess the viability data pertaining to these mutants. Clearly the isolation of confirmed *Oga* null alleles is of major importance. Radermacher et al. (2014) have isolated Oga^{P} and described it to be a putative protein-null allele, supported via a western blot showing an absence of protein in homozygotes. This allele should be investigated and confirmed or refuted as amorphic before other work to obtain *Oga* nulls is conducted. However, if the previously mentioned findings are not repeatable then an *Oga* protein null allele could be obtained by using various other methods, including homologous recombination to obtain gene knockout (Rong & Golic, 2001) or type II clustered regular interspersed short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system (Bassett et al., 2013; Port et al., 2014). The relevance of null alleles for basic analysis of the effects of compromised *Oga* function on viability is obvious. Nevertheless, my finding that global expression of some *UAS-Oga RNAi* transgenic lines results in moderate semi-lethality suggests that the *Oga* gene may be essential. My qPCR data for flies in which RNAi transgenes were expressed globally did not correlate consistently with the viability data. For example, global expression of *UAS-Oga RNAi 41822* is viable and my qPCR analysis of the appropriate flies showed a striking decrease in *Oga* transcript to 16% of the level found in control flies. On the other hand, the 23-5 transgene was the most effective in terms of semi-lethality, but the survivors exhibited a level of *Oga* mRNA equivalent to that of line 48122. However, it is important to note that I was measuring mRNA levels in eclosed survivors. Presumably, those flies that did not survive expression of the RNAi in 23-5 would have much lower levels of *Oga* mRNA. In any event, definitive confirmation of my RNAi results will require further experiments. Since the developmental profile for *Oga* expression shows a significant maternal contribution (modENCODE; FlyBase), it would be useful to test the effects of GAL4 drivers that evoke early embryonic expression with some of the *Oga* RNAi transgenes. Such studies could be used in conjunction with germline knockout analysis

(Bassett et al., 2013; Port et al., 2014) using confirmed *Oga* null alleles, in order to evaluate the effects of downregulation of *Oga* on early embryonic development.

Ubiquitous overexpression of *Oga* did not affect viability when the Oga^{+} transgene was only present in one copy; however, viability was affected in experiments where two copies of the transgene were included. A western blot to quantify global levels of *O*-GlcNAc modified proteins within the survivors would be crucial to ascertain the effects of overexpression in these experiments.

4.3. Are the essential functions of OGT due to its catalytic activity?

As mentioned in the introduction, using a mouse knockout mutation of *Oga*, Yang et al. (2012) have demonstrated that the gene is essential for completion of mouse embryonic development; they also showed that *Oga* is required for normal cell cycle regulation and genomic stability in mouse embryonic fibroblasts. My data suggest that the *Oga* gene may also be essential in flies; however, if the Oga^{P} allele, described by Radermacher et al. (2014), is confirmed to be a protein-null that is homozygous viable and fertile then that would support the gene being nonessential. The essential nature of the *Ogt* gene for embryonic development in vertebrates is well established (reviewed by Love et al., 2010); indeed tissue-specific knockout of *Ogt* is cell lethal. As mentioned earlier, *Ogt*/*sxc* is also essential for *Drosophila* development and the gene is a member of the Polycomb Group (PcG) (Ingham 1984; Gambetta et al. 2009; Sinclair et al. 2009); interestingly, flies devoid of OGT function associated with null alleles of *Ogt*/*sxc* survive to the pharate adult stage and exhibit homeotic phenotypes typical of the PcG (Sinclair et al., 2009). However, early pole-cell transplantation experiments (Ingham 1984), confirmed later by RNAi studies (Sinclair et al. 2009), have shown that *Ogt* is essential for fly embryonic development.

The exact nature of the essential requirements for *Ogt* is not completely clear, although it is generally assumed that the crucial function of OGT is catalyzing *O*-GlcNAcylation (O'Donnell et al., 2004). However, it is formally possible that the OGT protein has essential, non-catalytic functions. This was the underlying motivation for a

collaborate effort involving myself and workers from the Vocadlo laboratory. We decided to test the ability of a mutated transgenic version of the human *Ogt* cDNA to rescue lethality associated with *sxc* mutant alleles; importantly, the mutant transgene coded for OGA containing a substitution of an active-site residue, His558, thought to be critical for catalytic activity (Martinez-Fleites et al., 2008). My data showed that global expression of *UAS-Hs Ogt cDNA CSD* transgene did indeed rescue the *sxc* mutants; however, further investigation into the structure of human OGT revealed that the His558 residue was not required for *O*-GlcNAcylation (Lazarus et al. 2011). Instead, these authors proposed that His498 is the key catalytic base. However, recent work has suggested that neither His558 nor His498 are close enough from the acceptor hydroxyl to function as the catalytic base (Lazarus et al., 2012). Even more recently, it has been proposed that the catalytic base is provided by the α-phosphate of the donor substrate, rather than residues in the OGT enzyme itself (Schimpl et al., 2012). In light of these recent proposals, David Shen's finding that rescued flies had normal OGT activity levels is not surprising because the mutant version of the human *Ogt* transgene effectively has wild-type activity. Nonetheless, these experiments, combined with my control data, show that the effectively wild-type human *Ogt* cDNA, as well as the wild-type fly *Ogt* cDNA, rescue various combinations of *sxc* alleles that had not been tested previously (Sinclair et al. 2009).

4.4. Weight assay

As mentioned previously, two earlier studies used global and/or targeted knockdown of *Oga* and *Ogt* via RNAi to investigate the effects of altered *O*-GlcNAcylation on body size in *Drosophila* (Park et al., 2011; Sekine et al., 2010). Using a global driver, *Actin-GAL4*, Park et al. (2011) found that knockdown of *Oga* caused a 17% relative increase in body weight of adult female flies (an inhibitor of OGA activity also increased body growth of flies of both sexes by ~10%). Furthermore, Park et al., 2011 showed that the growth effect occurred by increases in cell size in imaginal discs, rather than by changes in cell proliferation. On the other hand, these authors found that both global downregulation under the control of the *Actin-GAL4* driver and targeted downregulation under the control of the *engrailed-GAL4* driver of *Ogt* reduced the size of

imaginal discs in a cell-autonomous fashion. Park et al. (2011) also correlated their observations in regards to changes in fly size with insulin signalling; they found that increased *O*-GlcNAcylation enhanced insulin signalling, whereas decreased *O*-GlcNAcylation had the opposite effect. For example, global downregulation of *Oga* in larvae significantly increased the site-specific phosphorylation of Akt, whereas global downregulation of *Ogt* had the opposite effect; they also showed that overexpression of Ogt^{+} in S2 cells caused an increase in insulin-induced Akt phosphorylation. Finally, they showed that Akt is an OGT substrate.

In their study, Sekine et al. (2010) used the *Drosophila* insulin-like peptide 2 GAL4 driver (*dILP2-GAL4*) to target RNAi-induced downregulation of *Ogt* and *Oga* to Insulin-Producing Cells (IPCs) of *Drosophila*, thereby linking any effects directly to insulin signaling. Consistent with the results of Park et al. (2011), they found that *Oga* knockdown increased larval and adult body size (a relative increase of 12% in each case; sexes were not distinguished), whereas, *Ogt* knockdown had the opposite effect (a relative decrease of ~10% in each case). Interestingly, when they carried out targeted overexpression of *Ogt* using a *UAS-Ogt cDNA*, this had the same effect as downregulation of *Oga*. Sekine et al. (2010) extended their study by showing that downregulation of *Oga* increased the expression of the *dilp2*, *dilp3* and *dilp5* genes, whereas down-regulation of *Ogt* decreased their expression. These authors found that while targeted downregulation of *Oga* stimulated site-specific phosphorylation of Akt in larvae and adults, targeted downregulation of *Ogt* had no significant effect on Akt phosphorylation in larvae, but caused a modest, albeit statistically significant, decrease in Akt phosphorylation in adults. Finally, they showed that downregulation of either gene evokes increased carbohydrate levels in adult hemolymph, but lessens insulin-induced Akt phosphorylation in *ex vivo* cultured larval fat bodies; the latter is also correlated with decreased triglyceride levels.

In an attempt to confirm and extend the abovementioned observations regarding the effects of altered *O*-GlcNAcylation on fly growth, I used *dILP2-GAL4* to target down-regulation of *Oga* or *Ogt* to the IPCs and examined the effects on adult body weight. I observed an average relative increase in weight of ~9.5% (+16% in females and +3% in males) in response to *Oga* RNAi transgene expression and this compares favourably with the data of Sekine et al. (2010) for a mixed gender sample (+10%). Furthermore,

my result is similar to that of Park et al. (2011) (+17% in female body weight), although they expressed the *Oga* RNAi using a strong, global *Act-GAL4* driver rather than the IPC specific driver used in my study (*dILP2-GAL4*). On the other hand, my data for *Ogt* RNAi transgene expression were contradictory as female weight increased by 7%, but male weight decreased by 4%, which is effectively no change if you average the two genders. In comparison and under similar conditions, Sekine et al. (2010) reported a 12% decrease in adult body weight. I also examined overexpression of *Oga* and *Ogt* cDNA transgenes separately, again with contradictory results. Oga^{+} overexpression showed a clear increase (+13%) in female weight, but no change in male weight; the increase for the females was opposite to the expectations that excess OGA would decrease overall *O*-GlcNAcylation and thus decrease body weight via effects on insulin signalling. Overall, Ogt^{+} overexpression caused only a slight increase in body weight, much less than the effect reported by Sekine et al. (2010). Clearly, my experiment should be repeated, preferably using optimally expressed transgenes such as those generated with phiC31 site-specific integration.

I also tested the effects of IPC targeted expression of a *Gfat1* RNAi transgene and a *Gfat1* cDNA, separately, on adult body weight. The GFAT enzymes catalyze the rate-limiting step in the hexosamine biosynthetic pathway (HBP), which synthesizes UDP-GlcNAc, the substrate for *O*-GlcNAcylation by OGT (Haltiwanger et al., 1990). In *Drosophila*, and most eukaryotes, there are two paralogous GFAT enzymes, GFAT1 and GFAT2, encoded by the respective genes *Gfat1* in 3R heterochromatin and *Gfat2* in 3R euchromatin (Graack, Cinque, & Kress, 2001). These enzymes appear to be functionally equivalent and our laboratory has isolated and characterized lethal mutations in each gene (unpublished). A plausible prediction is that overexpression of a wild-type copy of either gene in the IPCs would drive the HBP and thus *O*-GlcNAcylation of targets by OGT and, by inference cause an increase in body weight. However, once again, my data are rather contradictory; when *Gfat1* cDNA was driven by *dILP2-GAL4* it modestly increased the weight of females (+4%), but it clearly decreased the weight of males (-10%). I also looked at targeted knockdown of *Gfat1*, using an RNAi transgene. Perhaps surprisingly, this caused a marked increase in female weight and a more modest increase in male weight; on the other hand, one might attribute that to GFAT2 compensation for the downregulation of GFAT1. Obviously, the situation regarding

regulation of *Gfat* genes and their products is complicated and thus it is difficult to assess the data. Nevertheless, the *Gfat1* tests should be repeated and, if possible, it would be preferable to use a doubly transgenic line containing RNAi constructs for both *Gfat1* and *Gfat2*.

4.5. Future directions

Obviously the characterization of *Oga* and the encoded protein in *Drosophila* is nowhere near complete; however, the identification of the Oga^{P} allele, by Radermacher et al. (2014) looks like a promising conclusion to the debate as to whether *Oga* is essential in flies. The abovementioned body weight assay should be repeated with Oga^{P} to determine whether homozygotes are larger in size, as would be expected in a protein null. The western blots, performed by Radermacher et al. (2014), showing an absence of OGA should be repeated, and perhaps a western blot showing whether a *UAS-Oga cDNA* transgene driven ubiquitously in a homozygous Oga^{P} background can restore the OGA protein. Failing the confirmation of Oga^{P} as a protein null, CRISPR could be used to knockout gene function, as previously mentioned. qPCR was done on survivors of the RNAi knockdown experiments in which *UAS-Oga RNAi* transgenes were ubiquitously expressed with *tub-GAL4* in the presence of *UAS-Dcr-2.* One may expect the survivors to have more *Oga* transcript than the deceased flies, especially if the protein is essential; therefore, a more meaningful result may be obtained by conducting the qPCR experiments on embryos or larvae while using drivers that are expressed earlier, such as *Nanos-GAL4*. This procedural modification should also be done to measure *Oga* transcript levels in embryos/larvae in which two copies of the *UAS-Oga cDNA* transgenes are present. A western blot identifying an increase in global *O*-GlcNAc modification in larvae/embryos with Oga^{+} cDNA transgenes in two copies would be helpful to show whether an increase in global *O*-GlcNAc can be correlated with reduced viability. Work should be continued to generate a catalytically inactive form of OGT *in vivo* because knowing whether *O*-GlcNAcylation is the essential function of OGT in flies could be helpful for the continued characterization of OGA.

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389–402. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=146917&tool=pmcentrez&rendertype=abstract

Ball, L. E., Berkaw, M. N., & Buse, M. G. (2006). Identification of the major site of O-linked beta-N-acetylglucosamine modification in the C terminus of insulin receptor substrate-1. *Molecular & Cellular Proteomics : MCP*, *5*(2), 313–23. doi:10.1074/mcp.M500314-MCP200

Bassett, A. R., Tibbit, C., Ponting, C. P., & Liu, J. (2013). Highly Efficient Targeted Mutagenesis of Drosophila with the CRISPR/Cas9 System. *Cell Reports*, *4*, 220–228.

Bickel, P. E., Tansey, J. T., & Welte, M. a. (2009). PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica et Biophysica Acta*, *1791*(6), 419–40. doi:10.1016/j.bbalip.2009.04.002

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Cambridge, England)*, *118*(2), 401–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8223268

Brasaemle, D. L. (2007). Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of Lipid Research*, *48*(12), 2547–59. doi:10.1194/jlr.R700014-JLR200

Bullen, J. W., Balsbaugh, J. L., Chanda, D., Shabanowitz, J., Hunt, D. F., Neumann, D., & Hart, G. W. (2014). Crosstalk between two essential nutrient-sensitive enzymes: O-GlcNAc transferase (OGT) and AMP-activated protein kinase (AMPK). *The Journal of Biological Chemistry*, 0–25. doi:10.1074/jbc.M113.523068

Butkinaree, C., Park, K., & Hart, G. W. (2010). O-linked beta-N-acetylglucosamine (O-GlcNAc): Extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress. *Biochimica et Biophysica Acta*, *1800*(2), 96–106. doi:10.1016/j.bbagen.2009.07.018

Cheung, W. D., & Hart, G. W. (2008). AMP-activated protein kinase and p38 MAPK activate O-GlcNAcylation of neuronal proteins during glucose deprivation. *The Journal of Biological Chemistry*, *283*(19), 13009–20. doi:10.1074/jbc.M801222200

Comer, F. I., & Hart, G. W. (2001). Reciprocity between O-GlcNAc and O-phosphate on the carboxyl terminal domain of RNA polymerase II. *Biochemistry*, *40*(26), 7845–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11425311

Comtesse, N., Maldener, E., & Meese, E. (2001). Identification of a nuclear variant of MGEA5, a cytoplasmic hyaluronidase and a beta-N-acetylglucosaminidase. *Biochemical and Biophysical Research Communications*, *283*(3), 634–40. doi:10.1006/bbrc.2001.4815

Cooper, J. L., Greene, E. A., Till, B. J., Codomo, C. A., Wakimoto, B. T., & Henikoff, S. (2008). Retention of induced mutations in a Drosophila reverse-genetic resource. *Genetics*, *180*(1), 661–7. doi:10.1534/genetics.108.092437

Cooper, J. L., Till, B. J., & Henikoff, S. (2008). Fly-TILL: Reverse genetics using a living point mutation resource. *Fly*, *2*(6), 1–3. doi:10.1534/genetics.108.092437.1

Dennis, R. J., Taylor, E. J., Macauley, M. S., Stubbs, K. a, Turkenburg, J. P., Hart, S. J., … Davies, G. J. (2006). Structure and mechanism of a bacterial beta-glucosaminidase having O-GlcNAcase activity. *Nature Structural & Molecular Biology*, *13*(4), 365–71. doi:10.1038/nsmb1079

Denzel, M. S., Storm, N. J., Gutschmidt, A., Baddi, R., Hinze, Y., Jarosch, E., … Antebi, A. (2014). Hexosamine pathway metabolites enhance protein quality control and prolong life. *Cell*, *156*(6), 1167–78. doi:10.1016/j.cell.2014.01.061

Dias, W. B., Cheung, W. D., Wang, Z., & Hart, G. W. (2009). Regulation of Calcium/Calmodulin-dependent Kinase IV by O-GlcNAc modification. *The Journal of Biological Chemistry*, *284*(32), 21327–21337. doi:10.1074/jbc.M109.007310

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., … Dickson, B. J. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, *448*(7150), 151–6. doi:10.1038/nature05954

Dorfmueller, H. C., Borodkin, V. S., Schimpl, M., & van Aalten, D. M. F. (2009). GlcNAcstatins are nanomolar inhibitors of human O-GlcNAcase inducing cellular hyper-O-GlcNAcylation. *The Biochemical Journal*, *420*(2), 221–7. doi:10.1042/BJ20090110

Duffy, J. B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis (New York, N.Y. : 2000)*, *34*(1-2), 1–15. doi:10.1002/gene.10150

Duggirala, R., Blangero, J., Almasy, L., Dyer, T. D., Williams, K. L., Leach, R. J., … Stern, M. P. (1999). Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans. *American Journal of Human Genetics*, *64*(4), 1127–40. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1377837&tool=pmcentrez&rendertype=abstract

Forsythe, M. E., Love, D. C., Lazarus, B. D., Kim, E. J., Prinz, W. a, Ashwell, G., … Hanover, J. a. (2006). Caenorhabditis elegans ortholog of a diabetes susceptibility locus: oga-1 (O-GlcNAcase) knockout impacts O-GlcNAc cycling, metabolism, and dauer. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(32), 11952–7. doi:10.1073/pnas.0601931103

Gambetta, M. C., Oktaba, K., & Müller, J. (2009). Essential role of the glycosyltransferase sxc/Ogt in polycomb repression. *Science*, *325*(93), 93–6. doi:10.1126/science.1169727

Gao, Y., Wells, L., Comer, F. I., Parker, G. J., & Hart, G. W. (2001). Dynamic O-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic beta-N-acetylglucosaminidase from human brain. *The Journal of Biological Chemistry*, *276*(13), 9838–45. doi:10.1074/jbc.M010420200

Graack, H. R., Cinque, U., & Kress, H. (2001). Functional regulation of glutamine:fructose-6-phosphate aminotransferase 1 (GFAT1) of Drosophila melanogaster in a UDP-N-acetylglucosamine and cAMP-dependent manner. *The Biochemical Journal*, *360*, 401–12. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1222241&tool=pmcentrez&rendertype=abstract

Hallson, G., Hollebakken, R. E., Li, T., Syrzycka, M., Kim, I., Cotsworth, S., … Honda, B. M. (2012). dSet1 is the main H3K4 di- and tri-methyltransferase throughout Drosophila development. *Genetics*, *190*(1), 91–100. doi:10.1534/genetics.111.135863

Haltiwanger, R. S., Holt, G. D., & Hart, G. W. (1990). Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. Identification of a uridine diphospho-N-acetylglucosamine:peptide beta-N-acetylglucosaminyltransferase. *Journal of Biological Chemistry*, *265*, 2563–2568.

Hanover, J. A. (2001). Glycan-dependent signaling : O-linked N-acetylglucosamine. *The FASEB Journal*, *15*, 1865–76.

Hanover, J. A., Forsythe, M. E., Hennessey, P. T., Brodigan, T. M., Love, D. C., Ashwell, G., & Krause, M. (2005). A Caenorhabditis elegans model of insulin resistance: altered macronutrient storage and dauer formation in an OGT-1 knockout. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(32), 11266–71. doi:10.1073/pnas.0408771102

Hanover, J. A., Krause, M. W., & Love, D. C. (2012). Bittersweet memories: linking metabolism to epigenetics through O-GlcNAcylation. *Nature Reviews. Molecular Cell Biology*, *13*(5), 312–21. doi:10.1038/nrm3334

Hanover, J. A., Yu, S., Lubas, W. B., Shin, S. H., Ragano-Caracciola, M., Kochran, J., & Love, D. C. (2003). Mitochondrial and nucleocytoplasmic isoforms of O-linked GlcNAc transferase encoded by a single mammalian gene. *Archives of Biochemistry and Biophysics*, *409*(2), 287–97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12504895

Hart, G. W., Housley, M. P., & Slawson, C. (2007). Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature*, *446*(7139), 1017–22. doi:10.1038/nature05815

Hart, G. W., Slawson, C., Ramirez-Correa, G., & Lagerlof, O. (2011). Cross Talk Between O-GlcNAcylation and Phosphorylation: Roles in Signaling, Transcription, and Chronic Disease. *Annual Review of Biochemistry*, *80*, 825–858. doi:10.1146/annurev-biochem-060608-102511.Cross

Harwood, K. R., & Hanover, J. a. (2014). Nutrient-driven O-GlcNAc cycling - think globally but act locally. *Journal of Cell Science*, 1857–1867. doi:10.1242/jcs.113233

Heckel, D., Comtesse, N., Brass, N., Blin, N., Zang, K. D., & Meese, E. (1998). Novel immunogenic antigen homologous to hyaluronidase in meningioma. *Human Molecular Genetics*, *7*(12), 1859–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9811929

Henikoff, S., Till, B. J., Comai, L., Division, B. S., Hutchinson, F., & Washington, S. H. (2004). Perspectives on Translational Biology TILLING . Traditional Mutagenesis Meets Functional Genomics. *Plant Physiology*, *135*, 630–636. doi:10.1104/pp.104.041061.630

Ho, C., Popat, S. D., Liu, T., Tsai, K., Ho, M., Chen, W., … Lin, C. (2010). Development of GlcNAc-inspired iminocyclitiols as potent and selective N-acetyl-B-hexosaminidase inhibitors. *ACS Chemical Biology*, *5*(5), 489–497.

Holt, G. D., & Hart, G. W. (1986). The Subcellular Distribution of Terminal N-Acetylglucosamine Moieties. *The Journal of Biological Chemistry*, *261*(17), 8049–8057.

Hunter, S., Jones, P., Mitchell, A., Apweiler, R., Attwood, T. K., Bateman, A., … Yong, S.-Y. (2012). InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Research*, *40*(Database issue), D306–12. doi:10.1093/nar/gkr948

Ingham, P. W. (1984). A gene that regulates the bithorax complex differentially in larval and adult cells of Drosophila. *Cell*, *37*(3), 815–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6430566

Jackson, C. (2007). *GFAT1/ZEPPELIN IS AN ESSENTIAL HETEROCHROMATIC GENE INVOLVED IN CUTICLE FORMATION IN D . MELANOGASTER .* Simon Fraser University. Retrieved from http://summit.sfu.ca/system/files/iritems1/8179/etd3058.pdf

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., … Hunter, S. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics (Oxford, England)*, 1–5. doi:10.1093/bioinformatics/btu031

Keembiyehetty, C. N., Krzeslak, A., Love, D. C., & Hanover, J. a. (2011). A lipid-droplet-targeted O-GlcNAcase isoform is a key regulator of the proteasome. *Journal of Cell Science*, *124*(16), 2851–60. doi:10.1242/jcs.083287

Keleman, K., Micheler, T., & VDRC project members. (2009). RNAi-phiC31 construct and insertion data submitted by the Vienna Drosophila RNAi Center.

Kelly, W. G., Dahmus, M. E., & Hart, G. W. (1993). RNA Polymerase II is a Glycoprotein: Modification of the COOH-terminal domain by O-GlcNAc. *The Journal of Biological Chemistry*, *268*(14), 10416–10424.

Kenwrick, S., Amaya, E., & Papalopulu, N. (2004). Pilot morpholino screen in Xenopus tropicalis identifies a novel gene involved in head development. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *229*(2), 289–99. doi:10.1002/dvdy.10440

Kornfeld, S., Kornfeld, R., Neufeld, E. F., & O'Brien, P. J. (1964). The feedback control of sugar nucleotide biosynthesis in liver. *Biochemistry*, *52*, 371–379.

Koundakjian, E. J., Cowan, D. M., Hardy, R. W., & Becker, A. H. (2004). The Zuker collection: A resource for the analysis of autosomal gene function in Drosophila melanogaster. *Genetics*, *167*, 203–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1470872&tool=pmcentrez&rendertype=abstract

Kreppel, L. K., Blomberg, M. A., & Hart, G. W. (1997). Dynamic Glycosylation of Nuclear and Cytosolic Proteins. CLONING AND CHARACTERIZATION OF A UNIQUE O-GlcNAc TRANSFERASE WITH MULTIPLE TETRATRICOPEPTIDE REPEATS. *Journal of Biological Chemistry*, *272*(14), 9308–9315. doi:10.1074/jbc.272.14.9308

Kreppel, L. K., & Hart, G. W. (1999). ENZYMOLOGY : Regulation of a Cytosolic and Nuclear O-GlcNAc Transferase : Role of the tetratricopeptide repeats. *The Journal of Biological Chemistry*, *274*, 32015–32022.

Lazarus, B. D., Love, D. C., & Hanover, J. a. (2006). Recombinant O-GlcNAc transferase isoforms: identification of O-GlcNAcase, yes tyrosine kinase, and tau as isoform-specific substrates. *Glycobiology*, *16*(5), 415–21. doi:10.1093/glycob/cwj078

Lazarus, M. B., Jiang, J., Gloster, T. M., Zandberg, W. F., Whitworth, G. E., Vocadlo, D. J., & Walker, S. (2012). Structural snapshots of the reaction coordinate for O-GlcNAc transferase. *Nature Chemical Biology*, *8*(12), 966–8. doi:10.1038/nchembio.1109

Lee, T., & Luo, L. (1999). Mosaic analysis with a repressible neurotechnique cell marker for studies of gene function in neuronal morphogenesis. *Neuron*, *22*(3), 451–461.

Lehman, D. M., Fu, D., Freeman, A. B., Hunt, K. J., Leach, R. J., Johnson-pais, T., … O-glycosylation, E. (2005). A single nucleotide polymorphism in MGEA5 encoding O-GlcNAc-selective N-acetyl-B-D glucosamine is associated with type 2 diabetes in Mexican Americans. *Diabetes*, *54*(April), 1214–1221.

Lewis, B. a. (2013). O-GlcNAcylation at promoters, nutrient sensors, and transcriptional regulation. *Biochimica et Biophysica Acta*, *1829*(11), 1202–6. doi:10.1016/j.bbagrm.2013.09.003

Londos, C., Brasaemle, D. L., Schultz, C. J., Segrest, J. P., & Kimmel, a R. (1999). Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Seminars in Cell & Developmental Biology*, *10*(1), 51–8. doi:10.1006/scdb.1998.0275

Love, D. C., & Hanover, J. a. (2005). The hexosamine signaling pathway: deciphering the “O-GlcNAc code”. *Science’s STKE : Signal Transduction Knowledge Environment*, *2005*(312), re13. doi:10.1126/stke.3122005re13

Love, D. C., Kochran, J., Cathey, R. L., Shin, S.-H., & Hanover, J. A. (2003). Mitochondrial and nucleocytoplasmic targeting of O-linked GlcNAc transferase. *Journal of Cell Science*, *116*(4), 647–654. doi:10.1242/jcs.00246

Love, D. C., Krause, M. W., & Hanover, J. A. (2010). O-GlcNAc cycling: Emerging Roles in Development and Epigenetics. *Seminars in Cell & Developmental Biology*, *21*(6), 646–54. doi:10.1016/j.semcdb.2010.05.001.O-GlcNAc

Lubas, W. A., & Hanover, J. A. (2000). Functional Expression of O-linked GlcNAc Transferase. DOMAIN STRUCTURE AND SUBSTRATE SPECIFICITY. *Journal of Biological Chemistry*, *275*(15), 10983–10988. doi:10.1074/jbc.275.15.10983

Lubas, W. A., Smith, M., Starr, C. M., & Hanover, J. a. (1995). Analysis of nuclear pore protein p62 glycosylation. *Biochemistry*, *34*(5), 1686–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7849028

Marchler-Bauer, A., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., … Bryant, S. H. (2009). CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Research*, *37*(Database issue), D205–10. doi:10.1093/nar/gkn845

Marchler-Bauer, A., & Bryant, S. H. (2004). CD-Search: protein domain annotations on the fly. *Nucleic Acids Research*, *32*(Web Server issue), W327–31. doi:10.1093/nar/gkh454

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., … Bryant, S. H. (2011). CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Research*, *39*(Database issue), D225–9. doi:10.1093/nar/gkq1189

Marchler-Bauer, A., Panchenko, A. R., Shoemaker, B. a, Thiessen, P. a, Geer, L. Y., & Bryant, S. H. (2002). CDD: a database of conserved domain alignments with links to domain three-dimensional structure. *Nucleic Acids Research*, *30*(1), 281–3. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=99109&tool=pmcentrez&rendertype=abstract

Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., … Bryant, S. H. (2013). CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Research*, *41*(Database issue), D348–52. doi:10.1093/nar/gks1243

Marshall, S., Bacote, V., & Traxinger, R. R. (1991). Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *The Journal of Biological Chemistry*, *266*(8), 4706–12. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2002019

McCallum, C. M., Comai, L., Greene, E. A., & Henikoff, S. (2000). Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiology*, *123*, 439–42. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1539256&tool=pmcentrez&rendertype=abstract

Myster, S., & Peifer, M. (2003). Myster and Peifer lethals.

Na, J., Musselman, L. P., Pendse, J., Baranski, T. J., Bodmer, R., Ocorr, K., & Cagan, R. (2013). A Drosophila model of high sugar diet-induced cardiomyopathy. *PLoS Genetics*, *9*(1), e1003175. doi:10.1371/journal.pgen.1003175

Ng, P. C., & Henikoff, S. (2001). Predicting deleterious amino acid substitutions. *Genome Research*, *11*(5), 863–74. doi:10.1101/gr.176601

Ng, P. C., & Henikoff, S. (2002). Accounting for human polymorphisms predicted to affect protein function. *Genome Research*, *12*(3), 436–46. doi:10.1101/gr.212802

Ng, P. C., & Henikoff, S. (2003). SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research*, *31*(13), 3812–3814. doi:10.1093/nar/gkg509

Nicholson, P., Yepiskoposyan, H., Metze, S., Zamudio Orozco, R., Kleinschmidt, N., & Mühlemann, O. (2010). Nonsense-mediated mRNA decay in human cells: mechanistic insights, functions beyond quality control and the double-life of NMD factors. *Cellular and Molecular Life Sciences : CMLS*, *67*(5), 677–700. doi:10.1007/s00018-009-0177-1

Nolte, D., & Müller, U. (2002). Human O-GlcNAc transferase (OGT): genomic structure, analysis of splice variants, fine mapping in Xq13.1. *Mammalian Genome*, *13*, 62–64. doi:10.1007/s00335-001-2108-9

O'Donnell, N., Zachara, N. E., Hart, G. W., Marth, J. D., & Donnell, N. O. (2004). Ogt-Dependent X-Chromosome-Linked Protein Glycosylation Is a Requisite Modification in Somatic Cell Function and Embryo Viability. *Molecular and Cellular Biology*, *24*(4), 1680–1690. doi:10.1128/MCB.24.4.1680

Ozcan, S., Andrali, S. S., & Cantrell, J. E. L. (2010). Modulation of transcription factor function by O-GlcNAc modification. *Biochimica et Biophysica Acta*, *1799*(5-6), 353–64. doi:10.1016/j.bbagrm.2010.02.005

Park, S., Park, S.-H., Baek, J. Y., Jy, Y. J., Kim, K. S., Roth, J., … Choe, K.-M. (2011). Protein O-GlcNAcylation regulates Drosophila growth through the insulin signaling pathway. *Cellular and Molecular Life Sciences*, *68*(20), 3377–3384. doi:10.1007/s00018-011-0640-7

Port, F., Chen, H.-M., Lee, T., & Bullock, S. L. (2014). Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(29), E2967–76. doi:10.1073/pnas.1405500111

Radermacher, P. T., Myachina, F., Bosshardt, F., Pandey, R., Mariappa, D., Müller, H.-A. J., & Lehner, C. F. (2014). O-GlcNAc reports ambient temperature and confers heat resistance on ectotherm development. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(15), 5592–7. doi:10.1073/pnas.1322396111

Rahman, M., Stuchlick, O., Karim, E. G. El, Stuart, R., Kipreos, E. T., & Wells, L. (2010). Intracellular protein glycosylation modulates insulin mediated lifespan in C . elegans. *Aging*, *2*(10), 678–690.

Rao, F. V, Dorfmueller, H. C., Villa, F., Allwood, M., Eggleston, I. M., & van Aalten, D. M. F. (2006). Structural insights into the mechanism and inhibition of eukaryotic O-GlcNAc hydrolysis. *The EMBO Journal*, *25*(7), 1569–78. doi:10.1038/sj.emboj.7601026

Rao, F. V, Schüttelkopf, A. W., Dorfmueller, H. C., Ferenbach, A. T., Navratilova, I., & van Aalten, D. M. F. (2013). Structure of a bacterial putative acetyltransferase defines the fold of the human O-GlcNAcase C-terminal domain. *Open Biology*, *3*(10), 130021. doi:10.1098/rsob.130021

Rong, Y. S., & Golic, K. G. (2001). A targeted gene knockout in Drosophila. *Genetics*, *157*, 1307–1312. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1461559&tool=pmcentrez&rendertype=abstract

Rossetti, L. (2000). Perspective: Hexosamines and nutrient sensing. *Endocrinology*, *141*(6), 1922–5. doi:10.1210/endo.141.6.7566

Rubin, G. M., & Spradling, a C. (1982). Genetic transformation of Drosophila with transposable element vectors. *Science (New York, N.Y.)*, *218*(4570), 348–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6289436

Rulifson, E. J., Kim, S. K., & Nusse, R. (2002). Ablation of Insulin-producing neurons in flies: growth and diabetic phenotypes. *Science*. Retrieved from http://www.sciencemag.org/cgi/content/full/296/5570/1118/DC1

Ryder, E., Ashburner, M., Bautista-Llacer, R., Drummond, J., Webster, J., Johnson, G., … Russell, S. (2007). The DrosDel deletion collection: a Drosophila genomewide chromosomal deficiency resource. *Genetics*, *177*(1), 615–29. doi:10.1534/genetics.107.076216

Sekine, O., Love, D. C., Rubenstein, D. S., & Hanover, J. a. (2010). Blocking O-linked GlcNAc cycling in Drosophila insulin-producing cells perturbs glucose-insulin homeostasis. *The Journal of Biological Chemistry*, *285*(49), 38684–91. doi:10.1074/jbc.M110.155192

Shafi, R., Iyer, S. P., Ellies, L. G., O'Donnell, N., Marek, K. W., Chui, D., … Marth, J. D. (2000). The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), 5735–9. doi:10.1073/pnas.100471497

Shen, D. L. (2011). *Kinetic studies of O -GlcNAc processing enzymes : implications for enzyme regulation*. Simon Fraser University. Retrieved from http://summit.sfu.ca/system/files/iritems1/11901/etd6464_DShen.pdf

Shen, D. L., Gloster, T. M., Yuzwa, S. A., & Vocadlo, D. J. (2012). Insights into O-linked N-acetylglucosamine ([0-9]O-GlcNAc) processing and dynamics through kinetic analysis of O-GlcNAc transferase and O-GlcNAcase activity on protein substrates. *The Journal of Biological Chemistry*, *287*(19), 15395–408. doi:10.1074/jbc.M111.310664

Sinclair, D. a R., Syrzycka, M., Macauley, M. S., Rastgardani, T., Komljenovic, I., Vocadlo, D. J., … Honda, B. M. (2009). Drosophila O-GlcNAc transferase (OGT) is encoded by the Polycomb group (PcG) gene, super sex combs (sxc). *Proceedings of the National Academy of Sciences of the United States of America*, *106*(32), 13427–32. doi:10.1073/pnas.0904638106

Slawson, C., Copeland, R. J., & Hart, G. W. (2010). O-GlcNAc signaling: a metabolic link between diabetes and cancer? *Trends in Biochemical Sciences*, *35*(10), 547–55. doi:10.1016/j.tibs.2010.04.005

Slawson, C., & Hart, G. W. (2011). O-GlcNAc signalling: implications for cancer cell biology. *Nature Reviews. Cancer*, *11*(9), 678–84. doi:10.1038/nrc3114

Spradling, a C., Stern, D., Beaton, a, Rhem, E. J., Laverty, T., Mozden, N., … Rubin, G. M. (1999). The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes. *Genetics*, *153*(1), 135–77. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1460730&tool=pmcentrez&rendertype=abstract

St. Pierre, S., Ponting, L., Stefancsik, R., McQuilton, P., & the FlyBase Consortium. (2014). FlyBase 102 - advanced approaches to interrogating FlyBase. *Nucleic Acids Research*, *42*(D1), D780–D788. doi:10.1093/nar/gkt1092

Syrzycka, M. A. (2009). *Genetic and molecular characterization of essential heterochromatic genes in Drosophila melanogaster*. Simon Fraser University.

Toleman, C., Paterson, A. J., Whisenhunt, T. R., & Kudlow, J. E. (2004). Characterization of the histone acetyltransferase (HAT) domain of a bifunctional protein with activable O-GlcNAcase and HAT activities. *The Journal of Biological Chemistry*, *279*(51), 53665–73. doi:10.1074/jbc.M410406200

Torres, C.-R., & Hart, G. W. (1984). Topography and polypeptide distribution Topography and Polypeptide Distribution of Terminal N-Acetylglucosamine Residues on the Surfaces of Intact Lymphocytes. *The Journal of Biological Chemistry*, *259*(5), 3308–3317.

Vaidyanathan, K., Durning, S., & Wells, L. (2014). Functional O-GlcNAc modifications: implications in molecular regulation and pathophysiology. *Critical Reviews in Biochemistry and Molecular Biology*, *49*(2), 140–63. doi:10.3109/10409238.2014.884535

Webster, D. M., Teo, C. F., Sun, Y., Wloga, D., Gay, S., Klonowski, K. D., … Dougan, S. T. (2009). O-GlcNAc modifications regulate cell survival and epiboly during zebrafish development. *BMC Developmental Biology*, *9*, 28. doi:10.1186/1471-213X-9-28

Wells, L., Gao, Y., Mahoney, J. A., Vosseller, K., Chen, C., Rosen, A., & Hart, G. W. (2002). GLYCOBIOLOGY AND EXTRACELLULAR MATRICES: Dynamic O-Glycosylation of Nuclear and Cytosolic Proteins: Further characterization of the nucleocytoplasmic B-N-Acetylglycosaminidase, O-GlcNAcase. *The Journal of Biological Chemistry*, *277*, 1755–61. doi:10.1074/jbc.M09656200

Wells, L., Vosseller, K., & Hart, G. W. (2001). Glycosylation of Nucleocytoplasmic Proteins: Signal Transduction and O-GlcNAc. *Science*, *291*(5512), 2376–2378. doi:10.1126/science.1058714

Whelan, S. A., Lane, M. D., & Hart, G. W. (2008). Regulation of the O-linked beta-N-acetylglucosamine transferase by insulin signaling. *The Journal of Biological Chemistry*, *283*(31), 21411–21417. doi:10.1074/jbc.M800677200

Whisenhunt, T. R., Yang, X., Bowe, D. B., Paterson, A. J., Van Tine, B. a, & Kudlow, J. E. (2006). Disrupting the enzyme complex regulating O-GlcNAcylation blocks signaling and development. *Glycobiology*, *16*(6), 551–63. doi:10.1093/glycob/cwj096

Winkler, S., Schwabedissen, A., Backasch, D., Bökel, C., Seidel, C., Bönisch, S., … González-Gaitán, M. (2005). Target-selected mutant screen by TILLING in Drosophila. *Genome Research*, *15*(5), 718–23. doi:10.1101/gr.3721805

Yang, X., Ongusaha, P. P., Miles, P. D., Havstad, J. C., Zhang, F., So, W. V., … Evans, R. M. (2008). Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. *Nature*, *451*(21), 964–970. doi:10.1038/nature06668

Yang, X., Zhang, F., & Kudlow, J. E. (2002). Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein O-GlcNAcylation to transcriptional repression. *Cell*, *110*(1), 69–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12150998

Yang, Y. R., Song, M., Lee, H., Jeon, Y., Choi, E.-J., Jang, H.-J., … Suh, P.-G. (2012). O-GlcNAcase is essential for embryonic development and maintenance of genomic stability. *Aging Cell*, *11*(3), 439–48. doi:10.1111/j.1474-9726.2012.00801.x

Zeidan, Q., & Hart, G. W. (2010). The intersections between O-GlcNAcylation and phosphorylation: implications for multiple signaling pathways. *Journal of Cell Science*, *123*(Pt 1), 13–22. doi:10.1242/jcs.053678

Appendices

Appendix A.

Fly lines used in this study

Table AA1. A list of the *Drosophila melanogaster* mutant lines used in this study.

Mutant name	Mutagen	Gene affected	Source/Reference
Oga ^{415}	EMS	*Oga*	SDTP
Oga ^{292}	EMS	*Oga*	SDTP
Oga ^{773}	EMS	*Oga*	SDTP
Oga ^{1179}	EMS	*Oga*	SDTP
Oga ^{1021}	EMS	*Oga*	SDTP
Oga ^{520}	EMS	*Oga*	SDTP
Oga ^{89}	EMS	*Oga*	SDTP
sxc ^{1}	EMS	*sxc*	Ingham, 1984
sxc ^{3}	EMS	*sxc*	Ingham, 1984
sxc ^{4}	EMS	*sxc*	Ingham, 1984
sxc ^{5}	EMS	*sxc*	Ingham, 1984
sxc ^{6}	EMS	*sxc*	Myster and Peifer, 2003
sxc ^{2637}	P-element-based gene disruption study	*sxc*	Spradling et al., 1999

*SDTP refers to the Seattle *Drosophila* TILLING Project

Table AA2. A list of the *Drosophila melanogaster* stocks containing RNAi transgenes used in this study.

Stock number	Inserted Chromosome	Gene affected	OFF Targets	Source/Reference
23-1	3	*Oga*	0	Kristina Pohl, 2011*
23-2	3	*Oga*	0	Kristina Pohl, 2011*
23-4	3	*Oga*	0	Kristina Pohl, 2011*
23-5	3	*Oga*	0	Kristina Pohl, 2011*
23-6	3	*Oga*	0	Kristina Pohl, 2011*
23-7	3	*Oga*	0	Kristina Pohl, 2011*
23-8	3	*Oga*	0	Kristina Pohl, 2011*
23-9	2	*Oga*	0	Kristina Pohl, 2011*
23-10	2	*Oga*	0	Kristina Pohl, 2011*
23-11	3	*Oga*	0	Kristina Pohl, 2011*
23-12	3	*Oga*	0	Kristina Pohl, 2011*
41822	2	*Oga*	0	Dietzl et al., 2007
41823	1	*Oga*	0	Dietzl et al., 2007
106670	2	*Oga*	1: CG10574	Dietzl et al., 2007

Stock number	Inserted Chromosome	Gene affected	OFF Targets	Source/Reference
2824-1-4M	2	*Ogt*		Sinclair et al., 2009
2664-1-1M	2	*Gfat1*		Jackson, 2007**

*indicates that the data is unpublished
**indicates work completed in a graduate studies thesis

	Trans-formant ID	Construct ID	Library	CG Number	Nearest Genes	Synonyms	ON Targets	OFF Targets	s19	CAN Repeats	Viability	Inserted Chromo-some	Status
☐	41822	10644	GD	CG5871		Oga,O-GlcNAcase	1	0	1	2	viable	2	available

	Trans-formant ID	Construct ID	Library	CG Number	Nearest Genes	Synonyms	ON Targets	OFF Targets	s19	CAN Repeats	Viability	Inserted Chromo-some	Status
☐	41823	10644	GD	CG5871		Oga,O-GlcNAcase	1	0	1	2	viable	1	available

	Trans-formant ID	Construct ID	Library	CG Number	Nearest Genes	Synonyms	ON Targets	OFF Targets	s19	CAN Repeats	Viability	Inserted Chromo-some	Status
☐	106670	100928	KK	CG5871		Oga,O-GlcNAcase	1	1	1	2	viable	2	available

OFF-TARGETS FOR TRANSFORMANT ID 106670

Transformant ID	106670
CG Number	CG5871
Synonyms	Oga O-GlcNAcase
OFF-Targets	CG Number: CG10574 — 19-mer hits: 1 — total 19-mers: 367

Figure AA1. VDRC *Oga* RNAi lines showing insertion chromosomes and predicted OFF-targets (from the VDRC website).

Table AA3. A list of the transgenic *Drosophila melanogaster* lines containing a cDNA copy of a particular gene for the purposes of targeted overexpression used in this study.

Stock number	Inserted chromosome	Gene affected	Source/Reference
8302-3-1M	2	*Oga*	Stefanelli, 2014
8302-3-2M	2	*Oga*	Stefanelli, 2014
8302-3-3M	3	*Oga*	Stefanelli, 2014
8302-4-1M	2	*Oga*	Stefanelli, 2014
8302-4-2M	3	*Oga*	Stefanelli, 2014
8302-4-3M	2	*Oga*	Stefanelli, 2014
8302-4-4M	3	*Oga*	Stefanelli, 2014
8302-4-5M	2	*Oga*	Stefanelli, 2014
8302-4-6M	3	*Oga*	Stefanelli, 2014
8302-4-7M	3	*Oga*	Stefanelli, 2014
8302-4-8M	2	*Oga*	Stefanelli, 2014
8302-4-9M	3	*Oga*	Stefanelli, 2014
8302-4-10F	2	*Oga*	Stefanelli, 2014
3632-2-2M	3	*Ogt*	Sinclair et al., 2009
4145-1-3M	3	*Gfat1*	Jackson, 2007

All of the lines were developed from cloned plasmid constructs that were sent to BestGene for injection into w^{1118} embryos.

Table AA4. A list of various genotypes of *Drosophila melanogaster* lines used in this study.

Genotype/Reference	In this study	Stock #	Chr	Gene(s) affected	Mutagen/ Feature Type	Symbol
w; P{Ilp2-GAL4.R}2* (Rulifson et al., 2002)	*dILP2-GAL4*	37516	2	*dILP2*	transgenic transposon	Scer\GAL4[Ilp2.PR]
y^1w; P{tubP-GAL4} LL7/ TM3 Sb^1* (Lee & Luo, 1999)	*tub-GAL4*	5138	3	*tubP*	in vitro construct - regulatory fusion	Scer\GAL4[αTub84 B.PL]
w^{1118}; Df(3R)ED10845, P{3'.RS5+3.3'}ED10845 / TM6C, cu^1 Sb^1 (Ryder et al., 2007)	*Df(3R) ED10845*	9487	3	many	chromosomal deletion	Dmel\Df(3R)ED10845
P{UAS-Dcr-2.D}1, w1118 (Dietzl et al., 2007)	*UAS-Dcr-2*	24646	2	Dcr-2	in vitro construct - regulatory fusion	Dmel\Dcr-2[Scer\UAS.cDa]

All of the lines were obtained from Bloomington (Bloomington stock numbers listed) and have detailed descriptions on FlyBase that include the information listed here (www.flybase.org). *Chr refers to the chromosome the transgene was inserted into.

Appendix B.

Protein sequence of *Drosophila melanogaster* OGA from FlyBase

MADEAGSQADGKRQFICGVIEGFYGRPWTTEQRKDLFRKLKSMGMGSSPS
YMYAPKDDYKHRAYWRELYTVEEADHLSSLIAAAKEAGITFYYALSPGLD
MTYSSPKEIATLKRKLDQVAQFGCEAYALLFDDIESELSKADKEVFQTFA
NAHVSVTNEIYTHLGSPRFLFCPTQYCASRAVPTVQESEYLNTLGSKLNN
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Appendix C.

Drosophila melanogaster Oga CDS from FlyBase with qPCR primers identified

Forward primer: teal

Exon-exon boundary: bright green

Reverse primer: pink

Oga CDS from FlyBase
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Legend:

Forward primer: teal

Exon-exon boundary: bright green

Reverse primer: pink

Oga genomic DNA (decorated FASTA with qPCR primers highlighted (exon-exon boundary))
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Legend:
*gene span:*xxxx
*RNA:*XXXX
*CDS:*XXXX

Appendix D.

OGA enzymatic activity assay

Materials and Methods:

OGA activity was measured in adult female flies, aged one day, in separate reactions for each of the following eight genotypes: *w^{1118}, w^{1118}* fed 200 μl of 10mM solution of NButGT as larvae, *Df(3R)ED10845/+, Oga^{415}/Df(3R)ED10845, UAS-Dcr-2/+; UAS-Oga RNAi 23-5/TM3Sb, UAS-Dcr-2/+; UAS-Oga RNAi 23-5/tub-GAL4* driver*, UAS-Dcr-2/+; UAS-Oga RNAi 106670/TM3Sb, UAS-Dcr-2/+; UAS-Oga RNAi 106670/tub-GAL4*. Protein lysates of each genotype were prepared by grinding ten female flies in 100 μl PBS treated with a protease inhibitor tablet (Roche Applied Science). The preparations were spun at 13000 rpm for 15 minutes at 4°C and the supernatant collected will hereinafter be referred to as the protein lysate. 1:10 dilutions of the recovered protein lysate were used to assay protein concentration via endpoint reading of a DC assay (Bio-Rad) using SoftMax Pro microplate data software.

Scott Yuzwa and I designed an experiment to assay the enzymatic activity of OGA in the abovementioned fly lines by exploiting the fluorogenic substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4MU-GlcNAc or MUG; Sigma); OGA is able to hydrolyze the compound, thus causing the release of the fluorescent molecule 4-MU (4-Methylumbelliferone, also known as 7-hydroxy-4-methylcoumarin), which can be measured using a fluorescence spectrophotometer to deduce the rate of hydrolysis over time (Dorfmueller, Borodkin, Schimpl, & van Aalten, 2009; Ho et al., 2010; Wells et al., 2002). All reactions were run in triplicate at 37 ^{o}C on a 96-well microplate to calculate the rate of reaction of OGA from each protein lysate. The experimental wells contained 0.98 mg protein, 15 μl 10mM GalNAc-thiazoline (a lysosomal β-hexosaminidase inhibitor; generously provided by the Vocadlo lab), 7.5 μl 10mM 4MU-GlcNAc, and phosphate buffered saline (PBS) to 150 μl total reaction volume (Table AD1). Two sets of control reactions were preformed alongside the experimental wells; the first control contained 15 μl 10mM thiamet-G (TG; OGA inhibitor provided by the Vocadlo lab), 0.98 mg protein, 7.5 μl 10mM 4MU-GlcNAc, and PBS to 150 μl total reaction volume, and the second control contained only 0.98 mg protein, 7.5 μl 10 mM 4MU-GlcNAc substrate and PBS to 150 μl total reaction volume (Table AD1). A Varian CARY Eclipse Fluorescence Spectrophotometer was used to record the change in fluorescence over the course of the sixty-minute run. The software used to access the recorded data was SoftMax Pro and the settings used were as follows: 20 millisecond intervals, speed of 10, 60 minute run time, 27 second read intervals, kinetic reaction, Ex 355/ Em485 filter pair.

The raw data from the fluorescence spectrophotometer is reported in relative fluorescence units (RFUs); RFUs can be converted into reaction rates by plotting a standard graph of RFUs to known concentrations of the leaving group of the reaction of OGA on 4MU-GlcNAc, which is 4-MU (4-Methylumbelliferone, also known as 7-hydroxy-4-methylcoumarin) and then dividing that number by mg of protein used in the reaction to get a rate measured in μM/min/mg protein (Figure AD1).

Table AD1. Reagents used in the experimental and control reactions of the OGA enzymatic activity assay

	Experimental		Control A		Control B	
Inhibitor	15 μl	GalNAc-thiazoline	15 μl	Thiamet-G		None
Protein lysate	0.98 mg	Protein	0.98 mg	Protein	0.98 mg	Protein
Substrate	7.5 μl	4MU-GlcNAc	7.5 μl	4MU-GlcNAc	7.5 μl	4MU-GlcNAc
Buffer	To 150 μl total volume	PBS	To 150 μl total volume	PBS	To 150 μl total volume	PBS

GalNAc-thiazoline, 4MU-GlcNAc (4-methylumbelliferyl N-acetyl-β-D-glucosaminide), and thiamet-G stock solutions were 10mM. OGA activity assay experiment designed with help and tutelage from Scott Yuzwa. All reactions were run in triplicate and the change in fluorescence detected by a Varian CARY Eclipse Fluorescence Spectrophotometer.

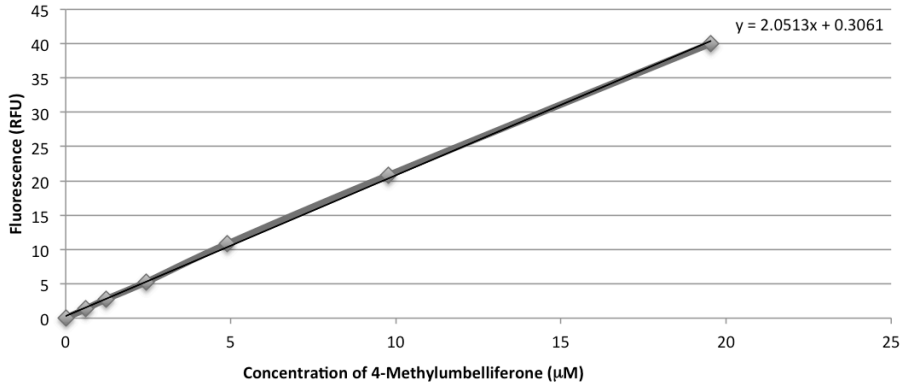


Figure AD1. Standard graph for determining relative fluorescence units (RFUs) measurement for known concentrations of 4-Methylumbelliferone fluorescence to be able to calculate the rate of OGA activity.

Results:

OGA enzymatic activity assay needs optimization to provide significant data

As previously mentioned, we found RNAi lines that knocked down *Oga* expression as well as one nonsense mutation that putatively results in the production of a truncated version of OGA; we were curious to see if there was OGA enzymatic activity in the surviving flies of the various genotypes of interest. To determine if OGA was being formed and functioning with an enzymatic activity comparable to the wild-type strain w^{1118}, an OGA activity assay was designed with help from Scott Yuzwa (Vocadlo lab). The average rate of OGA activity was measured by the increase in fluorescence upon liberation of the fluorescent leaving group, 4-MU (4-methylumbelliferone), over time. The rate of OGA activity in the lysosomal enzyme inhibitor GalNac-thiazoline (Vocadlo, unpublished results) treated w^{1118} sample was 26.4 μM/ min/ mg protein (Figure 10). The w^{1118} control sample that did not contain any inhibitors showed an increase in OGA activity, as did the control sample that was treated with an OGA inhibitor, NButGT, but only at the larval stage (Figure 10). The only other two samples that showed an increase in enzymatic activity were from the flies containing the *UAS-Oga RNAi* transgenes driven ubiquitously with *tub-GAL4* (Figure 10). The rest of the samples showed reaction rates lower than the 26.4 μM/ min/ mg protein activity level of the w^{1118} control sample (Figure 10).

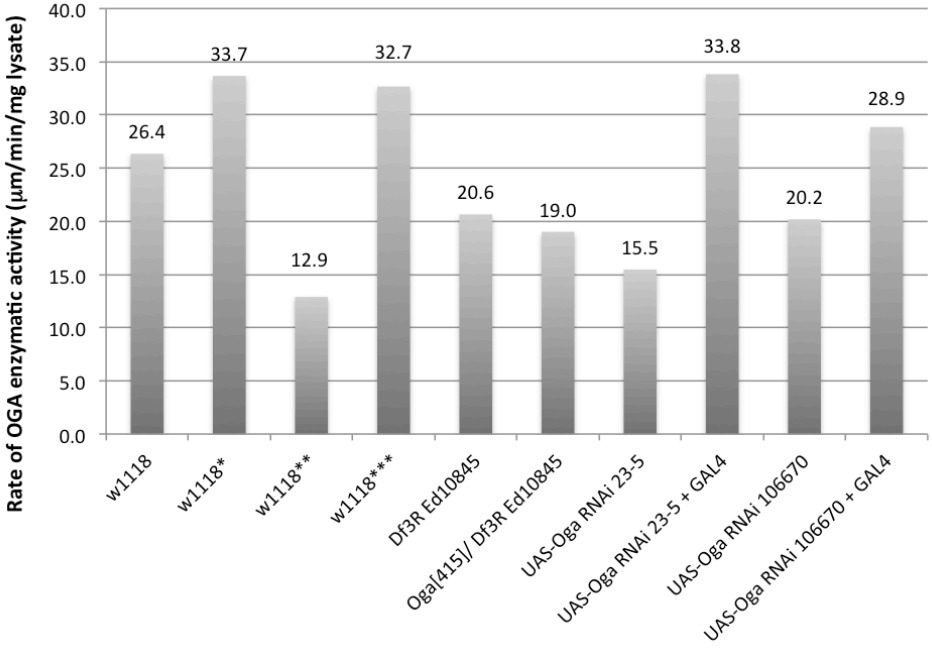


Figure AD2. Enzymatic activity of OGA.

The enzymatic activity assay was performed in triplicate on protein lysate samples made from one-day-old adult female flies. The reactions included 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4MU-GlcNAc) as a substrate and were assayed for increasing concentration of the fluorescent leaving group, 4-methylumbelliferone, over time via detection by a Varian CARY Eclipse Fluorescence Spectrophotometer
*no GalNAc-thiazoline (lysosomal enzyme inhibitor)
** with Thiamet G (OGA inhibitor), but no GalNAc-thiazoline (lysosomal enzyme inhibitor)
*** 200 μl of 10mM solution of NButGT (OGA inhibitor) fed to larvae

Discussion: OGA activity assay

The fly line with a nonsense mutation at amino acid position 761 of 1019, *Oga*^{415}, putatively codes for a truncated version of OGA; if the last quarter of OGA is not synthesized then it is possible that the protein is misfolding and being degraded, but it is also possible that the protein is still functional as the predicted active site is in the N-terminus, well before the premature stop codon. It could be that the truncated version of OGA functions as well as wild-type OGA, however, without some sort of assay to look for the presence of OGA in the *Oga*^{415}*/Df(3R)ED10845* flies, it is difficult to say. The enzymatic activity assay that was designed to show if OGA activity was affected within the mutant flies looked promising, but the results indicate that the assay is not optimal for fly tissue as is. *UAS-Oga RNAi 23-5* flies without the *tub-GAL4* driver had lower activity than the *UAS-Oga RNAi 23-5/tub-GAL4* flies, and the same for *Oga* RNAi line 106670 (Figure 10). I have shown that the RNAi line 23-5 reduces the *Oga* transcript level to 29% of wild-type upon ubiquitous expression with *tub-GAL4* and *UAS-Dcr-2* included (Figure 4), therefore, the activity should be lower than the sister progeny containing the transgene but no driver to initiate its expression. Perhaps the OGA activity assay does not work in flies, or the techniques need to be optimized, as the results do not make logical sense at this point.

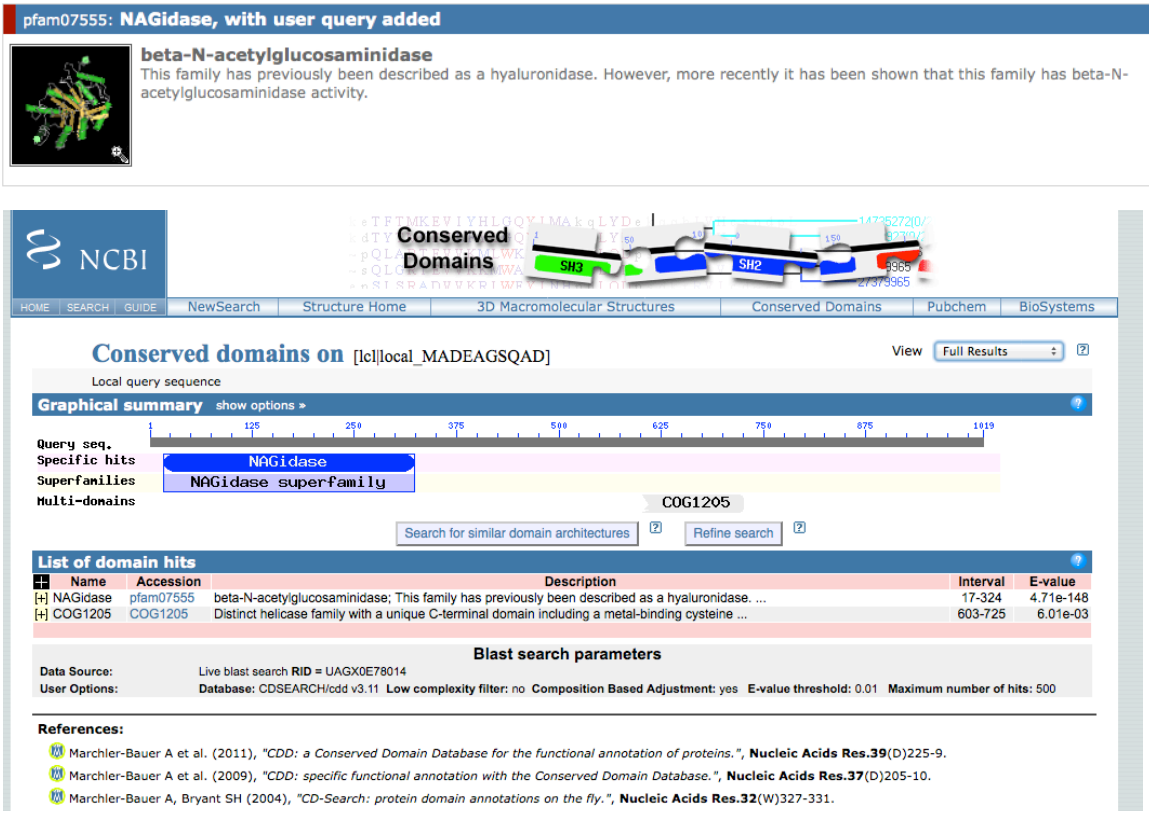
Appendix E.

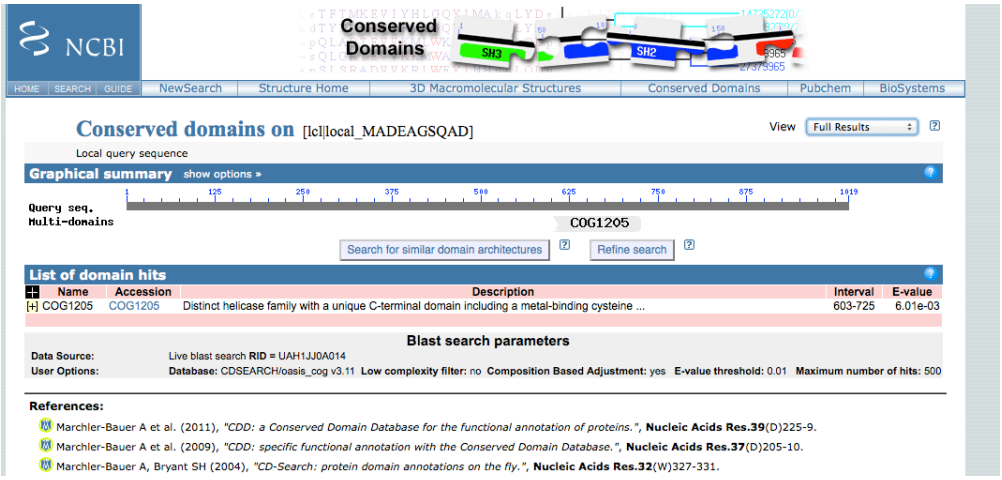
Online sequence analysis of *Drosophila melanogaster Oga* protein

Conserved domain search of Dmel OGA amino acid sequence, gi 74868464, against the NCBI Conserved Domain Database (CDD) showing the conserved domain to be NAGidase [pfam07555], beta-N-acetylglucosaminidase

http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

Pfam 07555 is a member of the superfamily cl06544

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Refine search
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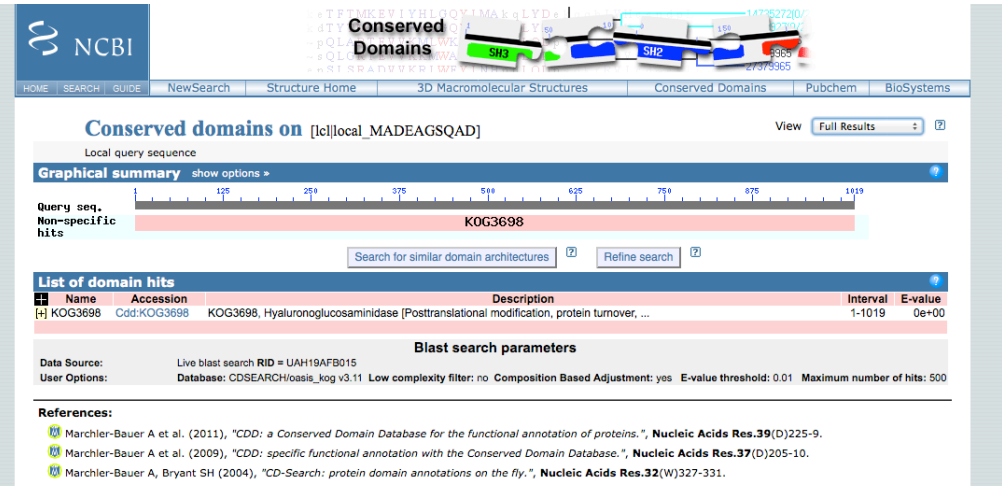
References:
Marchler-Bauer A et al. (2011), "CDD: a Conserved Domain Database for the functional annotation of proteins.", Nucleic Acids Res.39(D)225-9.
Marchler-Bauer A et al. (2009), "CDD: specific functional annotation with the Conserved Domain Database.", Nucleic Acids Res.37(D)205-10.
Marchler-Bauer A, Bryant SH (2004), "CD-Search: protein domain annotations on the fly.", Nucleic Acids Res.32(W)327-331.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Full Results
Local query sequence
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Search for similar domain architectures
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Marchler-Bauer A et al. (2011), "CDD: a Conserved Domain Database for the functional annotation of proteins.", Nucleic Acids Res.39(D)225-9.
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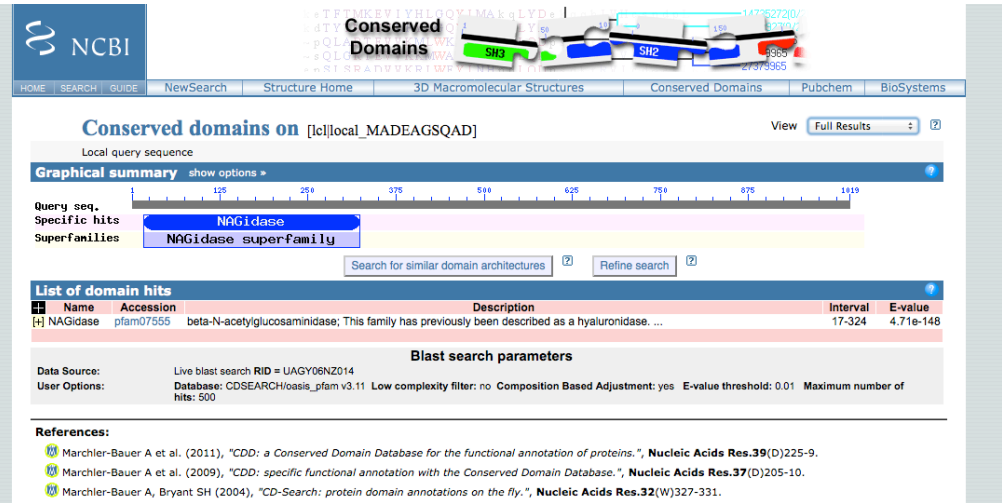
Marchler-Bauer A, Bryant SH (2004), "CD-Search: protein domain annotations on the fly.", Nucleic Acids Res.32(W)327-331.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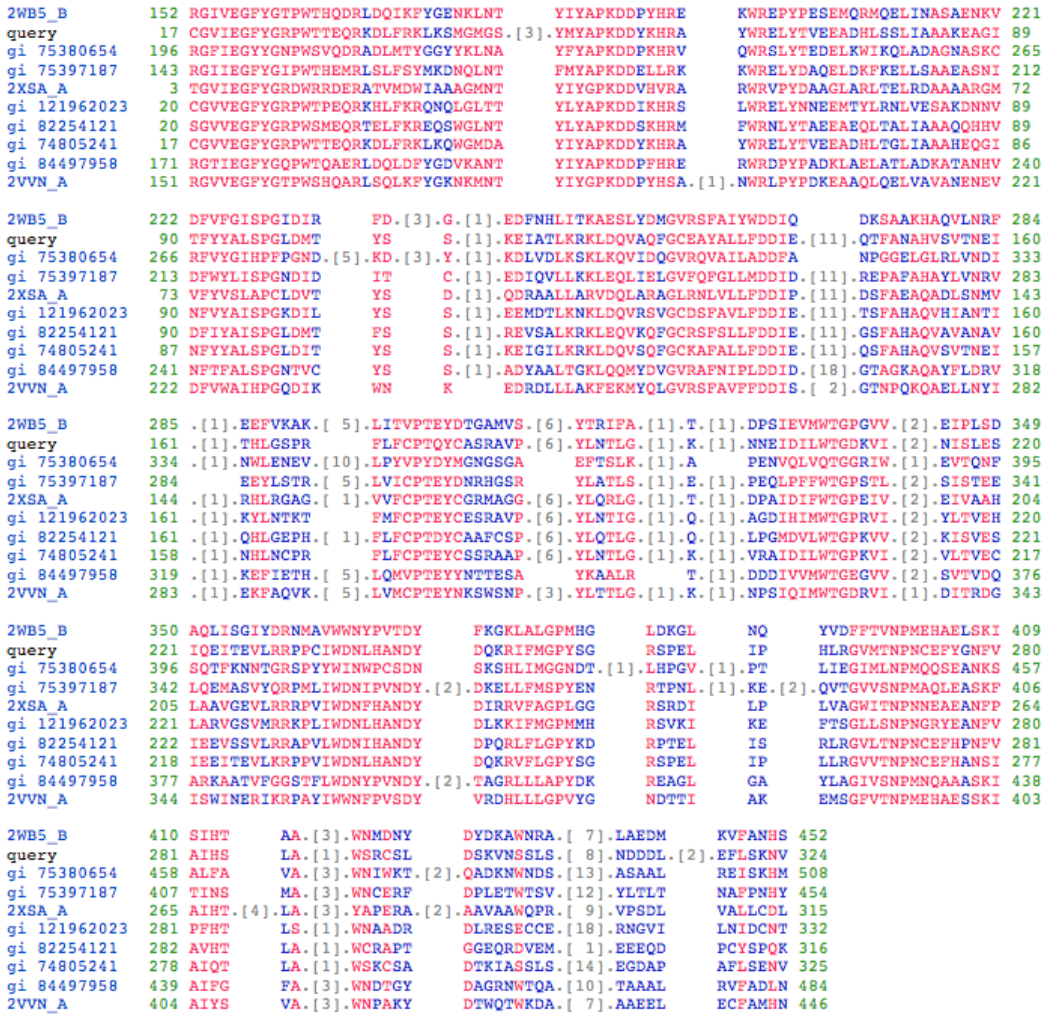
3D Macromolecular Structures
Conserved Domains
Pubchem
BioSystems
Conserved domains on [lcl|local_MADEAGSQAD]
View
Full Results
Local query sequence
Graphical summary
show options »
Query seq.
Specific hits
NAGidase
Superfamilies
NAGidase superfamily
Search for similar domain architectures
Refine search
List of domain hits
Name
Accession
Description
Interval
E-value
[+] NAGidase
pfam07555
beta-N-acetylglucosaminidase; This family has previously been described as a hyaluronidase. ...
17-324
4.71e-148
Blast search parameters
Data Source:
Live blast search RID = UAGY06NZ014
User Options:
Database: CDSEARCH/oasis_pfam v3.11 Low complexity filter: no Composition Based Adjustment: yes E-value threshold: 0.01 Maximum number of hits: 500
References:
Marchler-Bauer A et al. (2011), "CDD: a Conserved Domain Database for the functional annotation of proteins.", Nucleic Acids Res.39(D)225-9.
Marchler-Bauer A et al. (2009), "CDD: specific functional annotation with the Conserved Domain Database.", Nucleic Acids Res.37(D)205-10.
Marchler-Bauer A, Bryant SH (2004), "CD-Search: protein domain annotations on the fly.", Nucleic Acids Res.32(W)327-331.

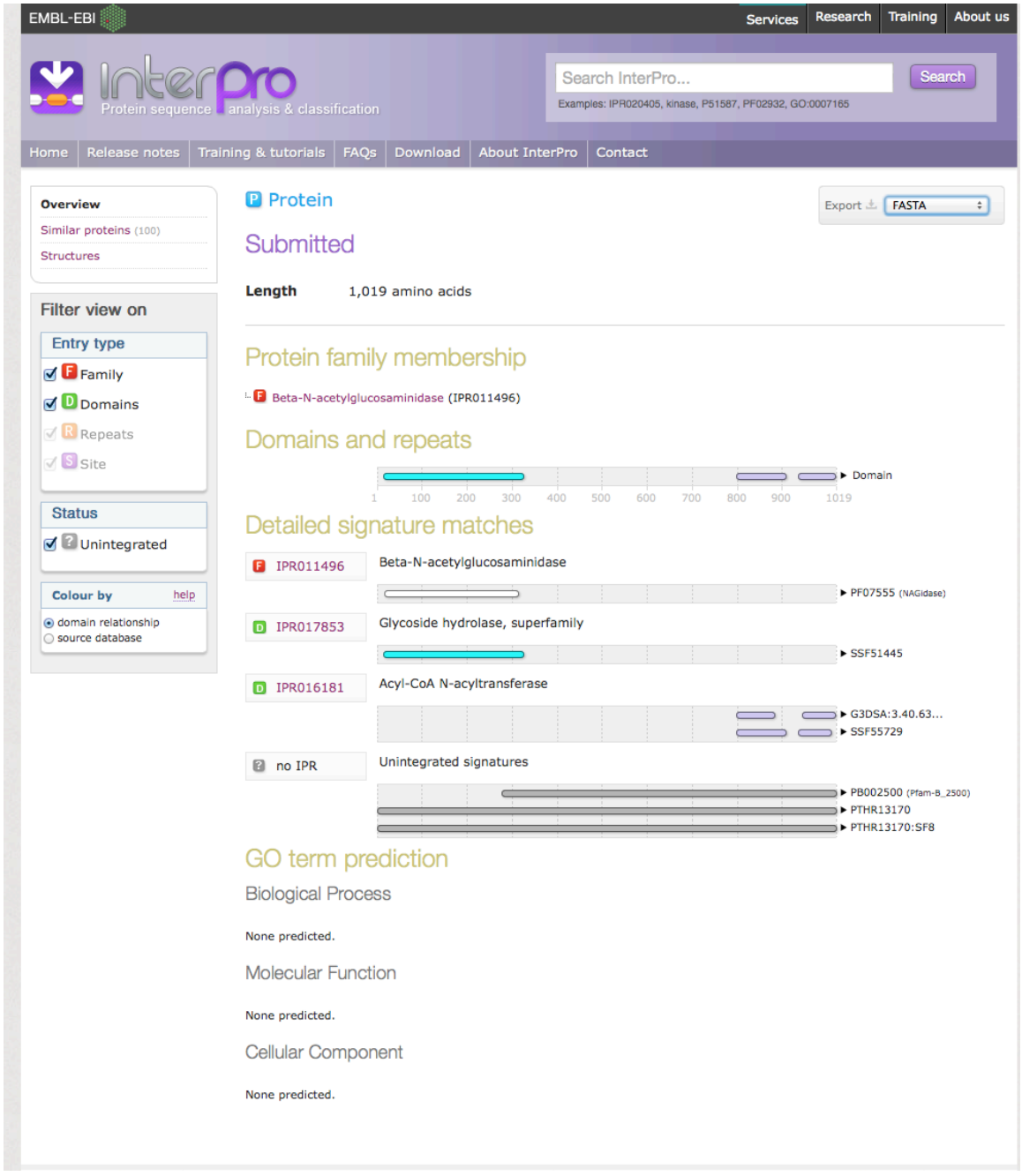


The above alignment shows protein sequences from the following organisms:

Clostridium perfringens	2WB5_B
Clostridium paraputrificum	gi 75380654
Enterococcus faecium	gi 75397187
Oceanicola granulosus HTCC2516	2XSA_A
Caenorhabditis elegans	gi 121962023
Tetraodon nigroviridis	gi 82254121
Anopheles gambiae str. PEST	gi 74805241
Janibacter sp. HTCC2649	gi 84497958

InterPro protein sequence analysis & classification demonstrated that *D. melanogaster Oga* protein sequence has a conserved N-terminal domain belonging to the glycoside hydrolase superfamily and two C-terminal acyl-CoA N-acyltransferase domains.

http://www.ebi.ac.uk/interpro/sequencesearch/iprscan5-R20140311-015904-0307-69194198-oy



Appendix F.

Oga transcript levels of *Oga*^{415}/ *Df(3R)ED10845* flies relative to wild-type

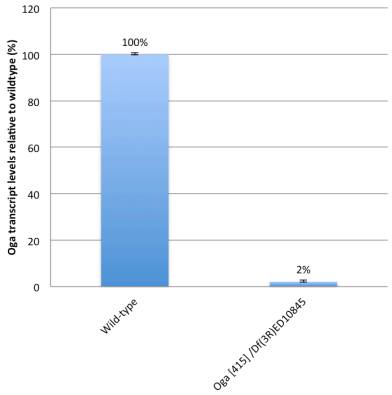


Figure AF1. **qPCR data with three reference genes shows *Oga* transcript to be reduced to 2% of wild-type expression in *Oga*^{415}/ *Df(3R)ED10845* flies.**