

Regulation of Adiposity in *Drosophila*

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Personal Declaration

The work in this thesis is entirely my own, except where clearly specified by acknowledgement or reference. All work was carried out in the Redox Metabolism group, led by Helena Cochemé, at the MRC London Institute of Medical Sciences. Any collaborations or contributions by others are clearly acknowledged and/or referenced accordingly throughout the text. I was able to complete the work in this thesis thanks to funding granted by the Medical Research Council.

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Abstract

Obesity is a complex pathophysiology that is influenced by both cellular and systemic processes, highlighting the importance of *in vivo* experimental models to study the regulation of adiposity and obesity-associated diseases. Using the genetic tractability of *Drosophila*, I explore the role of the metabolite asymmetric dimethylarginine (ADMA) in the regulation of adiposity. ADMA levels were experimentally increased *in vivo* by genetically reducing its metabolic clearance by knocking-out dimethylarginine dimethylaminohydrolase (*DDAH*). *DDAH* knock-out animals (*DDAH^{XP}*) display a range of metabolic phenotypes including a reduction in fat storage, increased mitochondrial content, elevated levels of respiration and altered sleep. Phenotypic differences between *DDAH^{XP}* and an independently generated *DDAH* knock-out allele led me to identify an uncharacterised neighbouring gene as a novel putative regulator of adiposity. Knockdown of this gene was associated with downregulation of the aminotransferase *dAGXT*. In humans, AGXT deficiency is associated with primary hyperoxaluria type 1 (PH1), an autosomal recessive disorder of glyoxylate metabolism. Despite its relevance to human disease, AGXT is almost completely unstudied in *Drosophila*. I found that knockdown of *dAGXT* resulted in the formation of renal stones, recapitulating the major pathological feature of PH1. I identified previously uncharacterised short *dAGXT* transcripts that lack the sequence encoding its putative N-terminal mitochondrial targeting signal. I suggest that differential transcript choice, and hence organelle targeting, could underlie an important regulatory mechanism of metabolic activity. Of relevance, the most common mutations associated with PH1 cause mitochondrial mistargeting. Finally, I develop a model of obesity-associated nephrolithiasis and explore the underlying aetiology. Overall, work here highlights the usefulness of *Drosophila* as a model to study adiposity and obesity-associated disease.

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List of Abbreviations

ACC	acetyl-CoA-carboxylase
ADMA	asymmetric dimethylarginine
AGEs	advanced glycation end-products
AGPAT	acylglycerol-3-phosphate acyltransferase
AGXT	alanine-glyoxylate aminotransferase
Akh	adipokinetic hormone
AkhR	Akh receptor
<i>attB</i>	bacterial attachment site
<i>attP</i>	phage attachment site
BCA	bicinchoninic acid
BDSC	Bloomington <i>Drosophila</i> Stock Center
bHLH-Zip	basic helix-loop-helix leucine zipper

Bmm	brummer
CAFE	capillary feeder
cAMP	cyclic adenosine monophosphate
CaOx	calcium oxalate
CB	central brain
CC	corpora cardiaca
Clk	clock
COVID-19	coronavirus disease 2019
cyc	cycle
DAG	diacylglyceride / diacylglycerol
dAGXT	<i>Drosophila</i> AGXT
DAMs	<i>Drosophila</i> Activity Monitors
DDAH	dimethylarginine dimethylaminohydrolase
DGAT	diacylglycerol acyltransferase
Dilp	<i>Drosophila</i> insulin-like peptide
DIOPT	DRSC Integrative Ortholog Prediction Tool
DMGV	α -keto- δ -(N, N-dimethylguanidino) valeric acid
dNOS	<i>Drosophila</i> NOS
ER	endoplasmic reticulum
ESRD	end-stage renal disease
FA	fatty acid
FAS	fatty acid synthase
FFA	free fatty acid
FLP	flippase
FlyPAD	fly proboscis and activity detector
FOXO	forkhead box subgroup O
G3P	glycerol-3-phosphate
GO	gene ontology
GOF	gain-of-function
GPAT	glycerol-3-phosphate acyltransferases
GPS2	gene-specific reverse primer
HFD	high fat diet
HKA	4-hydroxy-2-ketoglutarate aldolase
HPLC	high performance liquid chromatography
HSD	high sugar diet
InR	insulin receptor
IP3	inositol-1,4,5-trisphosphate
IPC	insulin producing cells
IR	infrared
ITP-R83A	inositol 1,4,5,-trisphosphate receptor
JH	juvenile hormone
KO	knock-out
L-NMMA	NG-monomethyl-L-arginine

LB	lysogeny broth
LC-MS/MS	liquid chromatography-mass spectrometry / mass spectrometry
LD	lipid droplet
LOF	loss-of-function
<i>loxP</i>	locus of X-over P1
LPA	lysophosphatidic acid
<i>mdy</i>	<i>midway</i>
MIGA2	mitoguardin 2
<i>mino</i>	<i>Minotaur</i>
mRNA	messenger RNA
mtDNA-helicase	mitochondrial DNA helicase
MTS	mitochondrial targeting sequence
NMD	nonsense-mediated mRNA decay
NO	nitric oxide
<i>nos</i>	<i>nanos</i>
NOS	nitric oxide synthase
ORF	open reading frame
PA	phosphatidic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
per	period
PH1	primary hyperoxaluria type 1
PI3K	phosphatidylinositol-3-phosphate kinase
Pi3k92E	phosphatidylinositol 3-kinase 92E
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PLP	pyridoxal 5'-phosphate
PRP38	Pre-mRNA-splicing factor 38
PTS1	peroxisomal targeting sequence
qPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNAi	RNA interference
RT	room temperature
s/l-LNvs	small and large ventral lateral neuron
S1P	site-1 protease
S2P	site-2 protease
SAGE	serial analysis of gene expression
SCAP	SREBP cleavage-activating protein
SOCE	store-operated calcium entry
SPAT	serine-pyruvate aminotransferase
SREBP	sterol regulatory element binding protein
SYA	sugar-yeast-agar

T2D	type-2 diabetes
TAG	triacylglyceride / triacylglycerol
<i>tim</i>	<i>timeless</i>
<i>tio</i>	<i>tiptop</i>
TS	template switching
TSS	transcription start site
UAS	upstream activating sequence
VDRC	Vienna <i>Drosophila</i> Resource Center
VNC	ventral nerve cord
<i>w^{Dah}</i>	<i>white Dahomey</i>
WT	wild-type

Chapter 1. Introduction

1.1. Obesity: a global epidemic

The obesity epidemic is now recognised as one of the most important public health problems facing the world today. The incidence of obesity nearly tripled between 1975 and 2016, affecting ~650 million people worldwide by the later date. Current projections suggest this number could rise to over 1 billion by 2025 (WHO, 2020). The rapidly increasing incidence of obesity has fuelled a growing interest in the study of the energy homeostasis and its dysregulation in obesity. Superficially, the cause of obesity can be reduced down to simple thermodynamics – a consequence of sustained elevated energy intake relative to energy expenditure. However, this explanation trivialises the complexity of energy homeostasis and the pathophysiology of obesity. In reality, the aetiology of obesity can be viewed as a product of genetic and environmental interactions, influencing countless physiological and metabolic processes that dictate energy intake, usage and storage.

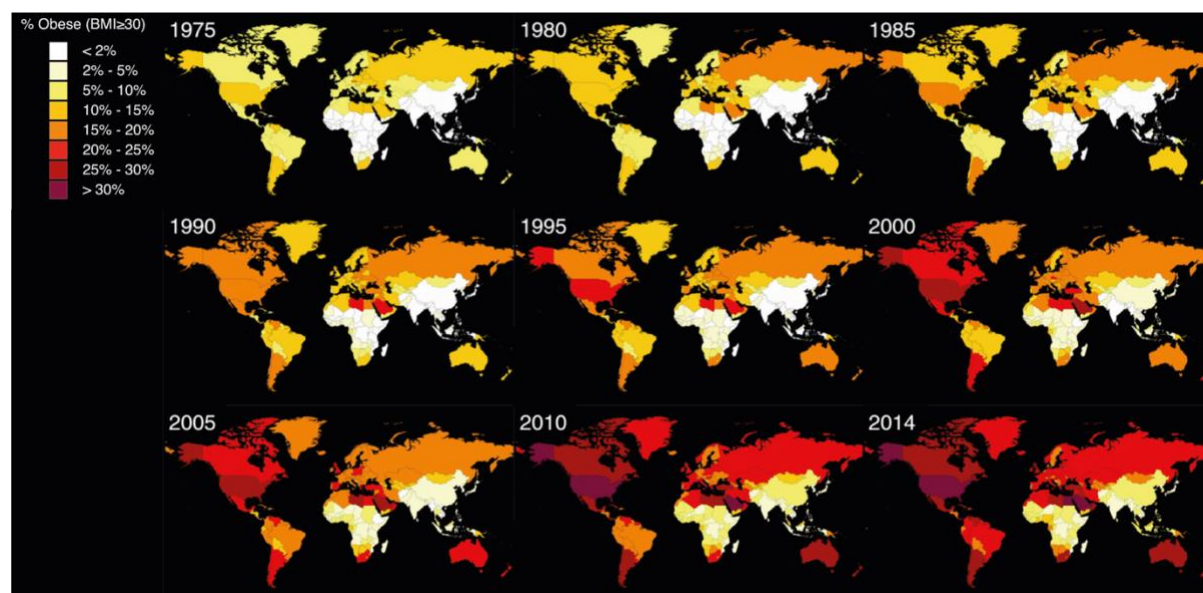


Figure 1.1 The incidence of obesity is increasing globally

Adapted from *metrocosm.com*. The original data were produced by Di Cesare et al., 2016.

Obesity is associated with an extensive list of health complications including metabolic disorders such as type-2 diabetes (T2D) and hyperlipidemia (both of which are associated with an increased risk of cardiovascular disease), nephropathy, neuropathy, and many types of cancers (Sharma et al., 2019). Increased susceptibility to infection is also associated with obesity and is of particular relevance given the current pandemic of coronavirus disease 2019 (COVID-19), with evidence suggesting obesity may be linked with severe COVID-19 outcomes (Huang et al., 2020).

Many of the metabolic complications associated with obesity can be attributed to ectopic lipid storage and the accumulation of harmful lipid species. As adipocytes exceed their storage capacity, fatty acids and cytokines are released into the circulation leading to local inflammation and cellular pathologies including mitochondrial dysfunction and endoplasmic reticulum (ER) stress (Kleinert et al., 2018). Thus, obesity is a complex pathophysiology that is influenced by cellular and systemic processes. This highlights the importance of experimental models to study energy homeostasis *in vivo*. Improved knowledge of the regulation of adiposity could contribute to a better understanding of metabolic disorders and obesity.

1.2. *Drosophila* as a model to study metabolism

The fruit fly *Drosophila melanogaster* has been used in biomedical science research since the early 1900s and remains one of the most commonly used model organisms (Tolwinski, 2017). The early years of *Drosophila* research contributed to major advances in the field of genetics. Notably, Thomas Hunt Morgan uncovered the role of chromosomes in heredity (Morgan & Bridges, 1916), earning him the first Nobel Prize for *Drosophila* research. Later, work using the *Drosophila* model provided insight into genetic control of embryo development (Nusslein-Volhard & Wieschaus, 1980), ultimately driving exceptional progress in developmental biology. Since then, *Drosophila* research has contributed to major discoveries in diverse fields and more recently, has become an attractive model for the study of metabolism and obesity (Heier & Kühnlein, 2018; Musselman & Kühnlein, 2018; Owusu-Ansah & Perrimon, 2014; Smith et al., 2014; Tennessen et al., 2014).

A simple yet fundamental reason why *Drosophila* is such a valuable model is its short lifecycle, ~10 days from embryo to adult (Fig. 1.2). This allows multiple generation crossing schemes and complex genetic manipulations to be carried out, which would not be feasible in mammalian models. The lifespan of a fly is short: ~3 months. For this reason, *Drosophila* is also an attractive model for studying ageing and survival, and is widely used in the ageing field (Piper & Partridge, 2018). Flies are also inexpensive to maintain and can be used in large numbers. Large-scale unbiased forward genetic screens have frequently been used by *Drosophila* researchers, in part for this reason.

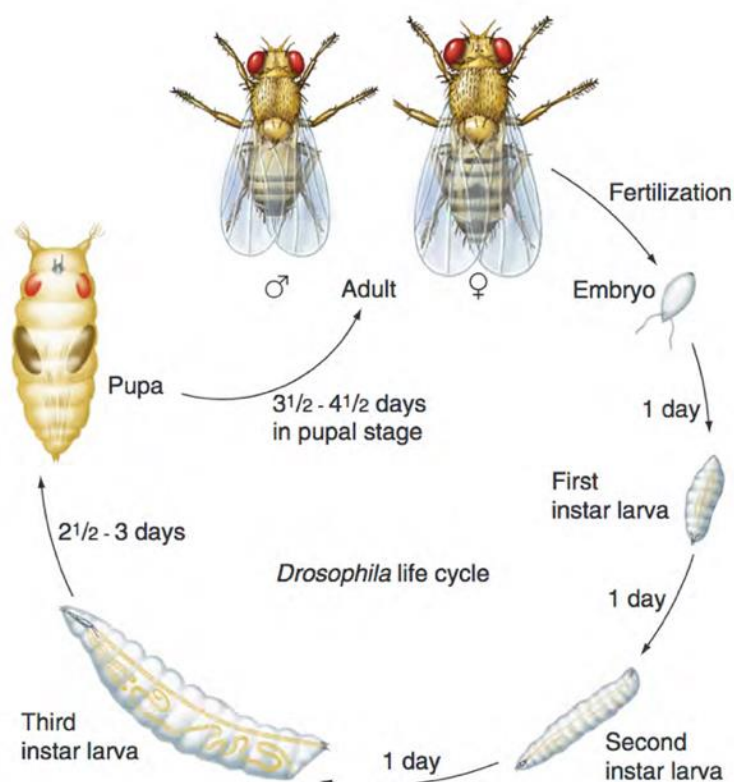


Figure 1.2 Schematic representation of the *Drosophila* life cycle

The *Drosophila* life cycle consists of four main stages: embryo, larva, pupa and adult. The larval stage is comprised of three sub-stages: the first instar (L1), second instar (L2) and third instars (L3). Development is temperature-dependent, and is faster at higher temperatures and slower at lower temperatures. All times shown are relevant to animals raised at 25°C. Schematic from Hartwell et al., 2011.

1.2.1. Evolutionary conservation between flies and mammals – from genes to metabolic pathways

*Am not I
A fly like thee?
Or art not thou
A man like me?*

William Blake, *The Fly* (1794)

William Blake mused about the similarities between humans and flies 200 years ago and he was not wrong. Humans and *Drosophila* are more similar than they may seem. In fact, up to 75% of human disease-related genes have functional homologues in *Drosophila* (Reiter et al., 2001). Beyond conservation at the genetic level, signalling and metabolic pathways are highly conserved. Master transcriptional regulators of lipid metabolism (e.g., SREBP), nutrient sensing pathways (e.g., PI3K/AKT/TOR pathway) and enzymes required for lipogenesis and triacylglycerol (TAG) mobilisation are all highly conserved, as discussed in detail below (Section 1.3).

1.2.2. *Drosophila* organs and their conserved functions in energy homeostasis

Many of the underlying processes governing energy homeostasis as well as the relevant organs and their functions are conserved between humans and *Drosophila* (Fig. 1.3). Nutrients are digested and absorbed in the gut before entering the hemolymph (Lemaitre & Miguel-Aliaga, 2013), the equivalent of mammalian blood. In contrast to mammals, *Drosophila* have an open circulation system with their organs bathed in hemolymph. The heart (dorsal vessel) stimulates circulation of the hemolymph, allowing nutrients to be absorbed and waste products to be excreted. *Drosophila* lack a pancreas; instead, two distinct subsets of neuroendocrine cells produce *Drosophila* insulin-like peptides (Dilps) and glucagon-like peptide adipokinetic hormone (Akh), which promote energy storage or mobilisation respectively. The fat body is the primary lipid storage organ and responds to these hormones to regulate fat storage and lipolysis. Together with the oenocytes (insect cells involved in lipid processing and detoxification), the fat body functions as the liver (Musselman & Kühnlein, 2018). The renal system of *Drosophila* includes two discrete organs anatomically and functionally: the Malpighian tubules and nephrocytes. Together, they

carry out the functions analogous to those of the mammalian kidney and are essential for filtration and excretion of waste products.

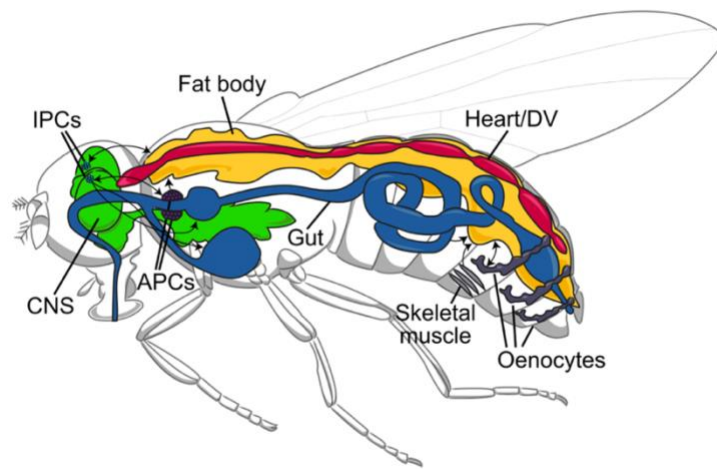


Figure 1.3 A schematic representation of *Drosophila* organs relevant to energy homeostasis

Depiction of adult organ architecture relevant to energy homeostasis. Adapted from Musselman & Kühnlein, 2018.

1.2.3. Genetic tools

Over the years, the fly community has generated a wealth of genetic tools and resources facilitating otherwise difficult and complex genetic manipulations, further enhancing the *Drosophila* model system.

1.2.3.1. GAL4-UAS expression system

The GAL4-UAS system allows ectopic gene expression with spatial control. Originally derived from *Saccharomyces cerevisiae*, the GAL4 transcriptional activator is expressed under the control of a specific promoter resulting in a defined expression pattern (Brand & Perrimon, 1993). Once expressed, the GAL4 protein binds to its cognate upstream activating sequence (UAS) in a separate transgene designed to activate either: a cDNA for overexpression, a microRNA/small interfering RNA for gene silencing, or a reporter (e.g., GFP). The original GAL4 lines were obtained by random insertion of a *P*-element containing a minimal promoter upstream of GAL4 coding DNA. GAL4 expression is then driven by local endogenous enhancers determined by the site of insertion (D'Avino & Thummel, 1999). Such lines were appropriately named enhancer traps. GAL4 enhancer traps have been incredibly valuable tools and make up a large proportion of the GAL4 lines used to this day. That

said, they do have their limitations. Random integration runs the risk of insertional mutagenesis, which, in the case of enhancer traps, can sometimes result in adverse effects specifically in the tissue of interest. These issues can be avoided by expressing GAL4 from a transgene controlled by a cloned, characterised promoter and inserted elsewhere in the genome. Recently, it has become possible to use precise targeted approaches to insert the GAL4 coding sequence downstream of endogenous promoters. This can be achieved using CRISPR cas9-triggered homologous recombination with GAL4 reintegration vectors, as described in Chapter 4. These lines use endogenous promoters in their native genomic environments giving them the most authentic expression patterns, although like enhancer traps, they usually affect the activity of the modified gene.

Initially, UAS transgenes were inserted randomly in the genome by *P*-element-mediated transformation (see Section 1.2.3.3). Nowadays however, UAS constructs are typically inserted in the genome by site-specific integration at so-called landing sites (see Section 1.2.3.4 and Chapter 4).

The impact of the GAL4-UAS system has been further increased by means of inducible (temporal) control (McGuire et al., 2004). One approach is to include a transgene expressing GAL80ts (a temperature sensitive inhibitor of GAL4) so that GAL4 can be activated by changing the temperature. Alternatively, the GeneSwitch system makes use of progesterone-dependent GAL4 to enable spatial and temporal control of UAS transgenes. The GAL4 UAS system is not the only means of ectopic expression. Another method makes use of transgenes that are expressed upon activation of the site-specific recombinase flippase (FLP), which removes an FRT-flanked stop cassette. This will not be described further as it was not used for this thesis.

1.2.3.2. RNAi libraries

RNA interference (RNAi), originally described in *Caenorhabditis elegans* (Fire et al., 1998), is an endogenous cellular mechanism that degrades messenger RNA (mRNA) transcripts in response to expression of complementary RNA species and the formation of double-stranded RNA fragments. RNAi can be used in conjunction with the GAL4/UAS system for tissue-specific and or inducible gene silencing. Over the past 20 years, extensive UAS-RNAi libraries (VDRC, TRiP and NIG) have been

generated, collectively covering more than 90% of coding genes in the *Drosophila* genome. The GD library, distributed by the Vienna *Drosophila* Resource Centre (VDRC), was one of the first *in vivo* RNAi libraries generated and remains the largest (in terms of the number of genes targeted) to date. This library is based on constructs containing gene-specific inverted repeats downstream of a UAS sequence, introduced randomly into the genome by *P*-element-mediated transformation. Random integrations resulted in variable knockdown efficiencies and a risk of insertional mutagenesis. More recently, RNAi constructs have been introduced into the genome by site-specific PhiC31-mediated integration into well characterised *attP* landing sites, avoiding expression variation. The KK library from VDRC and the TRiP collection from Harvard Medical School (distributed by the Bloomington *Drosophila* Stock Centre; BDSC) were both generated using this method (Heigwer et al., 2017).

The generation and efficient distribution of these resources is a great example of collaboration in the fly community. RNAi libraries facilitated large scale forward genetic screens which have provided a wealth of knowledge about the function of otherwise unknown genes across many fields including metabolism. As powerful as these tools are, they do have limitations. Even with the use of landing sites for the integration of RNAi constructs, knockdown can still be inefficient. This may not be a problem if incomplete knockdown is sufficient to cause a phenotype. However, for some genes, residual activity (e.g., 10%) suffices for function and a phenotype could be missed if one is solely reliant on RNAi. Furthermore, a significant number of KK lines (~25%) were found to have a second unintended insertion site in the 5' UTR of transcription factor *tiptop* (*tio*), resulting in ectopic UAS-driven expression of *tio*, which specifically modulates the Hippo pathway when used with certain GAL4 drivers (e.g., ubiquitous drivers) (Green et al., 2014; Vissers et al., 2016). Off-target effects from dsRNA hairpins targeting multiple genes can also be a problem. For these reasons multiple independent RNAi lines should ideally be used to ensure phenotypic consistency. Taken together, genetic manipulations by RNAi have their advantages and disadvantages, and will be more appropriate for some studies whereas true knock-outs (KOs) may be preferable for others.

1.2.3.3. *P*-element-mediated genetic manipulations

P-elements have played a huge role to the history of *Drosophila* genetics and are still widely used to this day. *P*-elements are based on transposable elements, DNA sequences that can change position or “jump” within the genome. As *P*-elements can insert randomly into the genome, they have been used to generate mutants, either by the insertion itself or following the imprecise excision of the element (Cooley et al., 1988; Hartwell et al., 2011). *P*-elements have also been engineered to introduce exogenous DNA sequences into the genome; the basis of the enhancer trap, which was used to generate most of the GAL4 driver lines still used to this day (Section 1.2.3.1). Random integration has also been taken advantage of to screen for phenotypes caused by ectopic expression. Here, the transgenes are constructed with a UAS-dependent promoter facing the flanking genomic sequences, so that the expression of nearby genes can be activated by GAL4. One such insertion was used during this thesis (Chapter 5).

1.2.3.4. Site-specific integration: phiC31-based integration system

While *P*-elements have been extremely useful for studies of gene function, random integration has its limitations. The PhiC31-based integration system enables site-specific integration into *attP* landing sites (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Venken & Bellen, 2007). The bacteriophage PhiC31 integrase mediates sequence-directed recombination between a bacterial attachment site (*attB*) and a phage attachment site (*attP*). As a result, *attB*-containing constructs are integrated in the genome at the *attP* site in the presence of the PhiC31 integrase leaving small *attR1* and *attL1* “scars” flanking the insert. PhiC31 integrase can be expressed under the control of the germline driver *nanos-GAL4* (*nos-GAL4*) or co-injected with the *attB* containing vector (see Chapter 4). *attP* sites were originally introduced randomly into the genome using *P*-elements before being characterised. Recently, CRISPR cas9-triggered homologous recombination has enabled targeted insertions of *attP* sites. This method can be used with ‘ends-out’ homologous recombination to replace specific genomic regions with *attP* sites. This strategy becomes increasingly powerful when used in conjunction with reintegration vectors that allow rapid subsequent genome modifications – where genomic regions can be swapped with specific constructs such as a modified gene or a GAL4 sequence (Baena-Lopez et al., 2013) (described in Chapter 4).

1.2.3.5. Site-specific recombination: Cre-Lox recombination system

Derived from P1 bacteriophage, the Cre-Lox recombination system is used for site-specific recombination of DNA (Sauer, 1987). The Cre recombinase catalyses the recombination of specific *loxP* sites (locus of X-over P1). The *LoxP* sequence is made up of two palindromic recognition regions flanking an asymmetric spacer region giving the *LoxP* site directionality. The orientation of the *LoxP* sites determines how the genetic material is to be rearranged. Deletions are generated if the *LoxP* sites are in the same orientation - the sequence between the two sites is excised as a circular piece of DNA. Other genetic manipulations are also possible: inversions if the *loxP* sites are in opposite orientations on the same strand of DNA or translocations if the sites are on separate DNA molecules. The Cre-Lox system is a useful complement to CRISPR-Cas9-mediated engineering. First, it allows genetic markers (e.g., Pax-Cherry cassette) to be removed after integration. Second, it enables the conversion of the CRISPR-modified allele without the need for re-injections (Baena-Lopez et al., 2013; Poernbacher et al., 2019). Practically, this is achieved by crossing the original allele bearing the *LoxP* cassette to a hsp80-Cre strain. The removal of the *LoxP* cassette is then selected in the progeny. This approach was used to convert a transcriptional reporter line (expressing GFP under the control of an endogenous promoter) to a line expressing an endogenously tagged protein (see Chapter 4).

1.2.3.6. CRISPR cas9-triggered homologous recombination

Although not specific to *Drosophila*, CRISPR cas9-triggered homologous recombination has been crucial for generating mutants and the genetic tools described in this thesis. This technique allows the deletion of genomic sequences and concurrent introduction of exogenous DNA with base-pair precision (described in detail in Chapter 4). In *Drosophila*, this is achieved by co-injecting embryos expressing Cas9 at the posterior end (where the germ cells form) with a cocktail comprising a construct encoding guide RNAs (gRNAs) and a targeting vector for homologous recombination. The gRNAs bring the cas9 endonuclease to the specific PAM sequences, resulting in double strand breaks at the locus. The targeting vector contains the DNA to be inserted flanked by two homology arms. It also typically contains a genetic marker (e.g., *Pax-Cherry*) for the selection of integrants. When the DNA is cut by the endonuclease, the donor template from the targeting vector is used to repair the

double strand breaks, introducing the fragment into the genome (Baena-Lopez et al., 2013; Poernbacher et al., 2019).

1.3. TAG metabolism in *Drosophila*

Triacylglycerol (TAG) metabolism is a central characteristic of energy homeostasis. The fine balance between synthesis and mobilisation of TAG is tightly regulated and is essential for animals to respond to changes in energy intake and demand. Although the focus of this section is TAG metabolism in *Drosophila*, many of the processes and pathways described are evolutionarily conserved. The relevant mammalian orthologues are referred to throughout. Components that are not conserved are also highlighted.

1.3.1. Overview of TAG metabolism

TAGs are fatty acid tri-esters of glycerol; they consist of three fatty acid (FA) chains bound to a glycerol backbone by ester bonds. TAG is the major energy storage molecule in many animals, including humans and *Drosophila*, and is stored in specialised organelles called lipid droplets (LDs) (Martin & Parton, 2006; Thiam et al., 2013). LDs represent a highly efficient form of stored energy. Since TAG is nonpolar, it can be stored in a nearly anhydrous form making it a highly concentrated form of stored energy (far greater than glycogen). FAs are highly reduced compared to carbohydrates; therefore, complete oxidation of FAs provides significantly more energy for a given mass. As well as energy storage, TAG is an important source of lipids used for the building blocks of structural membrane components and also signalling molecules (Berg et al., 2002).

TAG is derived from two sources: dietary lipids and *de novo* lipogenesis. Lipids from both sources are converted to a common currency, diacylglycerol (DAG), a key intermediate of TAG metabolism. DAG (in the form of lipoprotein complexes) is also the major transport form of neutral lipids (lacking charged groups) in the hemolymph (Palm et al., 2012). Dietary TAG is broken down into free FAs (FFAs), glycerol and acylglycerol intermediates by lipases in the lumen of the midgut before being absorbed by enterocytes and converted to DAG. Enterocytes also carry out *de novo* lipogenesis, where acetyl-CoA derived from the catabolism of ingested carbohydrates is converted

into FAs before being incorporated into DAG. DAG can then either be converted to TAG by esterification with excessive FAs for storage in the form of LDs (Carvalho et al., 2012) or exported to the hemolymph as part of specific lipoprotein complexes for lipid transport to other tissues (Palm et al., 2012). The *Drosophila* fat body is the major energy storing tissue and is the main recipient of circulating DAG. As well as importing DAG, adipocytes produce DAG by *de novo* lipogenesis and store TAG in bulk. During periods of starvation, TAG is mobilised and DAG is exported to supply other tissues, where FAs can eventually undergo β -oxidation and enter the tricarboxylic acid (TCA) cycle in the form of acetyl-CoA (Grönke et al., 2005, 2007). Although the fat body is the major site of TAG storage, most other tissues contain autonomous TAG reserves and express the necessary enzymes needed for catabolism of TAG for energy and synthesis of structural and signalling lipids from FAs.

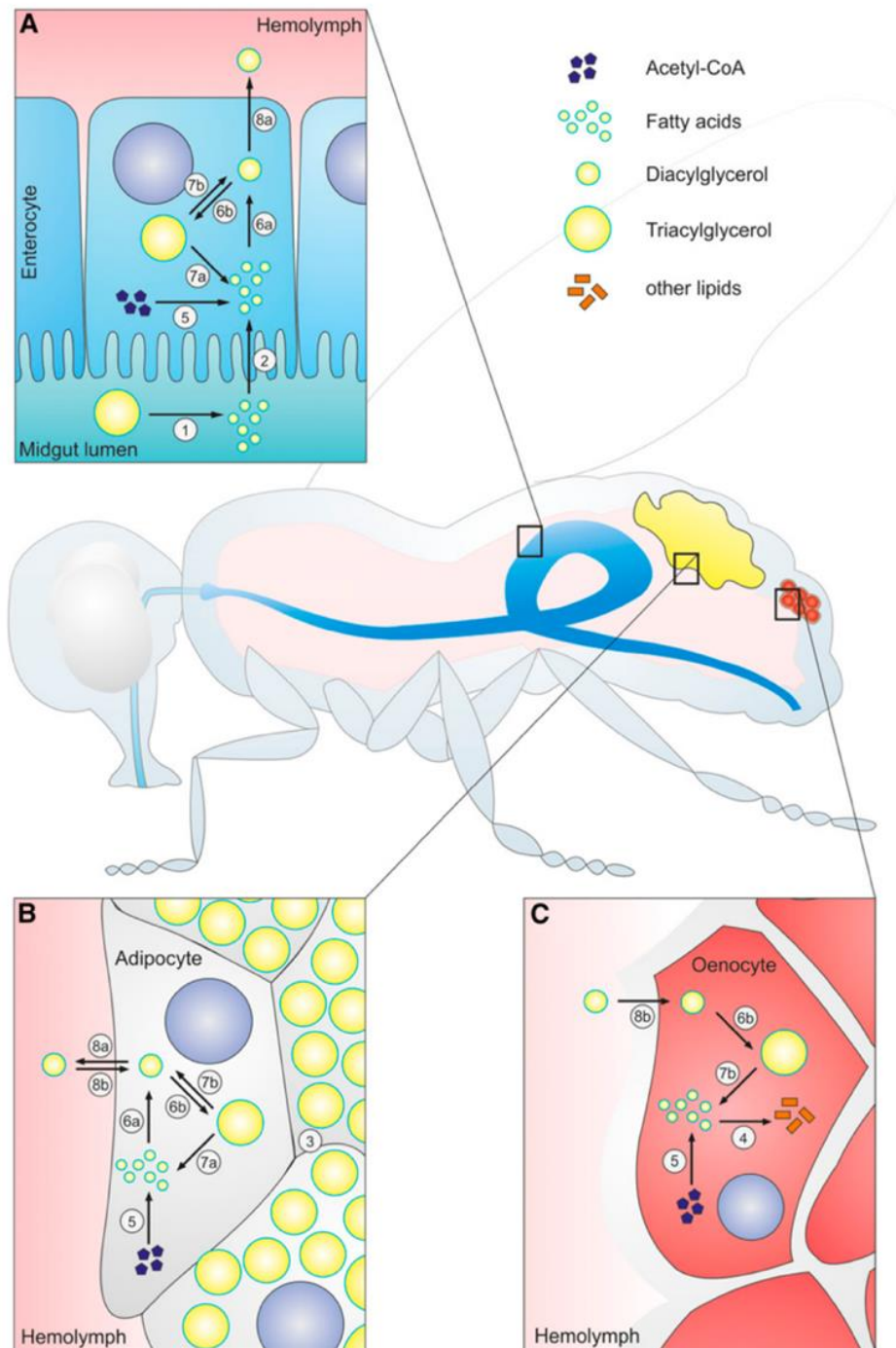


Figure 1.4 A schematic diagram summarising TAG metabolism in *Drosophila*

TAG metabolism in major fly organs of lipid absorption, storage, and utilisation. **(A)** Dietary TAG is broken down into lipolytic products (e.g., FAs) by lipases in the lumen of the midgut before being absorbed by enterocytes. Enterocytes produce additional FAs through *de novo* synthesis from acetyl-CoA. FAs from both sources are converted to DAG. DAG is exported to the hemolymph for lipid transport to other tissues or converted to TAG for storage. **(B)** The fat body is the major energy storing tissue and is the main recipient of circulating DAG. Adipocytes also produce DAG by *de novo* lipogenesis. DAG from either source is converted to TAG for storage. **(C)** Oenocytes are an exemplary site of lipid utilisation. FAs are converted to structural or signalling lipids. Oenocytes also store TAG. Note that TAG metabolism is not restricted to the organs displayed and the schematic serves as a general overview. Adapted from Heier & Kühnlein, 2018.

1.3.2. Endocrine regulation of TAG storage

Endocrine systems integrate information about nutritional state and energy demands at the organism level to coordinate a response at the cellular level. The major hormone pathways that regulate TAG storage in *Drosophila* utilise insulin, adipokinetic hormone (Akh), ecdysone and juvenile hormone (JH). The insulin and Akh pathways are described below.

1.3.2.1. Insulin

The insulin pathway is a central nutrient-sensing pathway controlling growth, ageing and reproduction, as well as being a key regulator of TAG metabolism. The *Drosophila* genome encodes eight insulin-like peptides, Dilps1-8. Dilp1, 2, 3 and 5 are functional homologues of human insulin and are expressed by insulin producing cells (IPCs), a group of neurosecretory cells in the brain (Brogiolo et al., 2001; Delanoue et al., 2016; Rulifson et al., 2002). The secretion of Dilps from IPCs is stimulated by a number of factors including nutrition-induced factors, neurotransmitters, neuropeptides and humoral factors (Ahmad et al., 2020). Dilps serve as ligands for a single insulin receptor (InR) which relays the signal through a highly conserved signal transduction cascade. Activated InR results in activation of Pi3k92E, the *Drosophila* orthologue of phosphatidylinositol-3-phosphate kinase (PI3K) (Leevers et al., 1996; Oldham et al., 2002), and ultimately the phosphorylation and activation of Akt1 (Cho et al., 2001; Verdu et al., 1999). Activated Akt1 phosphorylates a number of targets, including the transcription factor forkhead box subgroup O (FOXO). Akt1 phosphorylation of FOXO promotes cytoplasmic retention (Puig et al., 2003) and curtails expression of targets needed for mobilisation of TAG, including the essential lipase Brummer (bmm). Starvation results in reduced insulin signalling, promoting nuclear translocation of FOXO and increased expression of enzymes involved in TAG mobilisation (Vihervaara & Puig, 2008; B. Wang et al., 2011).

1.3.2.2. Adipokinetic hormone (Akh)

Glucagon is key mammalian hormone that works antagonistically with insulin to regulate the mobilisation of fat and glucose (Freychet, 1990; Ganong, 1991). The glucagon-like peptide Akh is produced by a subset of neurosecretory cells in the corpora cardiaca (Ahmad et al., 2020). Akh serves as a ligand for the G protein-

coupled Akh receptor (AkR), resulting in the activation of two distinct signal transduction pathways. One pathway leads to the activation of protein kinase A (PKA) via the second messenger cyclic adenosine monophosphate (cAMP), which promotes lipolysis by phosphorylation of various targets including transcription factors and LD proteins (Heier & Kühnlein, 2018). The second pathway involves the conversion of phosphatidylinositol 4,5- bisphosphate (PIP2) to inositol-1,4,5-trisphosphate (IP3) by phospholipase C, which in turn promotes Ca^{2+} efflux from the endoplasmic reticulum (ER) to the cytoplasm following activation of the IP3 receptor (ITP-R83A) at the ER. This store-operated calcium entry leads to transcriptional changes that promote TAG mobilisation (Baumbach et al., 2014).

1.3.3. TAG biosynthesis

1.3.3.1. Enzymatic control of TAG biosynthesis

As previously mentioned (Section 1.3.1), TAG is derived from two sources: dietary lipids and *de novo* lipogenesis. During *de novo* lipogenesis, acetyl-coenzyme A-carboxylase (ACC) catalyses the conversion of acetyl-CoA derived from the catabolism of carbohydrates and proteins to malonyl-CoA. Fatty acid synthase (FAS) catalyses the condensation of malonyl-CoA with acetyl-CoA, generating FAs (Heier & Kühnlein, 2018). FAS is encoded by three distinct *FASN* genes in *Drosophila*, *FASN1-3*, each with different expression patterns (Garrido et al., 2015). Dietary lipids and *de novo* lipogenesis converge at FAs in the synthesis of TAG as they enter the glycerol-3-phosphate (G3P) pathway. FA moieties are added to a G3P backbone by esterification in a number of enzymatic steps. The first esterification step is catalysed by glycerol-3-phosphate acyltransferases (GPATs), generating lysophosphatidic acid (LPA), which is further esterified by acylglycerol-3-phosphate acyltransferases (AGPATs) to form phosphatidic acid (PA). PA is then dephosphorylated to DAG by dLipin (Heier & Kühnlein, 2018; Lehmann, 2018; Ugrankar et al., 2011). Diacylglycerol acyltransferases (DGATs): midway (mdy) and possibly Dgat2, then catalyses the esterification of DAG and fatty acyl-CoA, the final and only committed step in TAG synthesis (Fig.1.5).

To date, many enzymes involved in TAG synthesis in *Drosophila* are yet to be experimentally characterised and/or need further evidence to support their proposed functions. There are three putative *Drosophila* GPATs that are structurally related to

mammalian GPATs, encoded by *Minotaur (mino)*, *CG15450* and *Gpat4*. Although there is some evidence supporting their role in TAG synthesis (Heier & Kühnlein, 2018), further characterisation is required. The *Drosophila* genome encodes a number of enzymes with predicted AGPAT function: *CG3812/Agpat1*, *fu12/Agpat2*, *CG4729/Agpat3*, *CG4753/Agpat4*, however putative molecular function is largely based on sequence homology and gene ontology (FlyBase). More is known about dLipin (Grillet et al., 2016; Schmitt et al., 2015; Ugrankar et al., 2011; Valente et al., 2010). The *Drosophila* genome has a single *dLipin* gene that encodes three unique polypeptides, each thought to occupy different intracellular locations, which may be relevant to their activity. As well as catalysing the dephosphorylation of PA to DAG, dLipin may also regulate lipid metabolism at the transcriptional level. *dLipin* is transcriptionally upregulated under starvation (Ugrankar et al., 2011) - which is rare for anabolic genes - and has been shown to translocate to the nucleus under starvation (Hood et al., 2020). A reduction in TORC1 signalling following RNAi-knockdown of *TOR* or *raptor* also induced nuclear translocation of dLipin (Schmitt et al., 2015). Although transcriptional targets of dLipin are not fully known, mammalian studies suggest nuclear lipin co-regulates the expression of genes involved in FA oxidation via PGC-1 α /PPAR α regulatory pathway, and indirectly represses lipogenic genes via SREBP inhibition (preventing the accumulation of nuclear SREBP) (Finck et al., 2006; Peterson et al., 2011). This dual function allows lipin to respond differently to changes in energy intake or demand. The final step, the conversion of DAG to TAG, is catalysed by DGATs (described above). *Mdy* encodes a protein with structural homology to DGAT1, with loss-of-function (LOF) mutations resulting in a strong reduction in TAG (Baumbach et al., 2014). Therefore, *mdy* has been assigned a principal role in TAG synthesis, although other uncharacterised DGAT orthologues also exist.

1.3.3.2. Transcriptional regulation of TAG biosynthesis

SREBPs are evolutionary conserved master transcriptional regulators of lipogenesis belonging to the membrane bound basic helix-loop-helix leucine zipper (bHLH-Zip) class of transcription factors (Shimano & Sato, 2017). They regulate the expression of *ACC* and *FASN*, essential enzymes in *de novo* lipogenesis, as well as the expression of genes involved in membrane biosynthesis. SREBPs respond to changes in the abundance of membrane lipids; sterols in mammals and phosphatidylethanolamine (PE) in *Drosophila* (*Drosophila* are cholesterol auxotrophs) (Dobrosotskaya et al.,

2002). When membrane lipids are abundant, SREBP is inactive and membrane-bound. When membrane lipids are depleted, SREBP is activated via proteolytic cleavage, allowing nuclear translocation of the active bHLH-Zip domain. Activation of SREBP requires lipid-sensing chaperone SREBP cleavage-activation protein (SCAP), as well as proteolytic cleavage enzymes, site-1 protease (S1P) and S2P – the *Drosophila* genome encodes single orthologues of each.

As well as responding to membrane lipids, SREBP activity is modulated by the PI3K/AKT/TOR pathway; a major nutrient sensing pathway that is activated in nutrient rich states promoting anabolic processes, such as lipogenesis. The PI3K/AKT/TOR pathway regulates SREBP activity by modulating its nuclear accumulation and therefore its transcriptional output. These effects are believed to be mediated by lipin1 in mammals - loss of mTORC1 mediated phosphorylation of lipin1 was found to promote its nuclear entry leading to a reduction in nuclear SREBP protein (Peterson et al., 2011). As previously mentioned, reduced TOR signalling was found to promote nuclear translocation of dLipin in *Drosophila* (Schmitt et al., 2015), suggesting the mechanism may be conserved.

The heterodimeric transcription factor Mondo-Mlx also responds to nutritional cues – specifically dietary sugars – to regulate lipogenesis as well as carbohydrate and amino acid metabolism. Mondo-Mlx positively regulates the transcription factor Sugarbabe to promote expression of lipogenic genes and negatively regulates dietary lipid digestion and lipid catabolism in response to dietary sugar (Mattila et al., 2015).

1.3.3.3. Cytosolic Ca²⁺ affects TAG biosynthesis

Store-operated calcium entry (SOCE) promotes TAG mobilisation and inhibits TAG biosynthesis concurrently (Baumbach et al., 2014). Genetic manipulations that increase cytoplasmic Ca²⁺ (knockdown of the calcium pump SERCA) decreases the expression of *mdy* (a DGAT which has a key role in TAG synthesis) while promoting TAG mobilisation (Bi et al., 2014). Conversely, *mdy* is upregulated when cytoplasmic Ca²⁺ is decreased in adipocytes by knockdown of IP3 receptor (ITP-R83A) or calcium sensor *Stim* (Baumbach et al., 2014).

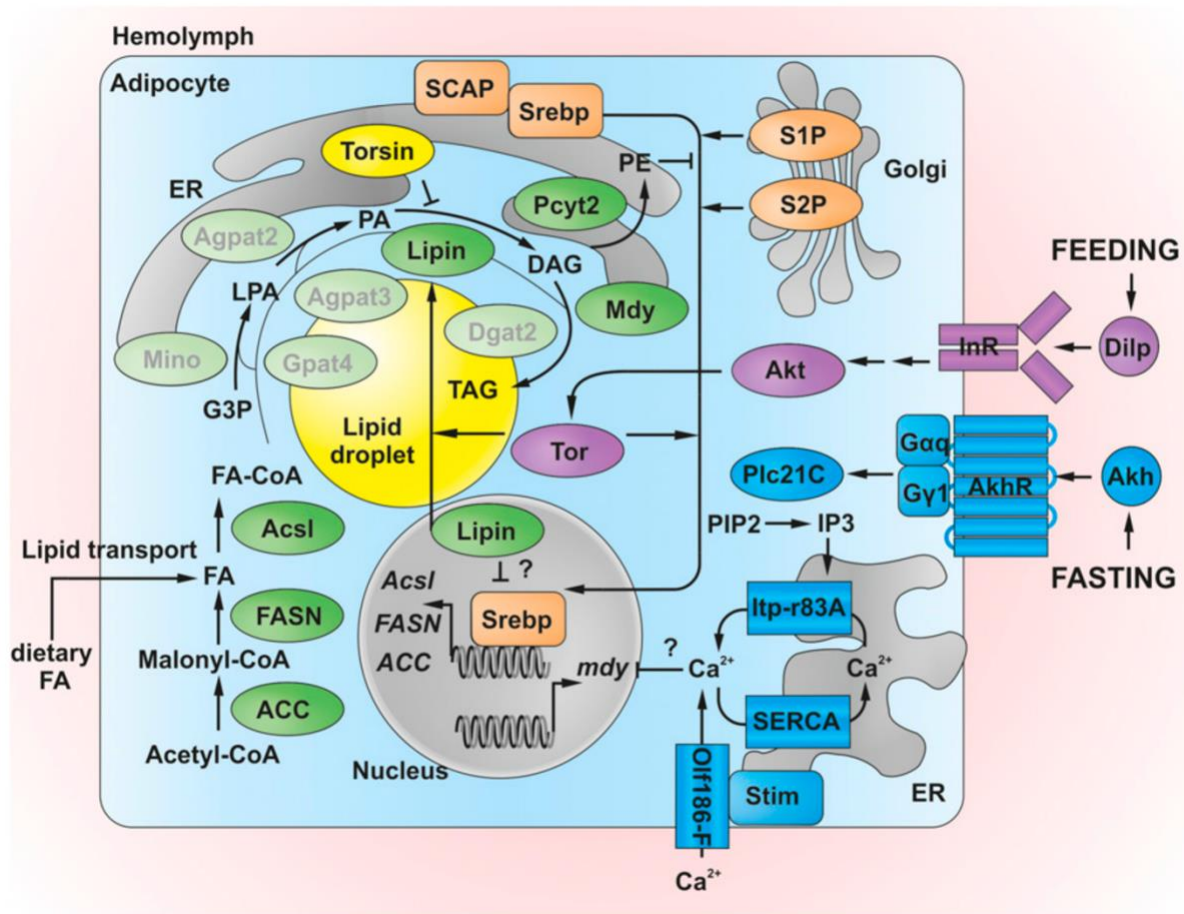


Figure 1.5 A schematic diagram summarising TAG synthesis in *Drosophila*

A summary of TAG synthesis in *Drosophila* including biochemical and regulatory pathways. Adapted from Heier & Kühnlein, 2018.

1.3.4. LD biosynthesis

LDs are specialised organelles used to efficiently store TAG in bulk. LDs consist of a hydrophobic core containing neutral lipids, mainly TAG, surrounded by a phospholipid monolayer. LDs contain membrane proteins involved a range of functions including lipid metabolism and make physical contact with other organelles (e.g., mitochondria and ER). LD formation is incompletely understood, however, a number of different models have been proposed (Choudhary et al., 2015; Ploegh, 2007; Robenek et al., 2004). The most widely accepted model describes the formation of an oil lens in the ER membrane and budding into the cytosol (Walther et al., 2017) (Fig. 1.6).

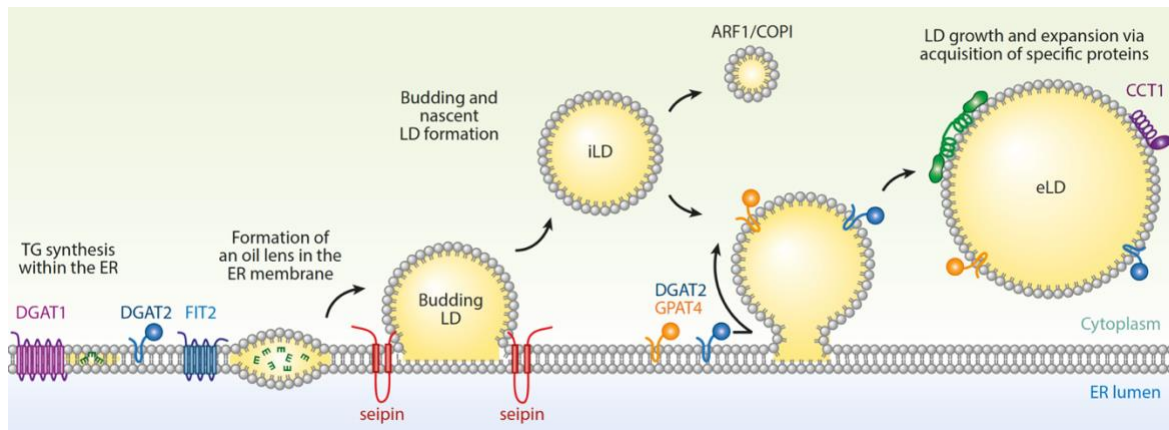


Figure 1.6 A schematic diagram illustrating the formation of LDs

An oil lens forms in the ER membrane before budding into the cytosol. Adapted from Walther et al., 2017.

1.4. *Drosophila* models of obesity

In the early 1960s, Winifred Doane hypothesised that repeated periods of famine could drive selection for mutations promoting efficient fat storage. This theory led to her discovery of *adipose*; the first obese *Drosophila* mutant, screened from a Nigerian wild population (Doane, 1960a, 1960b, 1961). This marked the beginning of obesity research using *Drosophila* as a model system (Musselman & Kühnlein, 2018). *Adipose* was not only the first obese mutant identified in *Drosophila*, but was later shown to be evolutionarily conserved and to regulate adiposity in mice (Suh et al., 2007). An allelic variant of the human *adipose* orthologue *WDTC1* was shown to strongly correlate with obesity (Lai et al., 2009), illustrating the value of *Drosophila* as a model for the study of adiposity.

1.4.1. Genetic studies of obesity

Since the discovery of *adipose*, substantial progress has been made in the field. Large scale unbiased forward genetic screens are a hallmark of *Drosophila* research; an approach that has contributed to our understanding of key metabolic pathways regulating adiposity and resulting in the identification of novel regulators of the insulin signalling pathway (Fuss et al., 2006; Wittwer et al., 2005). Other screens have also contributed to our understanding of adiposity regulation (Baumbach et al., 2014; Lee et al., 2014; Mosher et al., 2015; Pospisilik et al., 2010; Reis et al., 2010).

1.4.2. Diet-induced models of obesity

The fundamental cause of obesity is an energy imbalance between calories consumed and the calories expended (WHO, 2020). As with humans, high calorie diets, including diets high in sugar and fat, cause obesity and the associated pathophysiologies in *Drosophila*. As such, high fat (HFD) and high sugar diets (HSD) are considered models of diet-induced obesity in *Drosophila* (Hirabayashi, 2016; Musselman & Kühnlein, 2018).

As well as inducing obesity, high calorie diets are associated with a number of metabolic defects including insulin resistance and impaired glucose homeostasis (Morris et al., 2012; Musselman et al., 2011) as well as cardiac dysfunction (Birse et al., 2010; Diop et al., 2015; Na et al., 2013), all of which are hallmarks of metabolic syndrome. Renal dysfunction, another obesity associated complication, has also been described in HSD models (Na et al., 2015; van Dam et al., 2020). These studies demonstrate that *Drosophila* are an important translational model that can be used to improve our understanding of the molecular mechanism underlying the pathogenesis of obesity and its associated co-morbidities.

1.5. Thesis aims

Obesity is a complex pathophysiology that is influenced by cellular and systemic processes highlighting the importance of *in vivo* experimental models to study the regulation of adiposity and obesity-associated diseases. As such, the initial aim of this thesis is to use *Drosophila* to explore the role of the metabolite asymmetric dimethylarginine (ADMA) in the regulation of adiposity (Chapter 3). To this end, I generated a range of novel genetic tools to study ADMA metabolism (Chapter 4). From this work, I uncover a novel putative regulator of adiposity, *CG1622* (Chapter 5). Later, I focus on the aminotransferase, *dAGXT*, and explore its role in renal pathology. Finally, I use *Drosophila* to investigate the aetiology of obesity-associated nephrolithiasis (Chapter 6). Overall, work here highlights the usefulness of *Drosophila* as a model to study adiposity and obesity-associated disease.

Chapter 2. Materials and Methods

2.1. Fly husbandry

2.1.1. Fly strains

2.1.1.1. Genetic backgrounds

The inbred isogenic strain w^{1118} , which contains a LOF allele of the *white* gene, was used as the control background for most experiments. The outbred wild-type (WT) strain, *white Dahomey* (w^{Dah}), was used for some experiments. The w^{Dah} strain was derived from the parental *Dahomey* stock, originally collected in Benin in 1970, after incorporating the w^{1118} allele into the genome by backcrossing. Being an outbred strain, w^{Dah} are maintained in large population cages with overlapping generations.

2.1.1.2. Backcrossing

Backcrossing is the introgression of an allele/transgene into a specific genetic background. It is used to control for differences between genetic backgrounds, epistatic interactions between the focal allele/transgene and the genetic background, as well as potential heterosis/hybrid vigour. In this study, fly lines (Table 2.1) were backcrossed into the w^{1118} and/or w^{Dah} backgrounds for a minimum of six generations unless otherwise specified. The allele/transgene was selected at each generation using either a visible genetic marker (e.g. Pax-Cherry) or by PCR. Initially, w^{1118} or w^{Dah} females are crossed to males carrying the allele/transgene to ensure that the mitochondrial genome and other cytoplasmic factors (e.g. *Wolbachia* status) are inherited from the desired background. WT/control males were used for subsequent crosses.

2.1.1.3. Balancer chromosomes

Balancer chromosomes arose from early work on X-ray mutagenesis (Muller, 1928), and the observation that mutated chromosomes underwent little meiotic recombination due to the numerous rearrangements, including inversions, which prevented pairing. This lack of recombination in so-called balancer chromosomes allows the maintenance of mutations on the homologue (i.e., the mutation cannot be 'recombined away'). Indeed, balancer chromosomes are present in most laboratory strains that

harbour mutations or transgenes and were used throughout this thesis. Balancers typically carry a dominant marker which allows the mutant chromosome to be tracked in the progeny of crosses (by the absence of the marker).

2.1.1.4. Fly maintenance

Experimental flies were incubated at 25°C on a 12 h light:12 h dark cycle (9am:9pm) at constant humidity (65%) unless specified otherwise. Fly stocks were maintained longer term at 18°C.

Table 2. 1 Overview of *Drosophila* lines used in this thesis

Full genotype	Abbreviation	Origin
<i>w^{Dah}</i>	<i>w^{Dah}</i>	Broughton et al., 2005
<i>w¹¹¹⁸</i>	<i>w¹¹¹⁸</i>	
<i>w¹¹¹⁸;FB-GAL4</i>	<i>FB-GAL4</i>	
<i>w¹¹¹⁸;cg-GAL4</i>	<i>cg-GAL4</i>	
<i>w¹¹¹⁸::Lpp-GAL4/TM6B</i>	<i>Lpp-GAL4</i>	
<i>w¹¹¹⁸;to-GAL4</i>	<i>to-GAL4</i>	
<i>w¹¹¹⁸::da-GAL4</i>	<i>da-GAL4</i>	Bloomington <i>Drosophila</i> Stock Center
<i>w¹¹¹⁸;act5c-GAL4/CyO</i>	<i>act-GAL4</i>	
<i>w¹¹¹⁸;UAS-eGFP</i>	<i>eGFP</i>	
<i>w¹¹¹⁸FM7c/Lethal</i>	<i>FM7c</i>	
<i>w¹¹¹⁸;CyO/+</i>	<i>CyO</i>	
<i>w¹¹¹⁸::TM6B/+</i>	<i>TM6B</i>	
<i>w¹¹¹⁸::TM3Sb/+</i>	<i>TM3Sb</i>	
<i>w¹¹¹⁸;w⁺Cre^{hs}/CyO;T2ubx,e/T6Hm,e</i>	<i>Cre^{hs}</i>	Cyrille Alexandre, Francis Crick Institute

<i>yw;attP40{nos-Cas9}/cyo</i>	<i>nos-Cas9 (II)</i>	Kondo & Ueda, 2013
<i>y¹ w¹¹¹⁸;attP2{nos-Cas9}/Tm6C,Sb Tb</i>	<i>nos-Cas9 (III)</i>	
<i>y¹ w¹¹¹⁸;attP2{nos-Cas9Strep,paxGFP}/Tm6C,Sb Tb</i>	<i>nos-Cas9-mSA</i>	Poernbacher et al., 2019
<i>w^{Dah} DDAH^{-Δ153}</i>	<i>DDAH^{XP}</i>	Partridge & Leiper labs, (unpublished)
<i>w^{Dah} DDAH^[N-GFP;STOP]</i>	<i>DDAH^[N-GFP;STOP]</i>	This study (Chapter 4)
<i>w^{Dah} DDAH^[N-GFP]</i>	<i>DDAH^[N-GFP]</i>	
<i>w^{Dah} DDAH^{Δe2-5,attP}</i>	<i>DDAH^{Δe2-5}</i>	
<i>w^{Dah} DDAH-GAL4</i>	<i>DDAH-GAL4</i>	
<i>w¹¹¹⁸::;UAS-CG1764^{RNAi GD 20507}/TM3</i>	<i>CG1764^{RNAi GD}</i>	Vienna <i>Drosophila</i> Resource Center
<i>w¹¹¹⁸::;UAS-CG1622^{RNAi GD 38915}/TM3</i>	<i>CG1622^{RNAi GD}</i>	
<i>w¹¹¹⁸;UAS-CG1622^{RNAi KK 100715}</i>	<i>CG1622^{RNAi KK}</i>	
<i>y¹v¹;UAS-CG1622^{RNAi TRIP 60408}</i>	<i>CG1622^{RNAi TRIP 60408}</i>	Bloomington <i>Drosophila</i> Stock Center
<i>y¹sc[*]v¹sev²¹;UAS-CG1622^{RNAi TRIP 55648}</i>	<i>CG1622^{RNAi TRIP 55648}</i>	
<i>w¹¹¹⁸ PBac{WH}CG1764^{f07996}</i>	<i>CG1622^[PBac]</i>	Vienna <i>Drosophila</i> Resource Center
<i>w¹¹¹⁸::;UAS-dAGXT^{RNAi GD}</i>	<i>dAGXT^{RNAi GD}</i>	
<i>w¹¹¹⁸;UAS-dAGXT^{RNAi KK}</i>	<i>dAGXT^{RNAi KK}</i>	
<i>w¹¹¹⁸ AGXT^[N-GFP;STOP]</i>	<i>AGXT^[N-GFP;STOP]</i>	This study (Chapter 6)
<i>w¹¹¹⁸ AGXT^[N-GFP]</i>	<i>AGXT^[N-GFP]</i>	

2.1.2. Fly media

2.1.2.1. Sugar-yeast-agar (SYA)

The standard sugar-yeast-agar (SYA) diet was used for the maintenance of all stocks. Experimental flies were reared on SYA unless stated otherwise. SYA consists of 5% w/v sucrose (granulated sugar, Tate & Lyle), 10% w/v brewer's yeast (#903312, MP

Biomedicals) and 1.5% w/v agar (A7002, Sigma), supplemented with the microbial inhibitors nipagin (H5501, Sigma; 30 mL/L of 10% w/v nipagin in 95% EtOH) and propionic acid (0.3% v/v; P1386, Sigma) once the food reached a temperature below 60°C (Bass et al., 2007).

2.1.2.2. High fat diet (HFD)

Coconut oil is commonly used for HFD in the literature. Although due to its low melting point of 24°C, flies need to be maintained below this temperature to avoid the coconut oil melting (which results in wet media and high censoring as the flies get stuck in the food). This is not ideal for survival and ageing experiments that are generally performed at 25°C. Temperature has a strong effect on lifespan in *Drosophila*, with lower temperatures being associated with significantly longer lifespans. For this reason, a lard-based HFD was used in this thesis, since the melting point of lard is ~40°C. Diet-induced models of obesity are covered in detail in these reviews (Hirabayashi, 2016; Musselman & Kühnlein, 2018).

For the lard-based HFD, standard SYA medium was supplemented with lard (Sainsbury's basic) at a final concentration of 5-15% w/v. Lard was added once the medium had cooled to ~60°C.

2.1.2.3 Starvation media

Starvation media consisted of 1.5% w/v agar.

2.1.2.4 Defined media

Chemically defined media was prepared as described in Piper et al., 2014 and was used for L-arginine supplementation experiments. The concentration of L-arginine present in our standard SYA medium is 8.5 mM. Supplementation experiments included 2-, 6- and 11-fold this concentration.

2.1.3. Experimental animals

2.1.3.1. Generating experimental flies

Parental crosses/stocks were set up in cages with an apple juice agar base (2.3% w/v glucose, 2.3% w/v agar, 25% v/v apple juice and 15 mL/L of 10% w/v nipagin in 95% EtOH) containing live yeast to encourage egg laying. Eggs were collected after a 24 h

laying period using phosphate buffered saline (PBS). A standard density was achieved by transferring ~16 μ L egg suspension (equating to ~250 flies) using a wide-bore 200 μ L tip into 200 mL bottles containing standard SYA. Upon eclosion, flies were transferred to fresh SYA bottles (without anaesthesia) and allowed to mate for 48 h. The flies were then sorted by sex under mild CO₂ anaesthesia and transferred to narrow vials (25 x 95 mm; FlyStuff Genesee Scientific) containing the appropriate fly media for subsequent experimental procedures at a density of 15 females or 20 males per vial.

2.1.3.2. Freezing experimental flies

Experimental flies were frozen 7 days after eclosion except for dietary manipulation experiments, where flies were frozen after either 7 or 14 days of treatment. Freezing of flies was typically carried out between 3-4 pm to minimise potential circadian variation. Live flies were transferred directly from their vials to Eppendorfs on dry ice using a pre-chilled funnel and stored at -80°C until required.

2.2. Metabolic phenotyping

2.2.1. Body wet weight measurement

Frozen flies (Section 2.1.3.2) were sorted on weighing boats on dry ice into groups of 3-5 (with typically n=8 replicates per condition) and weighed on precision scales (Mettler Toledo XS105).

2.2.2. Triacylglyceride (TAG) assay

Frozen experimental flies (Section 2.1.3) were separated into cohorts of 3-5 (depending on the sex and genetic background) and weighed as described in Section 2.2.1. Cohorts of flies (n=8 biological replicates per condition) were then transferred to collection microtube strips (19560, QIAGEN) on ice and a stainless-steel bead (69989, QIAGEN) was added to each tube. 400 μ L of 0.05% (v/v) Tween-20 was added before the racked collection microtubes were placed into the 96-sample adapter (69984, QIAGEN) and homogenised for 2 min at 30 Hz using a QIAGEN TissueLyser II. The rack was rotated 180° after 1 min to ensure equal homogenisation of samples at both ends. Samples were incubated at 70°C using a water bath for 5 min to

inactivate enzymes. The racks were then centrifuged for 2 min at 4,000 rpm at room temperature (RT) before transferring ~250 μ L of the supernatant to new collection tubes. The samples were then centrifuged for 10 min at 4,000 rpm at RT. 20 μ L of the samples were loaded in duplicate into a 96-well plate along with glycerol standards. Each well was overlaid with 200 μ L of Infinity Triglycerides Liquid Stable Reagent (TR22421, Thermo Fisher Scientific) and incubated on a ThermoMixer for 10 min at 37°C at 300 rpm. The absorbance was then measured at 540 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech). A bicinchoninic acid (BCA) protein assay (23225, Thermo Fisher Scientific) (Section 2.7.6) was carried out in parallel. TAG levels were normalised either against total protein or fly wet weight.

2.2.3. Citrate synthase assay

The activity of citrate synthase was determined using a coupled colorimetric assay. In the assay, citrate synthase becomes the rate limiting step in the production of citrate and CoA-SH when oxaloacetate and acetyl-CoA are added to a sample in excess. DTNB (Ellmans reagent) reacts with sulphhydryl groups to yield a coloured product, TNB. The production rate of TNB is followed by measuring the absorbance of the sample at 412 nm and is proportional to the abundance of citrate synthase (Cochémé et al., 2012).

Oxaloacetic acid (10 mg free acid/mL Tris buffer [100 mM Tris-HCl pH 8.0, 0.1% (v/v) Triton-X-100]) and DTNB (4 mg/mL Tris buffer) solutions were prepared fresh on the day and kept on ice. Acetyl-CoA (10 mg/mL Tris buffer) was stored at –20°C for up to 6 months after preparation. A master mix containing 180 μ L Tris buffer, 2 μ L DTNB, 6 μ L acetyl-CoA and 1 μ L oxaloacetic acid per sample was pre-heated to 25°C. The same master mix was also prepared without oxaloacetic (replace with water) to measure the background production of TNB.

Frozen flies were sorted into cohorts of 5 (males) and homogenised as described in Section 2.2.2 (without the 70°C incubation). 10 μ L of sample were loaded into a 96-well plate before adding 189 μ L of master mix to each sample using a multichannel (dispensing needs to be fast as TNB accumulates quickly). The same was repeated for the background samples. Measurements were taken using a kinetic program on a spectrophotometer (FLUOstar Omega, BMG Labtech) with the following parameters:

wavelength 412 nm, positioning delay 0.2 s, 20 cycles, 22 s cycle time. The plate reader was pre-heated to 25°C. The rate of change was determined by linear regression. Background slopes were subtracted from the experimental slopes. This adjusted slope was used for relative comparison. Absolute values can be calculated using either a standard curve or Beer-Lamberts law for absorbance. Linear production of TNB is required for any comparisons.

2.2.4. *In vivo* metabolic rates

In vivo metabolic rates were assessed using metabolic chambers (Sable Systems, Las Vegas, NV, USA). Individual experimental flies (Section 2.1.3) were transferred (without anaesthesia) to metabolic chambers containing a food source (~1 mL SYA dispensed in a loose plastic cuvette cap as a container). The automated multiplexing program was used to successively measure the CO₂ production from 15 chambers (individual flies) and a baseline chamber over a period of 48 h. Data (voltage) were recorded using Sable Systems data acquisition hardware and software. The area under the recorded voltage peaks were calculated for relative comparisons.

2.3. Survival assays

2.3.1. Lifespan assays

Experimental flies (Section 2.1.3) were sorted into vials of the appropriate medium at day 2 and were transferred to fresh vials of food every 2-3 days at which point deaths/censors were scored. Typically, 10 vials (150-200 flies total) were set up per condition.

2.3.2. Starvation assays

Experimental flies (Section 2.1.3) were aged on SYA for 7 days, then transferred to starvation medium (Section 2.1.2.3). Starvation assays were scored ~2-3 times per day as required. Flies were transferred onto fresh starvation medium after ~5 days.

2.4. Behavioural phenotyping

2.4.1. Feeding assays

2.4.1.1. FlyPAD

FlyPAD (fly proboscis and activity detector) allows for the automated quantification of feeding behaviour. When the fly feeds, it completes a circuit leading to changes in capacitance between two electrodes which can be measured (Itskov et al., 2014).

Bottled SYA media was melted briefly using a microwave and poured into a small petri dish ~4 mm high and allowed to set at RT. The plates were then covered with parafilm and placed on ice to prevent drying out while setting up the experiment. To transfer solid SYA media from the plates to the electrodes, a p1000 tip was cut with a razor blade so that the diameter was equal to that of the central electrode. The tip was then used to 'punch' cylinders of food to place on the electrode using a straitened paperclip. This method of placing food on the electrodes was preferable over pipetting molten food directly (original protocol) as it allows a larger volume of food to be dispensed, preventing desiccation and thus allowing longer feeding experiments. Flies were transferred into the arenas without anaesthesia using a mouth aspirator. Feeding behaviour was then recorded undisturbed for up to 2 h at an ambient temperature of ~24-25°C. The capacitance data was collected using the 'FlyPAD library' in Bonsai software and was analysed using a MatLab (Mathworks) package (Itskov et al., 2014). Electrodes were cleaned with 70% EtOH before and after experiments.

2.4.1.2. Capillary Feeder (CAFE) assay

The Capillary Feeder (CAFE) assay allows the continuous quantitation of actual ingestion by measuring liquid food consumed from a graduated glass microcapillary (Ja et al., 2007). Individual experimental flies (Section 2.1.3) were transferred (without anaesthesia) to separate wells of a 24-well cell culture plate. To control for potential temperature or humidity fluctuation between wells, the flies were organised by alternating genotypes and blanks (containing no flies). Each fly was provided access to a 5 μ L glass micropipette (ringcaps #9600105, Hirschmann) containing liquid food (5% sucrose (w/v), and 0.1 mg/mL blue dye). The blue dye is used to aid visualisation. The changes in volumes were recorded at regular time intervals over a period of 5 days. The blank wells were used to estimate evaporative losses (typically ~10% of

ingested volumes) and were subtracted from experimental readings.

2.4.2. Activity and sleep measurements (Ethoscopes)

Ethoscopes (Geissmann et al., 2017) were used for activity and sleep measurements. Ethoscopes track *Drosophila* behaviour in using computerised (Raspberry Pi) video tracking units. The ethoscope behavioural profiling software based on a supervised machine learning algorithm generates information about activity and sleep.

Individual experimental flies (Section 2.1.3) were transferred (without anaesthesia) to separate glass tubes containing a food source (SYA). The tubes were loaded into a behaviour arena that slides and locks inside the lower part of the ethoscope chassis. All arenas feature fixed recognition marks on the corners that are used by the ethoscopes to automatically align and register the regions of interest. Ethoscopes were then placed into incubators maintained at 25°C and on a 12 h light:12 h dark cycle (9am:9pm). Videos were recorded over a period of 7 days and data were analysed offline using the ethoscope software (Python code on a Linux instance) (<https://lab.gilest.ro/ethoscope>). All data analysis was performed in R, using the Rethomics R package (<https://github.com/gilestrolab/rethomics>).

2.5. Immunohistochemistry and dissections

2.5.1. Adult fat body dissection

Live flies were anaesthetised on ice and dissected individually on a silicone plate. Legs and wings were removed using microscissors before securing the fly with a single pin through the thorax (ventral side up) and covering with ice-cold PBS. A small incision was made where the thorax meets the abdomen before gently tearing the ventral cuticle from the midsection down to the posterior end along one side of the fly. This was repeated on the opposite side allowing the intact ventral cuticle to be peeled back revealing the internal organs. Microscissors were then used to cut off the genitalia. At this point other internal organs were carefully removed without disturbing the fat body. Cutting the trachea helped to keep the fat body intact when removing other organs. Excess cuticle on the lateral edges was removed to reduce rounding of the cuticle, leaving the intact abdominal fat body attached to the dorsal cuticle. Dissected

fat bodies were transferred to ice-cold PBS (<1 h) before staining.

2.5.2. Brain and thoracic ganglion dissections

Live flies were prepared and secured onto a silicone plate as described above (Section 2.5.1). A small incision was made where the anterior legs met the thorax before gently tearing the ventral cuticle round to the dorsal cuticle (both sides) exposing the thoracic ganglion. Next, the proboscis was removed, and the head was opened along the anteroposterior axis exposing the brain. Excess cuticle was removed before transferring the brain and thoracic ganglion to ice-cold PBS (<1 h) for subsequent antibody staining.

2.5.3. Malpighian tubule dissections

Malpighian tubules were dissected for the scoring of renal stones. Live flies were anaesthetised on ice and secured to a silicone plate with a single pin through the thorax (ventral side up) and covered with ice-cold PBS. Flies were decapitated before the ventral cuticle of was removed as described above (Section 2.5.1). Malpighian tubules and ampulla were then removed by pulling the posterior tip of the abdomen. Stone deposition was scored based on severity using a scoring system ranging from 0 to 4 (van Dam et al., 2020; Fig. 6.1B).

2.5.4. Nile Red staining

Dissected tissue (Section 2.5.1) was fixed in 4% PFA/PBS for 30 min on ice, incubated with 10 µg/mL Nile Red stain (Thermo Fisher Scientific; N1142) in PBS for 30 min on ice followed by washing and subsequent mounting in Vectashield with DAPI.

2.5.5. Antibody staining

Dissected tissue (Section 2.5.1) was fixed in 4% PFA/PBS for 45 min at RT before washing in PBS, washing in PBS-T (0.5% Triton X-100) for 30 min and blocking in PBS-T/5% normal goat serum (PBTN) for 1 h at RT. Tissue was then incubated with primary antibody in PBTN overnight at 4°C, washed in PBS-T at RT, incubated with secondary antibody in PBTN at RT for 2 h, washed in PBS-T and briefly washed in PBS before mounting in Vectashield with DAPI. Primary antibodies used were goat

anti-GFP (ab5450; Abcam) and rat anti-elav (Rat-Elav-7E8A10 anti-elav; DSHB). Secondary antibodies used were donkey anti-goat FITC (A16006; Thermo Fisher Scientific) and goat donkey anti-rat Cy3 (AP189C; Sigma-Aldrich).

2.6. Microscopy and imaging

Whole animal images were acquired with a Leica M165 FC fluorescent microscope equipped with a Leica DFC 3000G digital camera. Confocal images were taken with a Leica SP5 II confocal microscope and Leica Application Suite Advanced Fluorescence software (Leica Microsystems).

2.6.1. Quantification of GFP intensity

Quantification of GFP intensity was performed using ImageJ software (version 2.0.0-rc-43). Images were converted to 8-bit before a selected region was added to the ROI (region of interest) manager. The area of selected region was consistent between samples. The mean gray value of the selection was calculated as the sum of the gray values of all the pixels in the selection divided by the number of pixels. All images were taken using the same microscope settings. All samples were mounted onto the same microscope slide.

2.7. Molecular biology

2.7.1 Genomic DNA extraction

Individual flies were transferred to 0.5 mL PCR tubes and homogenised in 50 µL of 'squishing' buffer (10 mM Tris-HCl (pH 8.2), 1 mM EDTA, 25 mM NaCl and 200 µg/mL freshly added proteinase K) using the end of a 200 µL pipette tip. The fly homogenates were then incubated at 37°C for 1 h followed by 10 min at 95°C to inactivate the proteinase K. DNA was stored at 4°C.

2.7.2. Primer design

Genotyping and sequencing primers were designed either using Primer3Plus (<https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) or manually. Manually designed primers were verified using PCR Primer Stats

(https://www.bioinformatics.org/sms2/pcr_primer_stats.html). The specificity of primers was checked using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The design of primers to be used for Gibson Assembly is described in Section 4.2.2.2.

2.7.3. PCR

PCRs were performed as directed using Q5[®] High-Fidelity DNA Polymerase (M0492L, New England BioLabs). The initial denaturation time used was 1 min. Annealing temperatures were calculated using the NEB Tm Calculator (<https://tmcalculator.neb.com/#!/main>). A gradient PCR was typically used during optimisation.

2.7.4. DNA gel electrophoresis

1-2% w/v agarose (A9539, Sigma-Aldrich) was dissolved in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; prepared by the LMS core media facility as a 50X stock solution), heated in a microwave and once sufficiently cooled down, 0.002% of ethidium bromide was added. The agarose solution was then poured in a gel-casting frame with a well comb and allowed to set at room temperature. Typically, 5 µL of a Quick-Load 100 bp or 1 kb DNA ladder (N0467S and N0468S, NEB) was loaded in the outermost wells. Gels were then run at 80-100 V for 40-80 min and imaged in a UV transilluminator chamber (Syngene InGenius).

2.7.5. Gel extraction

Gel extractions were performed as directed with the QIAquick Gel Extraction Kit (28704, Qiagen). Bands were excised on a UV transilluminator (Ultraviolet Products Inc).

2.7.6. Sequencing

Sanger sequencing was outsourced to Genewiz (<https://www.genewiz.com/en-GB/Public/Services/Sanger-Sequencing/>). Genomic or plasmid DNA was prepared and diluted as directed.

2.7.7. RNA extraction

RNA was extracted from either whole animals, isolated abdomens or pooled dissected fat body tissue using a TRIzol protocol. Frozen samples (typically n=4-5 flies) were homogenised in 1 mL of TRIzol in sterile 2 mL Ribolyser tubes using a tissue homogeniser (Precellys 24, Bertin Instruments). Samples were incubated for 5 min at RT before 200 µL of chloroform was added and mixed by vortexing for 15 s. After a 2 min incubation at RT, samples were centrifuged for 5 min at ~16,000 rpm using a bench-top centrifuge at 4°C. 500 µL of the aqueous top layer was then transferred to a fresh 1.5 mL tube and an equal volume of isopropanol was added. Samples were mixed by inversion, incubated at RT for 15 min and then centrifuged for 15 min at ~16,000 rpm at 4°C. After removing the supernatant, the pellets were washed a minimum of 3 times with 500 µL of 70% EtOH/DEPC-water. For each wash step, following the addition of 70% EtOH/DEPC-water, samples were centrifuged for 5 min at ~16,000 rpm at RT. Pellets were air dried until they appeared translucent and redissolved in an appropriate volume of RNase-free water. Samples were incubated at 60°C for 10 min to increase solubility. RNA was quantified on a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). Samples were stored at –80°C until required.

2.7.8. cDNA synthesis

First-strand cDNA was synthesised as directed with the iScript cDNA synthesis kit using the iScript Reverse Transcriptase (Bio-Rad; 1708890). Control reactions (minus reverse transcription) were set up by substituting the reverse transcriptase with nuclease free water.

2.7.9. Quantitative RT-PCR

Undiluted cDNA (50 ng/µL) was used to make a standard curve (1:5 dilutions). 2 µL of template cDNA (10-100 ng total) and 8 µL of the probe-master mix (TaqMan Probe (see below; Thermo Fisher Scientific) and TaqMan gene expression master mix (10525395; Thermo Fisher scientific)) were added to each well. The concentration of cDNA varied depending on the copy number of gene of interest. Analysis was carried out on a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific). 2

technical replicates were used. Expression values were normalised to *RpL32* or *alphaTub84B*. The probes used are listed in Appendix 1.2

2.7.10. RT-PCR

Diluted cDNA (Section 2.7.7) was used as a template for PCR using Q5[®] High-Fidelity DNA Polymerase (M0492L, New England BioLabs) as described in Section 2.7.3.

2.7.11. Protein quantification

The Pierce[™] BCA Protein Assay Kit (23225, Thermo Fisher Scientific) was used for protein quantification. 20 µL of samples or BSA standards were loaded in duplicate on a 96-well plate. Each well was then overlaid with a 50:1 mixture of reagents A and B of the BCA kit. Plates were incubated for 30 min at 37°C, before the absorbance was measured at 562 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech).

2.8. Metabolomics

2.8.1. Sample extraction and derivatisation for LC-HRMS analysis

Sample preparations for targeted metabolomics were carried out by A.J. Vincent. Metabolic analysis by LC-HRMS was performed by the LMS Proteomics and Metabolomics core facility. For sample preparation, 5 whole flies were used per biological replicate and placed into a 2 mL Eppendorf before adding 300 µL of 80% methanol (v/v) spiked with L-arginine ¹³C₆¹⁵N₄ (250 ng/mL) as an internal standard. Samples were homogenised at 20 Hz for 3 min using a QIAGEN TissueLyser II and centrifuged (13,000 g, 10 min, 5°C). The chemical derivatisation was carried out as reported by (Fleszar et al., 2018). Briefly, an aliquot of the supernatant (100 µL) was mixed with 50 µL of borate buffer (0.025 M Na₂B₄O₇·10H₂O, 1.77 mM NaOH, pH 9.2) and vortexed at 2,000 rpm for 1 min, 25°C, before adding 400 µL acetonitrile and 10 µL benzyl chloride (10% in acetonitrile). The reaction was incubated at 25°C in a ThermoMixer for 5 min before samples were vortexed and centrifuged (13,000 g, 10 min, 5°C). Samples were finally diluted 5-fold with water prior to injection. Quality controls (QCs) were created by pooling equal aliquots of each study sample and used to assess technical reproducibility across the batch.

2.8.2. Targeted analysis by LC-HRMS

Liquid chromatographic analyses were performed on a Vanquish Flex Binary UHPLC system (Thermo Scientific Inc., MA, USA) coupled to a benchtop hybrid quadrupole-Orbitrap Q Exactive mass spectrometer (Thermo Scientific Inc., Bremen, Germany). Chromatographic separation of hemolymph derivatized extracts was achieved using an Accucore aQ C18 Polar endcapped column (Thermo Scientific, 150 × 2.1 mm, 2.6 µm) equipped with a pre-column (Thermo Scientific, 30 × 2.1 mm, 2.6 µm), both held at a temperature of 40°C and a flow rate of 0.250 mL/min. Mobile phases were water with 0.1% (v/v) of formic acid (solvent A) and methanol with 0.1% (v/v) of formic acid (solvent B). The gradient elution started with 15% of solvent B increasing to 60% over 4 min and 90% of B at 4.5min. This condition was kept constant until 6.5 min and then returning to the initial conditions at 7 min. The column was equilibrated for 3 min, yielding a total run time of 10 min. Ionisation was performed in the positive mode using a heated electrospray ionisation source, with the following parameters: spray voltage 3.0 KV, heater temperature 330°C, capillary temperature 320°C, S-lens RF level 50, sheath and auxiliary gas flow rate, 35 and 10 units, respectively. The mass accuracy was calibrated prior to sample analysis. Mass spectrometric data were acquired at high-resolution (70,000 at m/z 200) in profile mode using a Full MS scan method (m/z 70 to 700). Automatic gain control (AGC) was set to 1e6 and maximum injection time 200 ms. Xcalibur version 4.1 was used for data acquisition and processing. Extracted Ion Chromatograms (EIC) were used in the data processing. Relative quantification is reported based on the peak area ratio of the analyte and the internal standard (L-arginine $^{13}\text{C}_6^{15}\text{N}_4$).

2.9. Cloning and genetics

2.9.1 Generation of guide RNA (gRNA) expression vectors

Suitable target sequences were identified using flyCRISPR target finder (<https://flycrispr.org/>; Gratz et al., 2013), checked for possible off-targets and cloned into the tandem gRNA expression vector pCFD4_U6:1-gRNA-U6:3-gRNA (addgene, #49411). gRNA expression vector cloning is covered in detail in Section 4.2.2.1.

2.9.2. Gibson Assembly

Gibson Assembly (E5510S, New England BioLabs) was used for the assembly of DNA fragments for the generation of gRNA expression vectors (Sections 4.2.2.1, 4.3.2.1 and 6.2.2.2) and targeting vectors (donor template) (Sections 4.2.2.2, 4.3.2.2 and 6.2.2.3). Gibson Assembly is covered in detail in Sections 4.2.2.1 and 4.2.2.2.

2.9.3. PhiC31-mediated integration

PhiC31-mediated integration (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Venken & Bellen, 2007) was used to generate the *DDAH-GAL4* driver line (Section 4.3.2.3) from the parental line, *DDAH^{Δe2-5 attP}* (Section 4.3.2). Further details can be found in Sections 1.3.4. and 4.3.2.3.

2.9.4. Cre-Lox recombination

Cre-Lox recombination was used for the excision of the *LoxP*-flanked *Pax-cherry* cassettes from parental lines *DDAH^[N-GFP;STOP]* (Section 4.2.2.) and *AGXT^[N-GFP;STOP]* (Section 6.2.2.1) generating the N-terminal GFP fusion protein expressing alleles *DDAH^[N-GFP]* (Section 4.2.3) and *AGXT^[N-GFP]* (Section 6.2.2.4) respectively. Cre-Lox recombination was also used for the excision of the *mini-white* and the *Pax-Cherry* cassettes from an intermediate line to generate the *DDAH-GAL4* driver line (Section 4.3.2.3). Cre-Lox recombination was achieved using the following crossing scheme:

F0 = (STOP allele or GAL4 parental allele)^{Cherry+} homozygotes (containing *loxP*-flanked *Pax-cherry* cassette) x y;^{w+}Cre^{hs}/CyO;T2ubx,e/T6Hm,e

F1 = (STOP allele or GAL4 parental allele)^{Cherry+(mosaic)} / y;^{w+}Cre^{hs}/+ (selected mosaic Cherry+ eyes) x w⁻FM7c/w⁺lethal virgin

F2 = N-terminal GFP fusion or *DDAH-GAL4*^{Cherry-} / w⁻FM7c x w⁻FM7c/y

Mosaic *Pax-Cherry* animals were selected for the F1. The mosaic pattern is a result of some somatic cells having undergone Cre-Lox recombination and others not. Cre-Lox recombination takes place in both somatic cells and germline therefore the progeny of the F1 cross consist of *Pax-Cherry* positive animals (no recombination) and *Pax-Cherry* negative animals (recombination has taken place).

2.9.5. Restriction digest

Restriction digests were carried out according to the manufacturer's protocols. Restriction enzymes used are listed below in the order that they first appear throughout this thesis: *BbsI*, *BsaI*, *SapI*, *Acc65*, *SpeI* (New England BioLabs) and *EcoRI*, *SphI* (Promega).

2.9.6. DNA ligation

DNA ligation was carried out as directed using T4 DNA ligase (New England BioLabs).

2.9.7. LB plates

LB (lysogeny broth) agar medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract and 1.5% agar, pH 7) was prepared the LMS core media facility. LB medium was autoclaved before ampicillin was added (once cooled to below 60°C) to a final concentration of 100 µg/mL. The LB agar was poured into Petri dishes and stored upside down at 4°C for a maximum of 6 weeks.

2.9.8. Transformation of competent cells

Both Stellar Competent cells (*E. coli*) (Takara Bio) and 5-alpha competent cells (*E. coli*) (New England BioLabs) were used for the transformation of plasmid DNA. Transformations were carried out as directed by the manufacturer's protocols. Low-density plates consisted of 20-50 µL SOC outgrowth medium following incubation. The remaining SOC medium was pelleted and resuspended in 20-50 µL fresh SOC medium. The resuspension was used for the high-density plate.

2.9.9. Isolation of plasmid DNA

Plasmid DNA was isolated as directed using either the QIAGEN Miniprep kit or QIAGEN Midiprep kit.

2.9.10. Germline microinjections

Injections were carried out by a collaborator, Joachim Kurth at the Francis Crick Institute.

2.9.11. GAL4/UAS System

2.9.11.1. Whole animal genetic manipulations

da-GAL4 flies were crossed with flies of the following genotypes: (1) *CG1622^{RNAi GD}*; (2) *CG1622^{RNAi KK}*; (3) *CG1622RNAi^{TRIP 60408}*; (4) *CG1622RNAi^{TRIP 55648}*. *act-GAL4* flies were crossed with flies of the following genotypes: (1) UAS-eGFP; (2) *dAGXT^{RNAi KK}*

2.9.11.2. Fat body genetic manipulations

FB-GAL4 flies were crossed with flies of the following genotypes: (1) *PBac[CG1622]*; (2) *CG1622^{RNAi GD}*; (3) *dAGXT^{RNAi KK}*; (4) *dAGXT^{RNAi GD}*; (4) UAS-eGFP.

cg-GAL4, *Lpp-GAL4* and *to-GAL4* flies were crossed with flies of the following genotypes: (1) UAS-eGFP; (2) *CG1622^{RNAi GD}*.

2.10. 5' RACE

2.10.1. First strand cDNA synthesis

In 5' RACE (rapid amplification of cDNA ends), SMARTScribe Reverse Transcriptase (Takara Bio) was used for first strand cDNA synthesis as directed from total RNA (Section 2.7.6). Further details can be found in Section 6.2.5.

2.10.2. Amplification of RACE products

The gene specific reverse primer (GPS2) was designed as described in Section 2.7.2. A homology region was included in the primer design to facilitate future cloning. RACE products were amplified from the RACE ready cDNA using the universal primer (SMARTScribe RACE 5' Kit, Takara Bio) and GPS2. Further details can be found in Section 6.2.5.

2.10.3. Characterisation of RACE products

Details on the characterisation of 5' RACE products can be found in Section 6.2.6.

2.11. Statistics and data analysis

Statistical analyses were carried out in GraphPad Prism 9. The distribution of the data

was determined using a number of normality tests (Anderson-Darling, D'Agostino-Pearson omnibus, Shapiro-Wilk, Kolmogorov-Smirnov) in order to choose the most appropriate statistical analysis. When parametrically comparing two groups, an unpaired t-test was used. For the parametric comparison of three or more groups, a one-way ANOVA with multiple comparisons and a Tukey post-hoc correction was used. For non-parametric testing of two groups, a Mann-Whitney test was used, for the comparison of three or more groups, a Kruskal-Wallis test with multiple comparisons and a Dunn's post-hoc correction was used. Calculations and data analysis were generally performed in Microsoft Excel. Data analysis for ethocope experiments were performed in R, using the Rethomics R package (<https://github.com/gilestrolab/rethomics>). For the analysis of relative gene expression from qPCR data, the comparative CT method was used (Schmittgen & Livak, 2008). Survival data (lifespans and starvation assays) were statistically analysed by Log-Rank test in Microsoft Excel. All graphical representations of the data were created in GraphPad Prism 9 with the exception of the ethoscope data, where graphs were generated in R. P values are represented with the following symbols: $P > 0.05$ = ns, $P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***.

Chapter 3. Investigating the role of ADMA in adiposity

3.1. Introduction

3.1.1. ADMA

Asymmetric dimethylarginine (ADMA), a naturally occurring amino acid, is generated through the irreversible post-translational methylation of arginine residues and released after proteolysis (Tran et al., 2003; Vallance & Leiper, 2004). This arginine metabolite was first isolated from human urine in 1970 (Kakimoto & Shigenori, 1970), but the significance of ADMA in disease was not appreciated until the 1990s, when elevated plasma ADMA levels were detected in chronic kidney disease patients (Vallance et al., 1992). It was observed that elevated ADMA was strongly associated with cardiovascular disease in these patients. The authors proposed that ADMA may contribute to the associated elevated risk of cardiovascular disease via inhibition of nitric oxide synthase (NOS), which uses L-arginine as a substrate, since nitric oxide (NO) is a key regulator of vascular tone. It was later confirmed that ADMA is a potent endogenous inhibitor of NOS and therefore, a key regulator of NO signalling. ADMA acts as a competitive inhibitor, competing with L-arginine, the requisite NOS substrate and structural analogue of ADMA. Thus, the ratio of L-arginine to ADMA (and other methylated arginines) determines NOS activity. Elevated plasma ADMA levels is now recognised as a hallmark of endothelial dysfunction, and as such, a strong independent risk factor for cardiovascular diseases (Cooke, 2005; L. Dowsett et al., 2020; Tran et al., 2003; Vallance & Leiper, 2004).

3.1.2. ADMA and metabolic disease

As the role of ADMA in cardiovascular dysfunction became more evident, a number of studies sought to understand the relationship between ADMA and other risk factors of cardiovascular disease including obesity. One such study found an association between elevated plasma ADMA levels and insulin resistance, and demonstrated that pharmacological interventions enhancing insulin sensitivity – using the anti-diabetic drug rosiglitazone – reduced plasma ADMA concentrations (Stühlinger et al., 2002).

Later, ADMA was found to be positively correlated with obesity and BMI (Eid et al., 2004; McLaughlin et al., 2006), as well as full metabolic syndrome (Palomo et al., 2011). Plasma ADMA levels were also found to decrease following weight loss (McLaughlin et al., 2006). Although several clinical studies have described positive correlations between ADMA and adiposity, a causal relationship has not been demonstrated.

In the mid 1990s, the enzyme dimethylarginine dimethylaminohydrolase (DDAH) was demonstrated to be required for the metabolic clearance of ADMA (Kimoto et al., 1995; MacAllister et al., 1996). Reducing the metabolic clearance of ADMA via DDAH inhibition/gene inactivation has since proved a powerful tool to experimentally manipulate ADMA levels *in vivo*. Previous work from the lab of James Leiper (formerly at the MRC LMS, currently at the University of Glasgow) aimed to use this system to investigate the role of ADMA in adiposity. Adipocyte-specific knock-out (KO) mice for DDAH1, one of two existing DDAH isoforms in mammals (see Section 3.1.3.2), were developed to manipulate ADMA levels exclusively in adipose tissue. These animals exhibited a significant increase in fat mass compared to DDAH1 floxed mice when maintained on standard chow diet (Dowsett, 2014 - PhD thesis). These results appeared to implicate DDAH – and therefore possibly ADMA – in the regulation of adiposity. These findings were corroborated by further work from the Leiper lab, investigating the action of ADMA on adipocyte physiology using fully differentiated 3T3-L1 cells as a model of mature adipocytes (Dowsett, 2014 - PhD thesis). ADMA treatment induced significant cellular hypertrophy and increased lipid content. The synthetic NOS inhibitors L-NAME and PBI-TU had no effect on adipocyte size or lipid content, suggesting that ADMA could be acting independently of NOS in the regulation of adipocyte hypertrophy (Dowsett, 2014 - PhD thesis). These results were particularly intriguing given the previous assumption that the metabolic effects of ADMA would be mediated by NOS inhibition. As for the mode of action, downstream effectors are yet to be identified, however, preliminary results have indicated transcriptional and post-translational changes in lipogenic pathways (Dowsett, 2014 - PhD thesis).

3.1.3. Regulation of ADMA

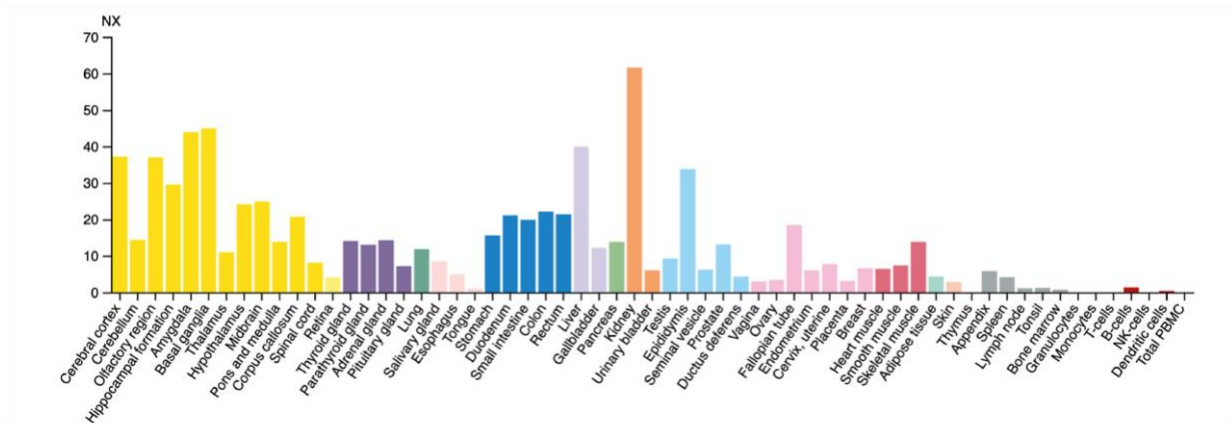
3.1.3.1. DDAH

As ADMA is continuously generated through protein turnover, efficient ADMA clearance is necessary to prevent its accumulation. DDAH is a highly conserved hydrolytic enzyme, which metabolises the methylated arginines ADMA and L-NMMA. DDAH is responsible for the majority of ADMA clearance. In humans, ~80% of ADMA generated is actively degraded within the cell by DDAH, with the remainder excreted in the urine after leaving the cell through cationic amino acid transporters (Achan et al., 2003; Teerlink, 2005). With ADMA and L-NMMA being strong endogenous competitive inhibitors of NOS (Leiper, 2005; Tran et al., 2003), DDAH has been implicated as a key modulator of the NO pathway.

3.1.3.2. DDAH isoforms

Two DDAH isoforms exist in humans, DDAH1 and DDAH2. They are encoded by two different genes arising from a duplication event that took place ~400 million years ago (Tran et al., 2000). Both are widely expressed, although with distinct tissue distributions. DDAH1 is highly expressed in the liver, kidneys and brain, while DDAH2 is highly expressed in the vasculature, thyroid gland and placenta, as well as in immune cells (Fig. 3.1) (<https://www.proteinatlas.org/>; Lambden et al., 2015; Tran et al., 2000). The expression patterns of the two DDAH isoforms overlap with the expression of different NOS isoforms. DDAH1 overlaps predominately with neuronal NOS and DDAH2 expression overlaps more with endothelial NOS and inducible NOS. Although both isoforms were shown to metabolise L-NMMA and ADMA to a similar degree *in vitro* (Leiper et al., 1999), the role of DDAH2 in ADMA metabolism *in vivo* has been debated (Altmann et al., 2012; X. Hu et al., 2011; Pope et al., 2009; D. Wang et al., 2007).

A



B

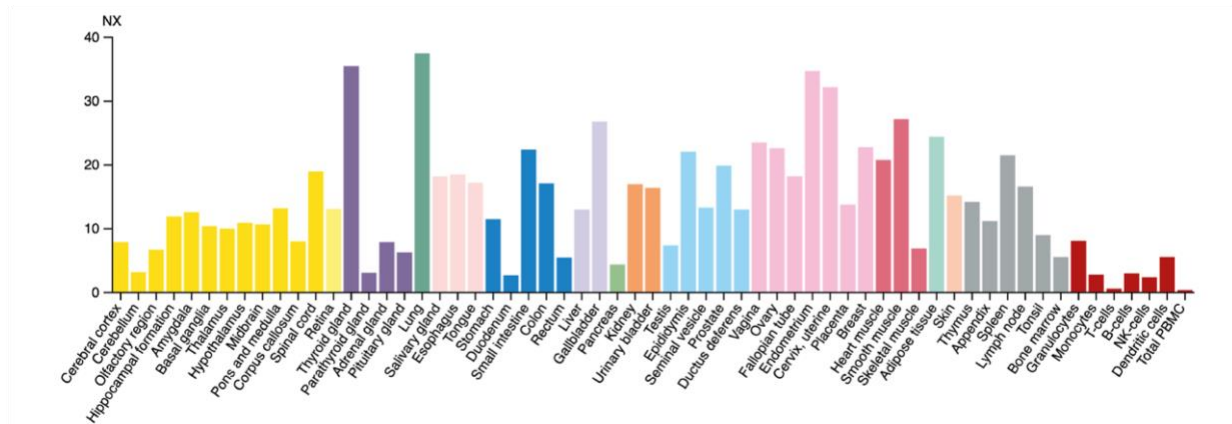


Figure 3.1 The tissue distribution of *DDAH1* and *DDAH2* RNA expression in humans

(A) *DDAH1* RNA expression is enriched in the liver, kidneys and brain, while *DDAH2* (B) is enriched in the vasculature thyroid gland and placenta (<https://www.proteinatlas.org/>).

3.1.3.3. DDAH enzyme activity and catalytic triad

DDAH hydrolyses ADMA and L-NMMA, producing citrulline and either dimethylamine or monomethylamine, respectively (Fig. 3.2). This enzymatic activity is dependent on a Cys-His-Glu catalytic triad identified after the crystal structure of the bacterial enzyme was solved (Murray-Rust et al., 2001). Equivalent cysteine and histidine residues and a glutamic or aspartic acid residue are conserved in all known DDAH orthologues. The presence of a reactive cysteine residue allows DDAH activity to be directly regulated through the S-nitrosylation of the cysteine by NO donors, thus allowing negative feedback (Leiper et al., 2002).

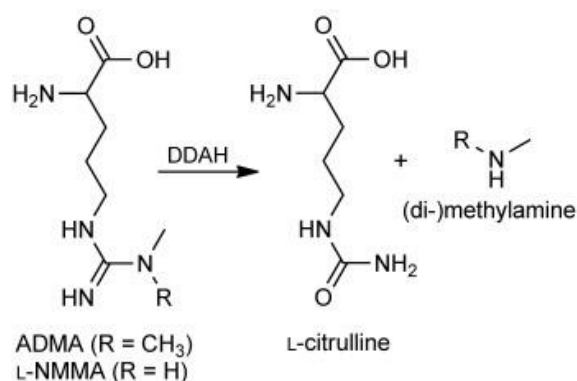


Figure 3.2 A schematic diagram illustrating the enzymatic activity of DDAH

DDAH hydrolyses ADMA and L-NMMA, producing citrulline and either dimethylamine or monomethylamine, respectively. Adapted from Altmann et al., 2012.

3.2. Results

3.2.1. Identification of *Drosophila* DDAH

To identify a candidate orthologue of DDAH in *Drosophila*, the DRSC Integrative Ortholog Prediction Tool (DIOPT) was applied (DRSC Integrative Ortholog Prediction Tool, version 8; Y. Hu et al., 2011; <http://www.flyrnai.org/diopt>). The protein encoded by *Drosophila* CG1764 was found to be the closest orthologue of human DDAH1, sharing 62% similarity and 40% identity as indicated from protein sequence alignments (Fig. 3.3) and received a top DIOPT score of 15/15. *Drosophila* CG1764 was also found to be orthologous to human DDAH2 (Fig. 3.3), although with a lower DIOPT score of 11 and sharing 56% similarity and 34% identity. Importantly, the amidinotransferase domain of DDAH is conserved in CG1764 (highlighted in green) (Fig. 3.3). The catalytic triad, consisting of a cysteine, histidine, and either a glutamic or aspartic acid residue, is essential for ADMA binding and catalysis. A similar triad, consisting of a cysteine, a positively charged arginine and aspartic acid residue, is present in *Drosophila* CG1764 (Fig. 3.3). Therefore, from the protein sequence alignments and shared catalytic domains, CG1764 is a strong candidate for the *Drosophila* orthologue of human DDAH and is most similar to DDAH1.

DDAH1_Human	1	MAGLGHPAAFGRATHAVVRALPESLGQHALRSAKGEEVDVARAERQHQLY	50
CG1764_Fly	1	-----MSPKYTHAIVARISDAL----LENGK---FDVQLAKQOHEQY	35
DDAH1_Human	51	VGVLGSKLGLQVVELPADESLPDCVFVEDVAVVCEETALITRPGAPSRRK	100
CG1764_Fly	36	CTLLRT-IGLDVIELPPDDQLPEGVVFENSAVICNGVALIGRSEHPKRQL	84
DDAH1_Human	101	EVDMMKEALEK-LQLNIVEMKDENATLDGGDVLFTGREFFVGLSKRTNQR	149
CG1764_Fly	85	EAESMAIILKKELDIPVIEIEDPNAQLDGGDVLFTGREFFVGISSFTNEE	134
DDAH1_Human	150	GAEILADTFKDYAVSTVPVADGLHLKSFCSMAGPNLIAIGSSESAQKALK	199
CG1764_Fly	135	GARAVAMAYPEYPVTPIRVNGTKRLKYVVTMAGPEVLCVSSSPTCQEIVK	184
DDAH1_Human	200	IMQQMSDHRDYLTPDDIAANCIYLNIPNKGHVLLHRTPEEYPESAKVY	249
CG1764_Fly	185	RMEREAICTYQKLTLPESAAANMLYIN-----GTIVHRSPTEIPEA---Y	226
DDAH1_Human	250	EKLKDHMLIP---VSMSELEKVDGLLTCCSVLIN--KKVDS-	285
CG1764_Fly	227	KTLKEKIDIPTRNINISEFSQYSSGLTSSCLLLRRWKSIRSL	268
DDAH2_Human	1	MGTPGEGLRGCSHALIRGVPESLASGEGAGAGLPALDLAKAQREHGVLGG	50
CG1764_Fly	1	-----MSPKYTHAIVARISDALLEN-----GKFQVQLAKQOHEQYC---	36
DDAH2_Human	51	KLRQRLGLQLLELPPEESLPLGPLLGD TAVIQGDTALITRPWSPARRPEV	100
CG1764_Fly	37	TLLRTIGLDVIELPPDDQLPEGVVFENSAVICNGVALIGRSEHPKRQLEA	86
DDAH2_Human	101	DGVRKAL-QDLGLRIVEIGDENATLDGTDVLFTGREFFVGLSKWTNHRGA	149
CG1764_Fly	87	ESMAIILKKELDIPVIEIEDPNAQLDGGDVLFTGREFFVGISSFTNEEGA	136
DDAH2_Human	150	EIVADTFRDFAVSTVPVSGPSHLRGLCGMGGPRTTVAGSSDAAQKAVRAM	199
CG1764_Fly	137	RAVAMAYPEYPVTPIRVNGTKRLKYVVTMAGPEVLCVSSSPTCQEIVKRM	186
DDAH2_Human	200	---AVLTDHPYASLTLPDDAAADCLFLRPGLPGVPPFLHRRGGGDLPSQ	246
CG1764_Fly	187	EREAICT---YQKLTLPESAAANMLYIN---GT---IVHRSPTEIPEAY	226
DDAH2_Human	247	EALQKLSDVTLVPVSCSELEKAGAGLSSLCLVLSTRPHS----	285
CG1764_Fly	227	KTLKEKIDIPTRNINISEFSQYSSGLTSSCLLL--RRWKSIRSL	268

Figure 3.3 Human DDAH1 and DDAH2 protein sequence alignments with *Drosophila* CG1764

Pairwise sequence alignments between human DDAH isoforms and *Drosophila* CG1764. Alignments were performed using EMBOSS Needle tool, EMBL-EBI. A conserved amidinotransferase domain is highlighted in green. The catalytic triad required for ADMA binding is highlighted in pink.

3.2.2. CG1764

To date, CG1764 has not been functionally characterised. The annotated molecular functions of CG1764 (amino acid binding and dimethylargininase activity) are currently based on gene ontology (GO) predictions (Gaudet et al., 2011). CG1764 is located on the X chromosome (13,294,473..13,302,415 [-]) and is reported to produce two transcripts, RA and RB, although the expression of RB is almost negligible (Leader et al., 2018, www.flyatlas2.org). Both transcripts produce the same polypeptide. High throughput transcriptional data suggest that CG1764 is moderately expressed throughout embryo development and pupation, with relatively low expression in adults (modENCODE Temporal Expression Data, Graveley et al., 2011). In adults, expression is highest in the head, brain and thoracic ganglion where *Drosophila* NOS (*dNOS*) is also expressed (Leader et al., 2018, www.flyatlas2.org). Other high throughput studies have confirmed expression in the head, however unlike FlyAtlas2, found clear sexual dimorphism, with expression being higher in males (modENCODE Tissue Expression Data, Brown et al., 2014; Anholt et al., 2003; Fujii & Amrein, 2002).

3.2.3. *DDAH^{XP}*: manipulating ADMA levels in *Drosophila*

A *Drosophila* CG1764 mutant was previously generated by imprecise excision of a *P*-element located 80 bp upstream of the putative start codon (Fig. 3.4). Mobilisation of this *P*-element resulted in a ~2 kb deletion removing the entire CG1764 open reading frame. The resulting KO allele is referred to as *DDAH^{XP}*. The expected deletion was confirmed by PCR (Fig. 3.5A)

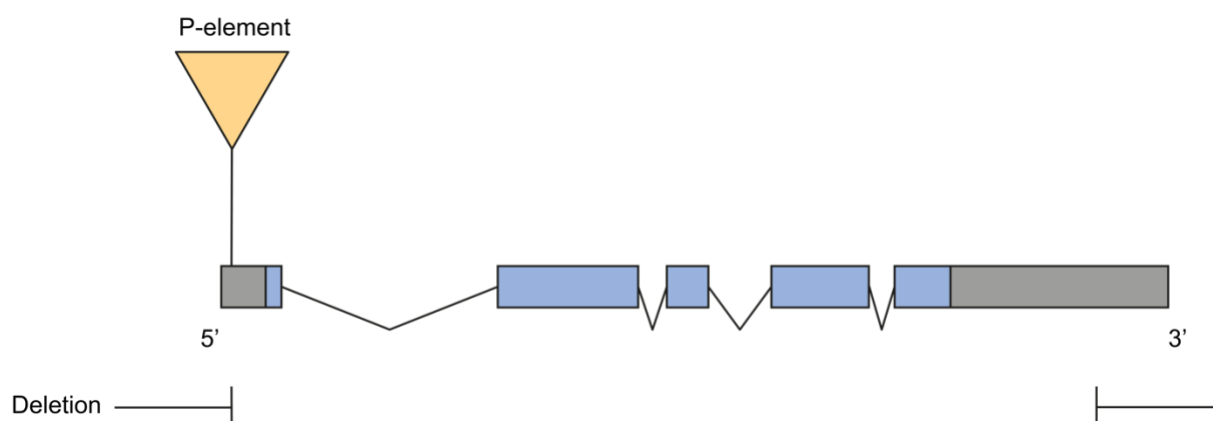


Figure 3.4 Schematic representation of the CG1764 locus illustrating the excision of a *P*-element in the generation of *DDAH^{XP}*

DDAH^{XP} was generated by the imprecise excision of a *P*-element resulting in a ~2 kb deletion. This line was previously generated by the Partridge/Leiper labs.

3.2.4. ADMA levels are elevated in *DDAH^{XP}* animals

The deletion of *CG1764* in animals is expected to increase ADMA levels as a result of reduced metabolic clearance. To confirm this, arginine metabolites were extracted from whole adult flies and analysed by liquid chromatography-mass spectrometry (LC-MS). As predicted, *DDAH^{XP}* animals had significantly elevated (~2-fold) ADMA levels (Fig. 3.5A). In addition to DDAH, the mitochondrial aminotransferase alanine-glyoxylate aminotransferase 2 (AGXT2) is also reported to metabolise ADMA. AGXT2 catalyses the transamination reaction between ADMA and glyoxylate, generating dimethylguanidino valeric acid (DMGV) and glycine. Given that *Drosophila* AGXT (*dAGXT*) has also previously been found to modulate ADMA levels (Leiper lab, unpublished), I measured the expression of *dAGXT* by qPCR. *dAGXT* was upregulated in *DDAH^{XP}* animals compared to controls (Fig. 3.5B), presumably as compensation for the loss of *CG1764* (addressed in more detail in Chapter 6).

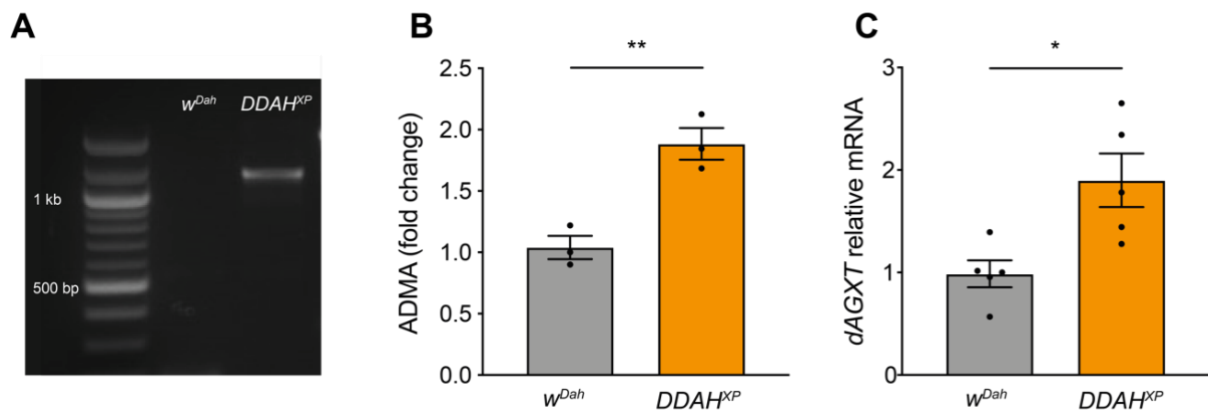


Figure 3.5 ADMA levels are elevated and *dAGXT* is transcriptionally upregulated in *DDAH^{XP}* animals

(A) PCR confirming the expected deletion in *DDAH^{XP}* animals using primers SOL894 and SOL895 (Appendix 1.3). Primer binding either side of the breakpoint produces a product in *DDAH^{XP}* but not *w^{Dah}*. (B) Targeted metabolomic analysis from *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7). *n*=3 per condition. All sample preparation for metabolomic data was performed by A J. Vincent. All metabolomic analyses were performed by J. Tomlinson (LMS) (C) *dAGXT* expression in *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7), as determined by qPCR. *n*=5 per condition. Samples are normalised to alphaTub84B. Results are shown as mean relative fold change $\pm$ SEM and analysed by unpaired t-test in both datasets. *, $P \leq 0.05$; **, $P \leq 0.01$.

3.2.5. Reduced fat storage in *DDAH^{XP}* animals

Having shown that ADMA levels are elevated in *DDAH^{XP}* animals, I assessed their lipid storage. By comparison to wild-type (WT) controls, *DDAH^{XP}* adults had significantly reduced (~50%) total TAG levels (Fig. 3.6A). To assess lipid storage

specifically in the fat body, dissected tissue was stained with the neutral lipid dye Nile Red. Lipid droplets were visibly smaller in *DDAH^{XP}* animals compared to controls (Fig. 3.6B), consistent with whole animal TAG levels.

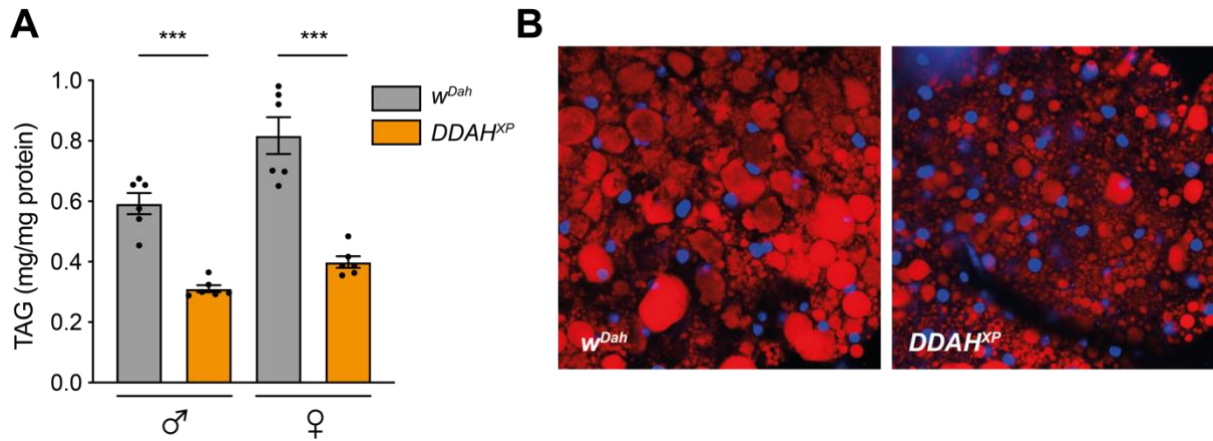


Figure 3.6 Reduced fat storage in *DDAH^{XP}* animals

(A) TAG levels from *w^{Dah}* and *DDAH^{XP}* whole adults (male and female, day 7). *n*=6 per condition. TAG levels were normalised to protein (mg/mg). Results are shown as mean ± SEM. Statistical analyses were carried out with an unpaired t-test. ***, *P* ≤ 0.001. **(B)** Lipid staining (red) and DAPI (blue) of dissected fat body tissue from *w^{Dah}* and *DDAH^{XP}* adult males (day 7). Lipid droplets were not quantified.

3.2.6. *DDAH^{XP}* animals exhibit no change in feeding behaviour

Next, I assessed whether changes in feeding behaviour could contribute to the reduced TAG levels observed in *DDAH^{XP}* animals. Two independent methods were used to measure feeding - the FlyPAD (fly proboscis and activity detector) and the Capillary Feeder (CAFE) assay. The FlyPAD allows for the automated quantification of feeding behaviour. When the fly feeds, it completes a circuit leading to changes in capacitance between two electrodes which can be measured (Itskov et al., 2014). This is typically used for short-term monitoring of feeding behaviour (~1 h). The CAFE assay, which allows the continuous quantitation of actual ingestion by measuring liquid food consumed from a graduated glass microcapillary (Ja et al., 2007), was used to monitor feeding over longer periods of time (~ several days). No significant difference in feeding was observed using either assay (Fig. 3.7A-C).

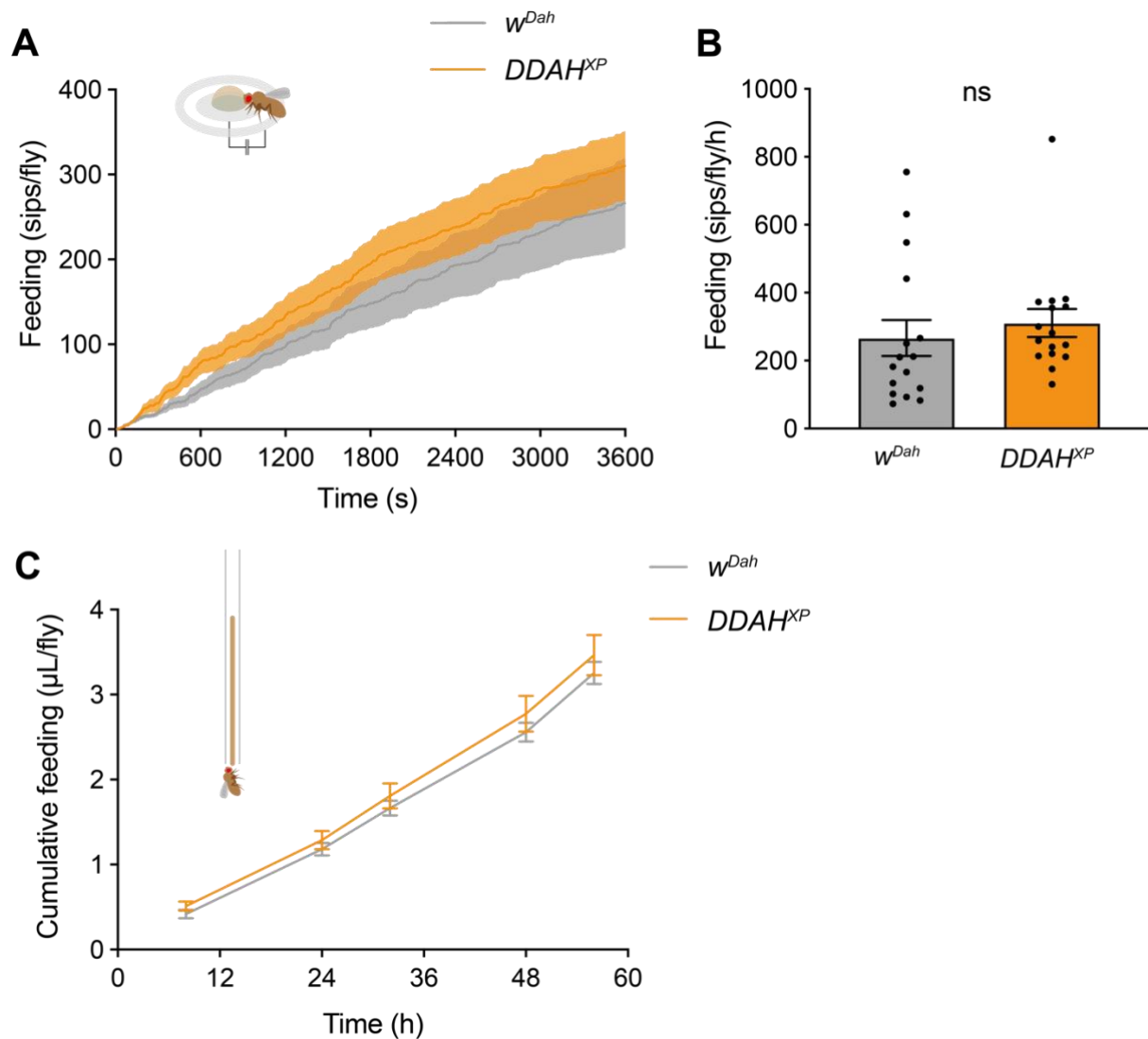


Figure 3.7 *DDAH^{XP}* animals exhibit no change in feeding behaviour

Feeding behaviour of adult (day 7) w^{Dah} and $DDAH^{XP}$ males assessed by (A, B) FlyPAD and (C) CAFE assay. $n=16-22$ per condition. Results are shown as cumulative frequencies (A, C) or as mean $\pm$ SEM (B). Statistical analyses were carried out with an unpaired t-test. ns, $P>0.05$.

3.2.7. *DDAH^{XP}* animals are sensitive to starvation

As the major form of energy storage, TAG is mobilised upon starvation in order to maintain energy homeostasis. Considering the dramatic reduction of TAG observed in $DDAH^{XP}$ animals, starvation sensitivity was assessed. Following eclosion, adults were maintained on a standard sugar-yeast-agar (SYA) medium for 7 days before being transferred to starvation medium (1.5% w/v agar). Thereafter, deaths were scored between 3-4 times per day. $DDAH^{XP}$ males were found to be severely starvation sensitive (median survival of 3.3 days, compared to 5.9 days for WT controls) (Fig. 3.8), consistent with their lower levels of TAG reserves.

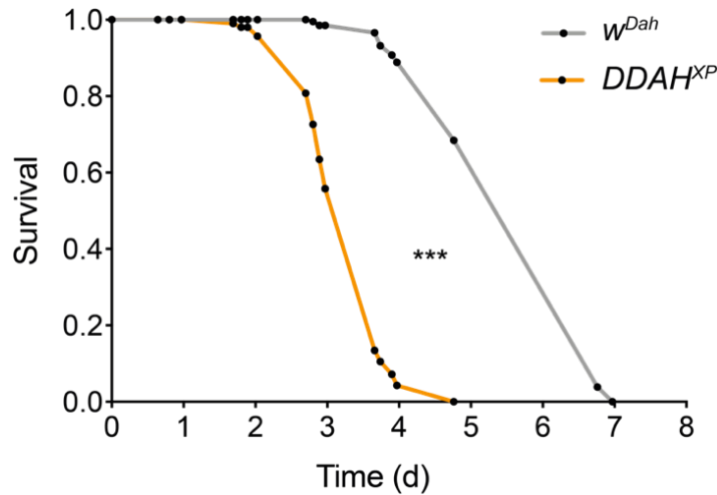


Figure 3.8 $DDAH^{XP}$ animals are sensitive to starvation

Survival of w^{Dah} and $DDAH^{XP}$ adult males (aged to day 7 before treatment). n=210 per condition. Statistical analysis was carried out with a Log-Rank test on survivorship data. ***, $P \leq 0.001$.

3.2.8. $DDAH^{XP}$ animals do not gain fat and are partially protected from reduced lifespan on a HFD

Given the lean phenotype of $DDAH^{XP}$ animals, I aimed to explore their response to a HFD. Animals were maintained on diets containing 0, 5, 10 and 15% lard. As expected, TAG levels increased in a dose-dependent manner in WT controls. In contrast, $DDAH^{XP}$ mutants raised on the same diets had no significant increase in TAG levels (Fig. 3.9A). Given that a HFD is associated with reduced lifespan in *Drosophila* (Liao et al., 2020), I investigated whether the reduced fat accumulation observed in $DDAH^{XP}$ animals could be protective in terms of lifespan. In line with previous studies, control animals had shortened lifespans on the HFD. This reduction in lifespan was also dose-dependent. $DDAH^{XP}$ animals were partially protected against shortened lifespan on HFD, being significantly longer lived on all concentrations of HFD compared to WT controls (Fig. 3.9C-E). There was no difference in lifespan between $DDAH^{XP}$ and WT animals on the control diet (Fig. 3.9B).

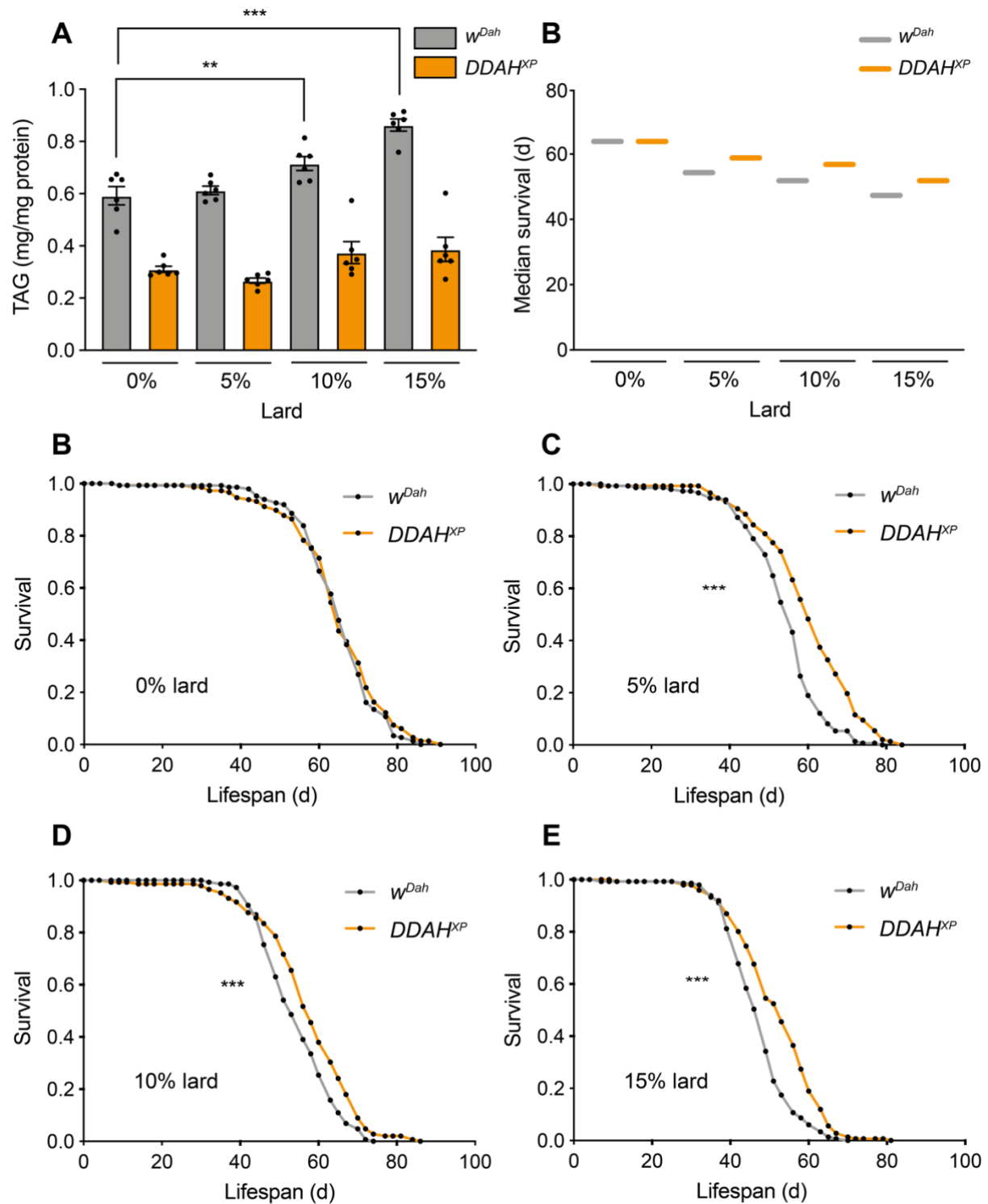


Figure 3.9 *DDAHXP* animals do not gain fat and are partially protected from reduced lifespan on HFD

(A) TAG levels from *wDah* and *DDAHXP* whole adult males (day 14) raised on HFD (0, 5, 10 and 15% lard) normalised to protein (mg/mg). n=6 per condition. Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with one-way ANOVA and a Dunnett's multiple comparisons test. (B-E) Survival of *wDah* and *DDAHXP* adult males fed a range of HFDs (0, 5, 10 and 15% lard). n=200-210 per condition. Statistical analysis was carried out with a Log-Rank test on survivorship data. ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001.

3.2.9. Transcriptional changes in lipid metabolism pathways suggest reduced TAG turnover in *DDAH^{XP}* animals

To explore how lipid metabolism is altered in *DDAH^{XP}* animals, I assessed the expression of selected key genes involved in both lipogenesis and TAG mobilisation. *SREBP*, *FASN1*, *mino*, *Agpat2*, *Lipin* and *mdy* are all involved in different stages of lipogenesis while *bmm*, *dob* and *Hsl* are involved in TAG mobilisation (covered in detail in Section 1.3.).

SREBP, a master transcriptional regulator of lipogenesis, was significantly upregulated in *DDAH^{XP}* animals (Fig. 3.10). SREBPs regulate lipogenesis in response to changes in the abundance of membrane lipids. Therefore, a reduction in the TAG reserves of *DDAH^{XP}* animals, to the extent that membrane lipids are depleted, could lead to the transcriptional upregulation of *dSREBP* observed. However, it is important to note that transcriptional regulation of lipogenesis by SREBP requires its activation via proteolytic cleavage. Therefore, without evidence of nuclear translocation of SREBP's bHLH-Zip domain, the significance of its transcriptional upregulation remains unclear. Whether *dSREBP* is indeed activated in *DDAH^{XP}* animals can be inferred by assessing the expression of a known target gene, as outlined below.

FASN1, a transcriptional target of *dSREBP*, encodes an essential enzyme required for *de novo* lipogenesis. No significant change in *FASN1* expression was observed (Fig. 3.10), suggesting that although *dSREBP* is transcriptionally upregulated, its transcriptional output may not be elevated. *mino*, *Agpat2*, *dLipin* and *mdy* all encode enzymes from the G3P pathway. *mino* (encodes a GPAT) and *Agpat2* catalyse the esterification of FA moieties to G3P backbones. No significant change in expression was observed for either gene (Fig. 3.10). *dLipin*, which catalysis the dephosphorylation of PA to DAG, the essential precursor of TAG, was downregulated in *DDAH^{XP}* animals (Fig. 3.10). Downregulation of *dLipin* could indicate a reduction in TAG biosynthesis as a result of reduced synthesis of DAG.

As for genes involved in TAG mobilisation, no transcriptional changes were found with the essential lipase *bmm* or its paralogue *dob* (Fig. 3.10). However, downregulation of the lipase *Hsl* was observed (Fig. 3.10). Overall, there appears to be either no change or downregulation of genes in both lipogenic and lipolytic pathways in *DDAH^{XP}* animals. Although *dSREBP* was upregulated, the expression of transcriptional target

FASN1 was not significantly different, suggesting the transcriptional program downstream of *dSREBP* is relatively unaffected. Altogether, these data seem to indicate reduced TAG turnover in *DDAH^{XP}* animals – the lean phenotype may therefore be a result of reduced TAG biosynthesis rather than increased catabolism. This could be explained by altered energy homeostasis, where high levels of energy expenditure would limit the need to store TAG (energy is burned before being stored).

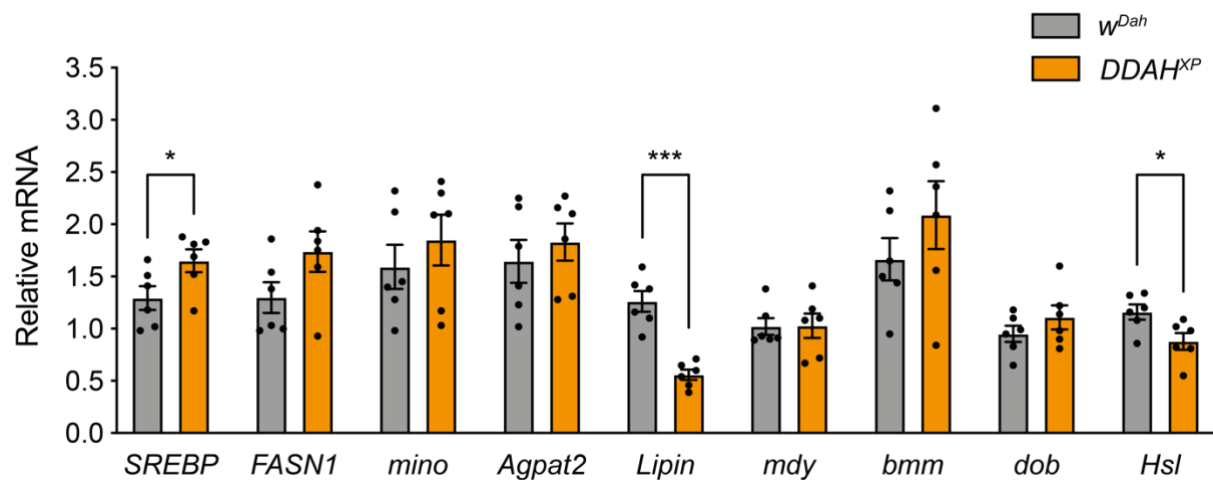


Figure 3.10 Transcriptional changes in lipid metabolism pathways suggest reduced TAG turnover in *DDAH^{XP}* animals

Expression levels of genes involved in lipid metabolism in whole *w^{Dah}* and *DDAH^{XP}* adult males (day 7), as determined by qPCR. n=6 per condition. Samples are normalised to alphaTub84B. Results are shown as mean relative fold change $\pm$ SEM and analysed using an unpaired t-test. *, $P \leq 0.05$; ***, $P \leq 0.001$.

3.2.10. *DDAH^{XP}* animals have increased mitochondrial content

Mitochondria play a central role in energy metabolism. To explore the possibility of increased energy expenditure in *DDAH^{XP}*, I assessed mitochondrial content - a quantitative indicator of oxidative capacity. Citrate synthase catalyses the condensation reaction between acetyl-CoA and oxaloacetate in the first step of the TCA cycle which takes place in the mitochondrial matrix. Citrate synthase activity is therefore used as an estimate of mitochondrial content, and was determined using a coupled colorimetric assay (Cochemé et al., 2012). *DDAH^{XP}* animals had significantly higher levels of citrate synthase activity compared to controls (Fig. 3.11A,B), suggesting increased mitochondrial content.

As a second independent assay for mitochondrial content, I measured expression of mitochondrial DNA (mtDNA) helicase, which is essential for mtDNA maintenance and

correlates with mtDNA copy number (Ikeda et al., 2015; Tynismaa et al., 2004). Consistent with the citrate synthase activity, mtDNA-helicase was upregulated in *DDAH^{XP}* animals (Fig. 3.11D). Taken together, these data are indicative of increased mitochondrial content in *DDAH^{XP}* animals, supporting the hypothesis of increased energy expenditure.

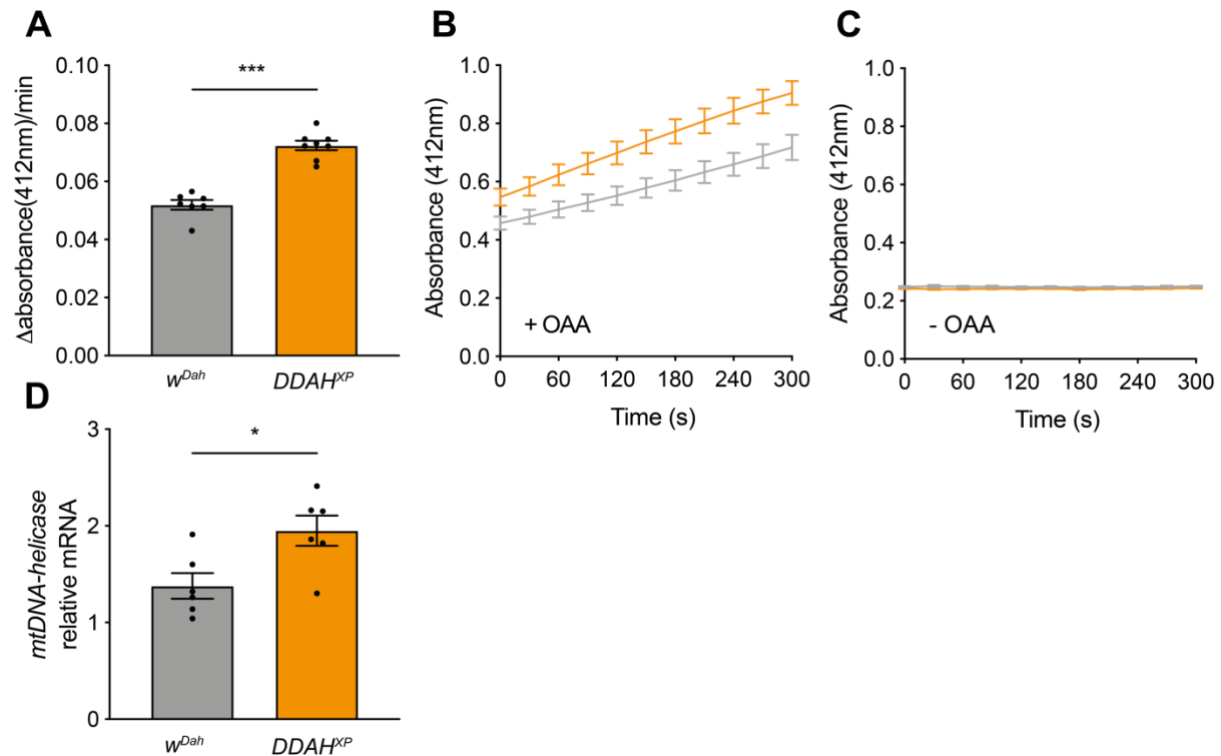


Figure 3.11 Elevated levels of citrate synthase activity and transcriptional upregulation of *mtDNA-helicase* are indicative of increased mitochondrial content in *DDAH^{XP}* animals

(A, B) Citrate synthase activity of adult *w^{Dah}* and *DDAH^{XP}* males (day 7) determined using a coupled colorimetric assay where both substrates – acetyl-CoA and oxaloacetate – are added in excess and citrate synthase becomes rate limiting in the production of the coloured product TNB. The production rate of TNB (measure by absorbance) is proportional to the activity of citrate synthase (when linear). (C) Background production of TNB in the absence of the oxaloacetate. (D) *mtDNA-helicase* expression in *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7), as determined by qPCR. n=6 per condition. Samples are normalised to alphaTub84B. Results are shown as mean relative fold change ± SEM and analysed by unpaired t-test. *, P≤0.05; ***, P≤0.001.

3.2.11. *In vivo* metabolic rates: *DDAH^{XP}* animals produce more CO₂ and lose diurnal patterns of CO₂ production

To further explore the hypothesis of increased energy expenditure, it was pertinent to measure *in vivo* metabolic rates. The TCA cycle and oxidative decarboxylation of pyruvate – central processes of cellular respiration – produce CO₂ as a waste product.

Therefore, CO₂ production can be used as an indication of energy expenditure. *In vivo* metabolic chambers (Sable Systems) were used to measure the CO₂ production of individual flies over a time course. Total CO₂ production was elevated in *DDAH^{XP}* animals (Fig. 3.12B), indicative of increased energy expenditure and is consistent with increased mitochondrial content.

Drosophila, like all insects, can largely be classified as ectotherms. Without the energetically costly need to generate heat to regulate body temperature, locomotor activity accounts for the majority of energy usage. Consequently, mitochondrial respiration and locomotor activity are tightly linked. This is elegantly demonstrated in Fig. 3.12, where the CO₂ production of WT animals correlates with the pattern of locomotor activity. *Drosophila* exhibit a bimodal activity distribution; they are highly active early morning and late evening. WT animals have peaks of CO₂ production corresponding to these time points (Fig. 3.12), and conversely produce low CO₂ levels at night when locomotor activity is low (i.e., sleeping). *DDAH^{XP}* animals display a striking difference in the pattern of CO₂ production, and no longer exhibit a diurnal pattern of CO₂ production (Fig. 3.12). Given the relationship between locomotor activity and mitochondria respiration in *Drosophila*, it is tempting to predict that *DDAH^{XP}* animals have altered locomotor activity patterns.

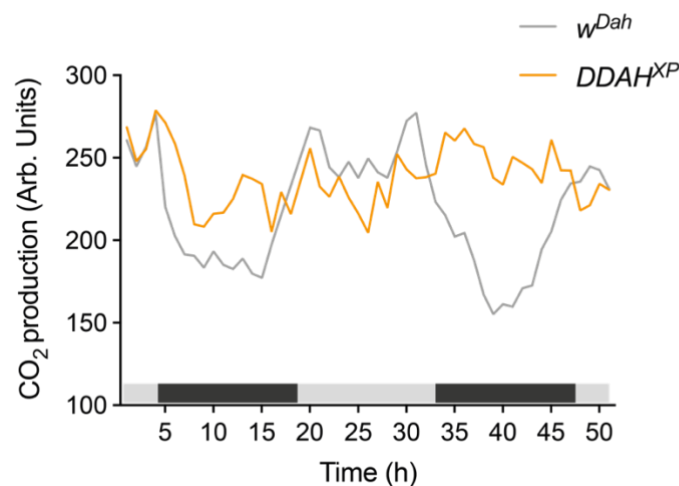


Figure 3.12 *DDAH^{XP}* animals produce more CO₂ and lose diurnal patterns of CO₂ production

Mean CO₂ production of *w^{Dah}* and *DDAH^{XP}* males (day 7) over time shown in arbitrary units. n=7. Mean total CO₂ production is elevated in *DDAH^{XP}* compared to *w^{Dah}*. Data were recorded using Sable Systems data acquisition hardware and software.

3.2.12. *DDAH^{XP}* animals are less active and sleep more

DDAH^{XP} animals produce high levels of CO₂ throughout the duration of the night, which seems to account for the majority of the difference in CO₂ production. This raises the question of whether altered activity or sleep patterns (high levels of locomotor activity at night) could account for the elevated levels of CO₂ production in *DDAH^{XP}* animals. To investigate whether altered locomotor activity contributes to the increased CO₂ production of *DDAH^{XP}* animals, I assessed their sleep and activity patterns. The *Drosophila* Activity Monitor (DAM) system (TriKinetics) has traditionally been used to study circadian rhythms and sleep by monitoring activity through the use of infrared (IR) beams. When a fly crosses the IR beam, the signal is interrupted and recorded. Although DAMs have been instrumental to circadian research, they generate binary data. Recently, new tools were developed by the group of Giorgio Gilestro (Faculty of Life Sciences, Imperial College London) for the study of activity and sleep in *Drosophila* (Geissmann et al., 2017). The so-called ethoscopes are used to track *Drosophila* behaviour using computerised (Raspberry Pi) video tracking units (Fig. 3.13A,B). Behavioural profiling software based on a supervised machine learning algorithm generates data on activity and sleep (Geissmann et al., 2017). Continuous tracking provides more resolution and the ability to distinguish between different behaviours (e.g., moving vs micro-moving and velocity).

Ethoscopes were used to assess the sleep and activity patterns of *DDAH^{XP}* animals in collaboration with Quentin Geissmann from the Gilestro lab. Surprisingly, *DDAH^{XP}* animals were found to sleep significantly more than controls. *DDAH^{XP}* animals had higher levels of overall sleep and a dramatic increase in daytime sleep during the so-called 'siesta' (Fig. 3.13C,D). These data suggest that elevated CO₂ production of *DDAH^{XP}* animals is not due to increased locomotor activity. Intriguingly, these data instead support the notion of elevated uncoupled oxidative phosphorylation by seemingly separating mitochondrial respiration from locomotor activity. Mitochondrial uncoupling reduces the amount of ATP that can be generated from oxidative metabolism via 'proton leak', a process that allows protons to cross the mitochondrial membrane without generating ATP.

The increase in sleep of *DDAH^{XP}* animals could be way of conserving energy in response to their highly active metabolic states and low TAG reserves. The low energy

storage status of *DDAH^{XP}* animals could be viewed as analogous to a starvation state. Increased sleep, particularly during the siesta, is an evolutionary response to chronic starvation (Slocumb et al., 2015). The siesta may be more susceptible to changes in sleep, as activity bouts need to be maintained for foraging and night time sleep is already high. However, with *CG1764* being transcriptionally implicated in circadian networks (Kadener et al., 2006), further characterisation would be necessary to elucidate the role of *CG1764* in sleep.

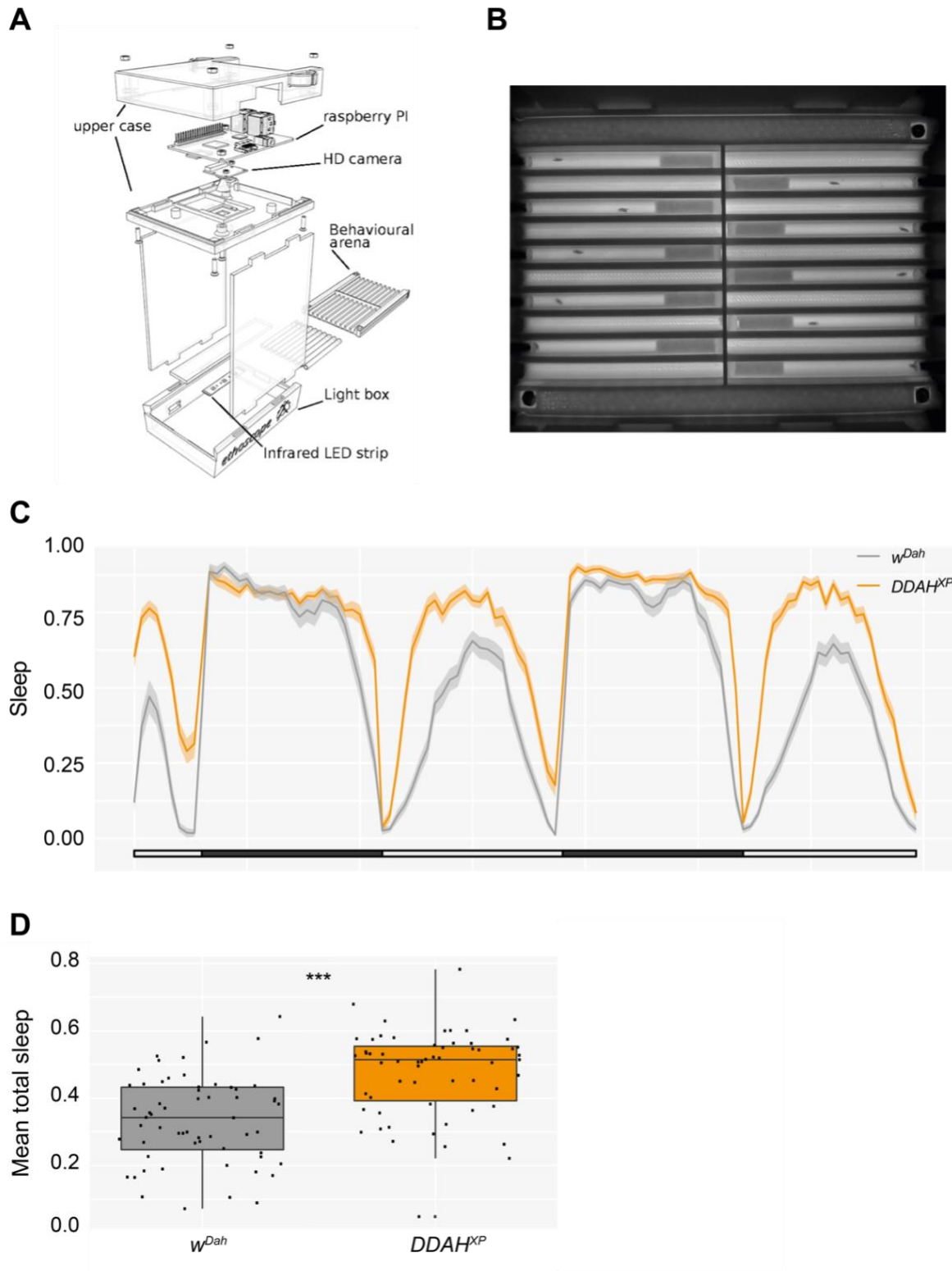


Figure 3.13 *DDAH^{XP}* animals are less active and sleep more than control

(A) Exploded drawing of an ethoscope (Geissmann et al., 2017). (B) Screenshot of a video recording. (C) Sleep profiles (proportion asleep) of w^{Dah} and $DDAH^{XP}$ males in conditions of 12 hour:12 hour, light and dark cycles measured using ethoscopes. (D) Mean total sleep of w^{Dah} and $DDAH^{XP}$ males (day 7). $n=60$. Statistical analysis was carried out using a Wilcoxon rank sum test with continuity correction. Ethoscope experiments were performed by A J. Vincent with the guidance of Q. Geissmann. All data analyses were performed by Q. Geissmann. ***, $P \leq 0.001$.

3.2.13. *DDAH^{XP}* animals lack morning anticipation, a classical circadian behaviour

The *Drosophila* clock network has been described at a near single neuron resolution and is arguably the most well characterised central brain network governing behaviour in *Drosophila* (Dubowy & Sehgal, 2017). Morning anticipation, the ramping up of locomotor activity in advance of sunrise (i.e. 'lights on') (Fig. 3.14B), is a classical circadian behaviour regulated by two small populations of clock neurons - small and large ventral lateral neurons (s/l-LNvs) (Flourakis & Allada, 2015). *DDAH^{XP}* animals lack morning anticipation. Instead, they exhibit an abrupt shift in behaviour, only becoming active at lights on (Fig. 3.14A). The loss of this well-studied circadian behaviour in *DDAH^{XP}* animals lends support for a role of *CG1764* in the regulation of circadian behaviour. Given the extensive characterisation of clock neurons and known anatomical positions (Fig. 3.14C), I was interested in whether the protein encoded by *CG1764* could be expressed in clock neurons, particularly in LNvs, which have been assigned an essential role in the regulation of morning anticipation. Protein localisation was assessed using an N-terminal GFP fusion line (GFP-DDAH) (generation of this strain is described in Chapter 4). GFP-DDAH was highly expressed in the ventral nerve cord (VNC) (Fig. 3.14D). The VNC plays a crucial role in the reception and integration of sensory information and generates the majority of locomotor actions that underlie behaviour. GFP-DDAH expression in the VNC therefore seems consistent with the reduced locomotor activity observed in *DDAH^{XP}* animals. In addition, GFP-DDAH expression in the brain may overlap with parts of the clock network (Fig. 3.14E), including LNvs, although co-localisation experiments would be necessary to draw any detailed conclusions. GFP-DDAH expression was consistent with publicly available high-throughput RNA expression data, where *CG1764* was found to be predominantly expressed in the brain and thoracic ganglion (www.flyatlas2.org, Leader et al., 2018; modENCODE Tissue Expression Data, Brown et al., 2014; Anholt et al., 2003; Fujii & Amrein, 2002).

An accidental shift in the light cycle of the incubators from 12 h:12 h ON/OFF to constant light (ON/ON) serendipitously revealed another interesting behavioural phenotype. *DDAH^{XP}* animals still went to sleep in their subjective night, even though the lights remained on, while WT controls remained awake during this time (Fig. 3.14A). Circadian clocks synchronise behaviour and physiology with the day and

night cycle, and are a product of both internal molecular pacemakers and environmental cues such as light (Dubruille & Emery, 2008). It is tempting to infer that *DDAH^{XP}* animals have difficulty integrating the environmental light or dark cues that are needed for sleep or wakefulness. NO, which is negatively regulated by ADMA, is suggested to play a role in integrating external information to the internal clock. Pharmacological inhibition of nNOS in mammalian models blocks the circadian response to light (Golombek et al., 2004). Therefore, elevated ADMA levels in *DDAH^{XP}* animals could conceivably inhibit NOS leading to a perturbed response to light. However, the abrupt increase in activity of *DDAH^{XP}* animals at lights on during the standard ON/OFF cycle suggests they are integrating light cues and would contradict this theory. Alternatively, *DDAH^{XP}* animals may simply require more sleep to conserve energy, and therefore sleep regardless of external cues.

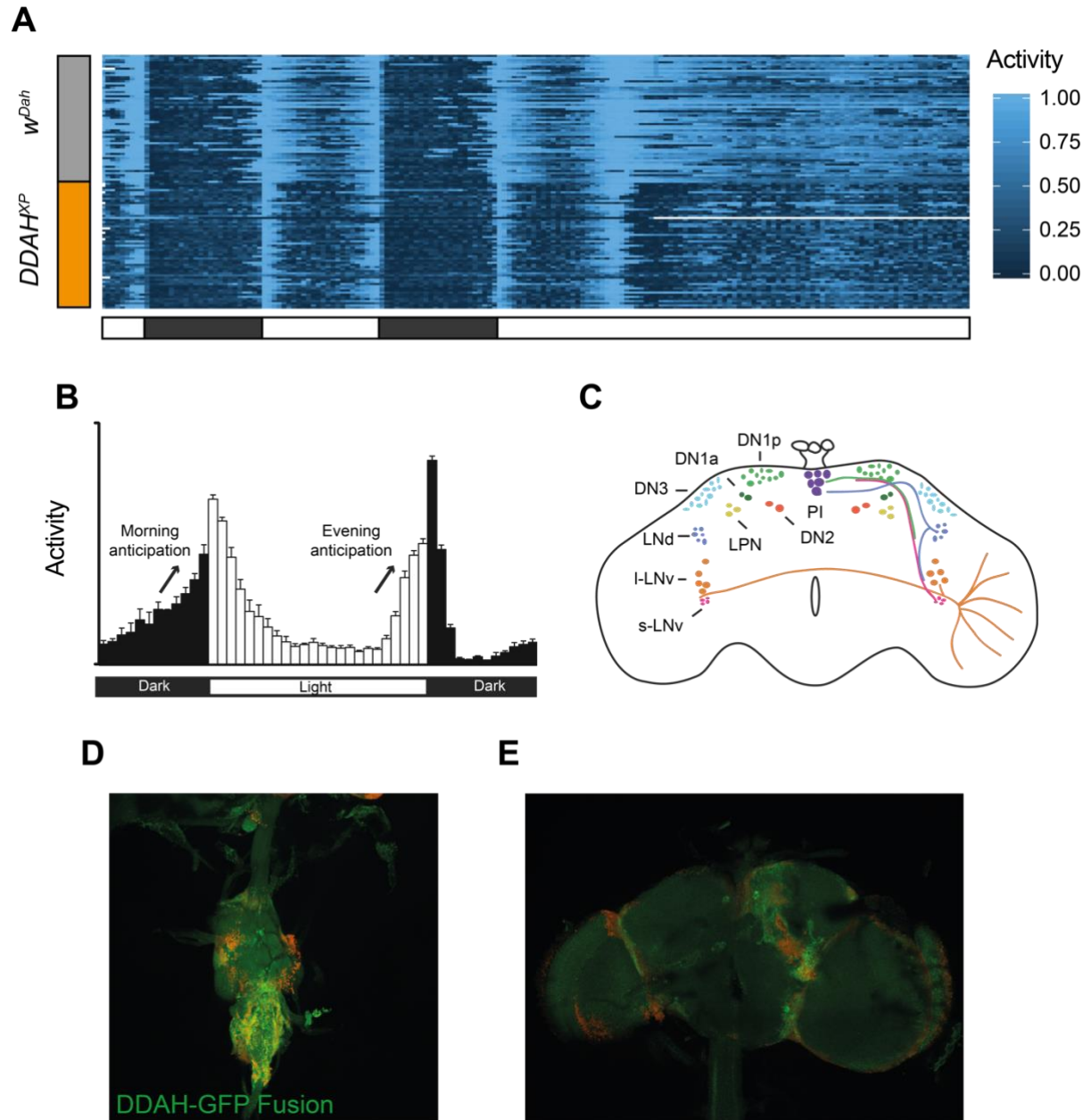


Figure 3.14 *DDAH^{XP}* animals lack morning anticipation, a classical circadian behaviour

(A) Activity of *w^{Dah}* and *DDAH^{XP}* males measured using ethoscopes. Flies were maintained in a 12 h:12 h light and dark cycle before a shift to constant light following the 2nd night (as indicated by the lower bar). Each row represents the activity of an individual fly, n=60. (B) A representative locomotor activity plot of flies maintained in a 12 h:12 h light and dark cycle (Flourakis & Allada, 2015). (C) Schematic representation of the circadian pacemaker network in the adult fly brain (Flourakis & Allada, 2015). (D) Confocal imaging of dissected ventral nerve cord (VNC) and (E) central brain (CB) of *DDAH^{IN-GFP}* adult males (~day 7) with immunostaining for GFP (green) and Elav (red).

3.2.14. *CG1764* mRNA expression is cyclic

Circadian clocks are endogenous pacemakers that align our physiology and behaviour to the day-night cycles. They are generated by interlocked molecular feedback loops that oscillate with a period of ~24 h. Feedback is generated as transcription factors clock (*Clk*) and cycle (*cyc*) activate the expression of their own repressors, period (*per*) and timeless (*tim*). Maintenance of the feedback loop requires additional components that are not covered here for brevity, but are reviewed extensively in the literature (Dubowy & Sehgal, 2017). Given that mRNA levels of most genes involved in circadian regulation are known to oscillate, I measured expression of *CG1764* over time. Total RNA was isolated from fly heads that were snap frozen at 2 h intervals over a 16 h period, and expression was determined by qPCR. *CG1764* mRNA levels appear to cycle and seem to be in antiphase to *Clk*, exhibiting a similar amplitude (Fig. 3.15A,B). *CG1764* expression seems to follow a ~4-6 h shift (delay) relative to the *per/tim* phase, which peaks in early evening (not shown). However, measurements did not cover a full 24 h period therefore exact peaks and troughs cannot be determined. These data could support the notion that *CG1764* is involved in regulation of circadian behaviour, although it is important to consider that the expression of metabolic genes can also be cyclic.

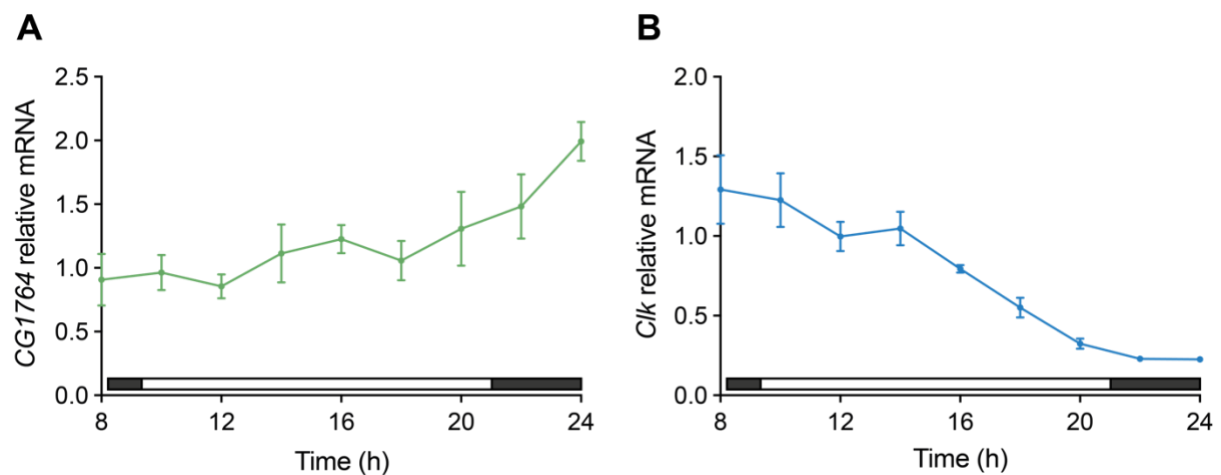


Figure 3.15 *CG1764* mRNA expression is cyclic

Expression levels of (A) *CG1764* and (B) *Clk* in WT (*w^{Dah}*) adult male heads (day 7) over a 16 h period, as determined by qPCR. n=4-5. Results are shown as mean $\pm$ SEM.

3.2.15. Dietary L-arginine supplementation has no effect on TAG levels in *DDAH^{XP}* animals

Given that ADMA is a strong endogenous competitive inhibitor of NOS, elevated ADMA levels in *DDAH^{XP}* animals is expected to reduce NO synthesis and bioavailability. It is therefore necessary to exclude NO from having a functional role in the metabolic phenotypes observed in *DDAH^{XP}* animals. NO is synthesised from the amino acid L-arginine by NOS. The ratio of L-arginine to ADMA (and other methylated arginines) determines NOS activity. As such, L-arginine supplementation can be used to increase NO synthesis in the context of elevated ADMA. *DDAH^{XP}* animals were therefore raised on a chemically defined synthetic medium, described in (Piper et al., 2014), where L-arginine levels could be manipulated. 1x (8.5 mM) is equivalent to the concentration of L-arginine present in our standard media (SYA). Dietary supplementation of L-arginine had no significant effect on TAG levels at any concentration (1x, 2x, 6x and 11x) (Fig. 3.16). These data suggest that the metabolic phenotypes of *DDAH^{XP}* animals are independent of NO. Although L-arginine is expected to elevate NO levels, NOx measurements were not performed and would be required to draw any definitive conclusions.

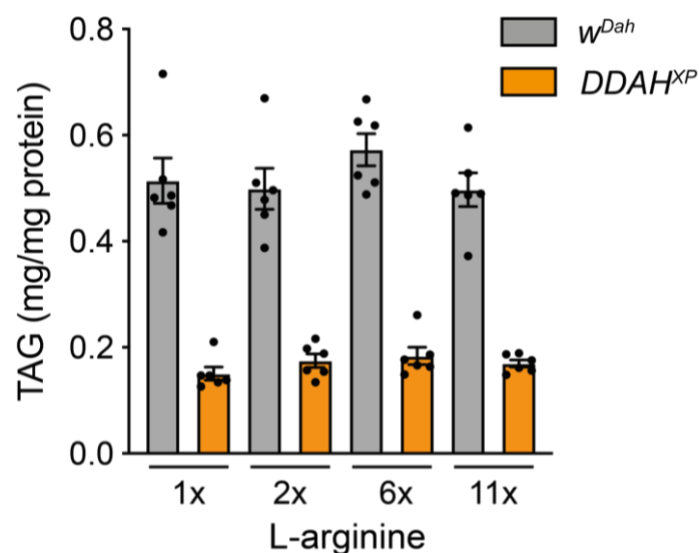


Figure 3.16 Dietary L-arginine supplementation has no effect on TAG levels in *DDAH^{XP}* animals

TAG levels from *w^{Dah}* and *DDAH^{XP}* whole adult males (day 14) raised on diets supplemented with L-arginine (1x, 2x, 6x, 11x,) where 1x (8.5 mM) is equivalent to the concentration of L-arginine present in our standard media (SYA). TAG levels are normalised to total mg protein. n=6 per condition. Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with one-way ANOVA and a Dunnett's multiple comparisons test. ***, $P \leq 0.001$ for each comparison.

3.3. Discussion

A number of clinical studies have described an association between plasma ADMA levels and obesity/insulin resistance (Eid et al., 2004; McLaughlin et al., 2006; Palomo et al., 2011; Stühlinger et al., 2002). Although positive correlations between ADMA and adiposity have been described, a causal relationship has not been demonstrated. To address this, adipocyte-specific *DDAH1* knock-out mice were developed by the Leiper lab to increase ADMA levels *in vivo* by reducing its metabolic clearance. These mice exhibited a significant increase in fat mass compared to controls, implicating ADMA in the regulation of adiposity. ADMA was also found to induce adipocyte hypertrophy and increased lipid storage *in vitro* (Dowsett, 2014 - PhD thesis), corroborating these findings. In light of these results, the initial aim of this thesis was to utilise the genetically amenable model organism, *Drosophila*, to explore the role of ADMA in the regulation of adiposity.

In this first results chapter, I identify *CG1764* as the likely orthologue of mammalian *DDAH1*. The deletion of *CG1764* in *DDAH^{XP}* animals results in elevated ADMA levels suggesting that: (i) *CG1764* is likely the *Drosophila* orthologue of *DDAH1* and (ii) *DDAH^{XP}* animals could be used as a model to explore the role of elevated ADMA in adiposity. Throughout this chapter, comprehensive phenotypic characterisation of *DDAH^{XP}* animals was carried out revealing striking metabolic phenotypes including a reduction in fat storage, increased mitochondrial content and elevated levels of respiration (indicated by elevated CO₂ production). The altered metabolism of *DDAH^{XP}* animals also appears to be protective against HFD-induced shortened lifespan. Many health complications associated with high calorie diets can be attributed to ectopic lipid storage and the accumulation of harmful lipid species as adipocytes exceed their storage capacity. Therefore, reduced ectopic lipid storage could potentially contribute to the improved survival of *DDAH^{XP}* animals on HFD given their low TAG levels.

The reduced TAG storage observed in *DDAH^{XP}* was somewhat surprising, given that elevated ADMA was predicted to increase adiposity. I propose an explanation for this apparent phenotypic inconsistency in the third results chapter. Although these observations were unexpected, given the striking changes in energy homeostasis observed in *DDAH^{XP}*, I wished to investigate the underlying mechanism.

To begin to address this question, I assessed the expression of key genes involved in lipid metabolism in *DDAH^{XP}* animals. This revealed either no transcriptional change or down regulation of genes involved in both lipogenic and lipolytic pathways indicating reduced TAG turnover. These findings led me to speculate that increased energy expenditure could limit the need to store TAG, as energy is burned before it can be stored. This idea was supported by findings indicating increased mitochondrial content and high levels of CO₂ production in *DDAH^{XP}* animals. Given that mitochondrial respiration and locomotor activity are predicted to be tightly linked in *Drosophila*, these observations naturally raised the question of whether increased locomotor activity could contribute to increased energy expenditure. Contrary to this expectation however, *DDAH^{XP}* animals were in fact less active. These findings appear to separate mitochondrial respiration from locomotor activity, supporting the notion of elevated uncoupled oxidative phosphorylation. This reduced efficiency of oxidative phosphorylation could lead to increased mitochondrial biogenesis and elevated levels of energy expenditure in order to maintain sufficient ATP production. Markers of mitochondrial biogenesis (e.g., downstream targets of PGC-1 α) could be assessed in the future to draw further conclusions.

CG1764 was previously identified in a study from the Rosbash lab exploring the function of circadian neurons (Kadener et al., 2006). The authors ablated a major population of clock neurons by expressing a neurotoxic protein under the control of a *tim-gal4* (*timeless*) driver. *CG1764* was among the highly upregulated targets identified, suggesting a potential role in circadian signalling. *DDAH^{XP}* animals exhibit a number of phenotypes that could appear to implicate *DDAH* in circadian regulation including the loss of a diurnal pattern of CO₂ production, altered sleep and the lack of morning anticipation (a classical circadian behaviour). In addition, the expression of *CG1764* (*DDAH*) was found to be cyclic. However, it could be argued that many of these phenotypes are instead a product of their highly active metabolic states and low TAG reserves. Higher mitochondrial respiration could explain elevated levels of CO₂ production regardless of locomotor activity and the increase in sleep and lack of morning anticipation could simply be a way of conserving energy. With the data at hand, this metabolic first, sleep/activity second hypothesis seems most plausible. However, with *CG1764* being transcriptionally implicated in circadian networks (Kadener et al., 2006), more work is required to draw definitive conclusions.

In summary, data from Chapter 3 draws the following key conclusions:

- (i) A *Drosophila* model, $DDAH^{XP}$, is established for studying the role of elevated ADMA in adiposity.
- (ii) $DDAH^{XP}$ animals exhibit metabolic phenotypes including a reduction in fat storage, increased mitochondrial content and elevated levels of respiration.
- (iii) Elevated uncoupled oxidative phosphorylation could contribute to increased energy expenditure and low TAG levels in $DDAH^{XP}$ animals.

Chapter 4. Generation of novel transgenic lines to study DDAH in *Drosophila*

4.1. Introduction

In Chapter 3, I established a *Drosophila* model, *DDAH^{XP}*, to study the effects of elevated ADMA on adiposity. I found that *DDAH^{XP}* animals exhibit a strong reduction in fat storage and presented evidence supporting the idea that *DDAH^{XP}* animals have elevated levels of mitochondrial respiration. I proposed that elevated uncoupled oxidative phosphorylation could lead to the increased mitochondrial respiration and reduced TAG storage observed in *DDAH^{XP}* animals. Here, I develop genetic tools to help gain a mechanistic understanding of role of *DDAH* in energy homeostasis. These include a transcriptional reporter, an N-terminal GFP fusion expressing allele, a *DDAH-GAL4* driver and two different LOF alleles; one knock-in only (i.e., no deletion) and one open reading frame (ORF) deletion. Given the unexpected lean phenotype of *DDAH^{XP}* (where an increase in adiposity was predicted), it was deemed important to generate independent LOF alleles to ensure phenotypic consistency. *DDAH^{XP}* was generated by the imprecise excision of a *P*-element resulting in a ~2 kb deletion removing the majority of *CG1764*. The proximity of the breakpoint of *DDAH^{XP}* to a nearby adjacent gene, *CG1622*, was of concern (Fig. 4.1). In Chapter 4, I use targeted approaches to generate LOF alleles designed to minimise any potential impact on the expression of *CG1622* by distancing the insertion/breakpoint from this gene. Only small changes in fat storage were observed following the knockdown of *CG1764* by RNAi. Although this could be explained by insufficient knockdown (residual enzyme sufficient for ADMA clearance), it highlights the importance of generating new LOF alleles for the study of *DDAH* gene function. I devised a strategy based on CRISPR/Cas triggered homologous recombination to generate multiple tools from a single site-specific integration event. This strategy removes the need for the multiple independent integration events that traditional CRISPR-based strategies would require, significantly reducing the time and cost needed to generate these transgenic lines.

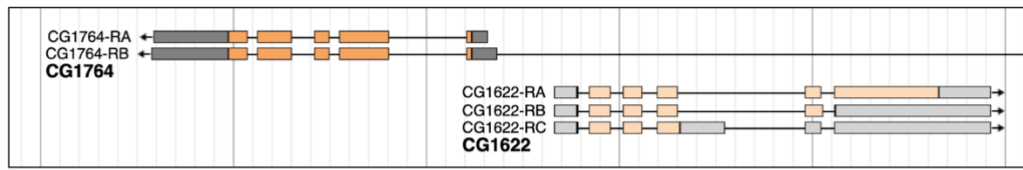
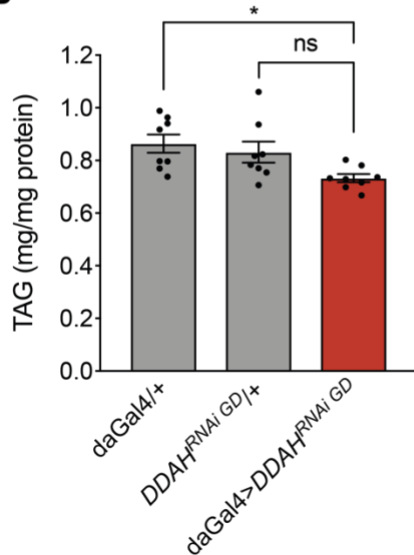
A**B**

Figure 4.1 Rationale for generating an independent *DDAH* LOF allele

(A) A schematic representation of the genomic locus showing the proximity between *CG1764* and the neighbouring gene *CG1622*. Map downloaded from FlyBase (JBrowse). (B) RNAi knock-down of *DDAH* resulted in only small changes in fat storage. TAG levels from da-GAL4>*DDAH*^{RNAi GD} and control (*w*¹¹¹⁸, da-GAL4/+, *DDAH*^{RNAi GD}/+) whole adult males (day 7). n=8 per condition. Results are shown as mean ± SEM. Statistical analyses were carried out with one-way ANOVA and a Dunnett's multiple comparisons test. ns, P>0.05; *, P≤0.05.

4.2. Results

DNA fragments, including sequences encoding protein tags and integration sites, were introduced in the *Drosophila* genome by Cas9-triggered homologous recombination. Subsequent genome modifications were made using PhiC31-mediated integration (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Venken & Bellen, 2007) and Cre-Lox recombination. The donor plasmids were generated by Gibson Assembly (Gibson et al., 2009).

4.2.1. Generating a *DDAH* loss-of-function allele combined with a GFP transcriptional reporter, *DDAH*^[N-GFP;STOP], and an N-terminal DDAH-GFP fusion, *DDAH*^[N-GFP], from a single site-specific insertion

A strategy based on CRISPR/Cas triggered homologous recombination was devised to generate a LOF mutant, a transcriptional reporter, and GFP-tagged protein, all from a single site-specific integration event (Fig. 4.2). Importantly, this strategy generates multiple genetic tools with a single round of injections and is therefore time- and cost-effective. With this strategy, no genomic DNA is deleted, minimising the risk of disrupting adjacent genes. A donor template and a gRNA expression vector (both described below) were co-injected into the germline of a Cas9 expression strain (*nos-Cas9*). The guide RNAs bring the Cas9 endonuclease to the 5'-NGG-3' motifs (PAMs), where cleavage occurs. The donor template (targeting vector) contains the desired insert flanked by two homology arms, ~1 kb each (Fig. 4.2). Homology-directed repair is initiated by the presence of the double strand breaks in the DNA, resulting in the introduction of the insert into the genome. *DDAH*^[N-GFP;STOP], which serves as a LOF allele and transcriptional reporter, can subsequently be converted into an N-terminal DDAH-GFP fusion allele by the excision of a LoxP-flanked Pax-Cherry cassette (Fig. 4.2).

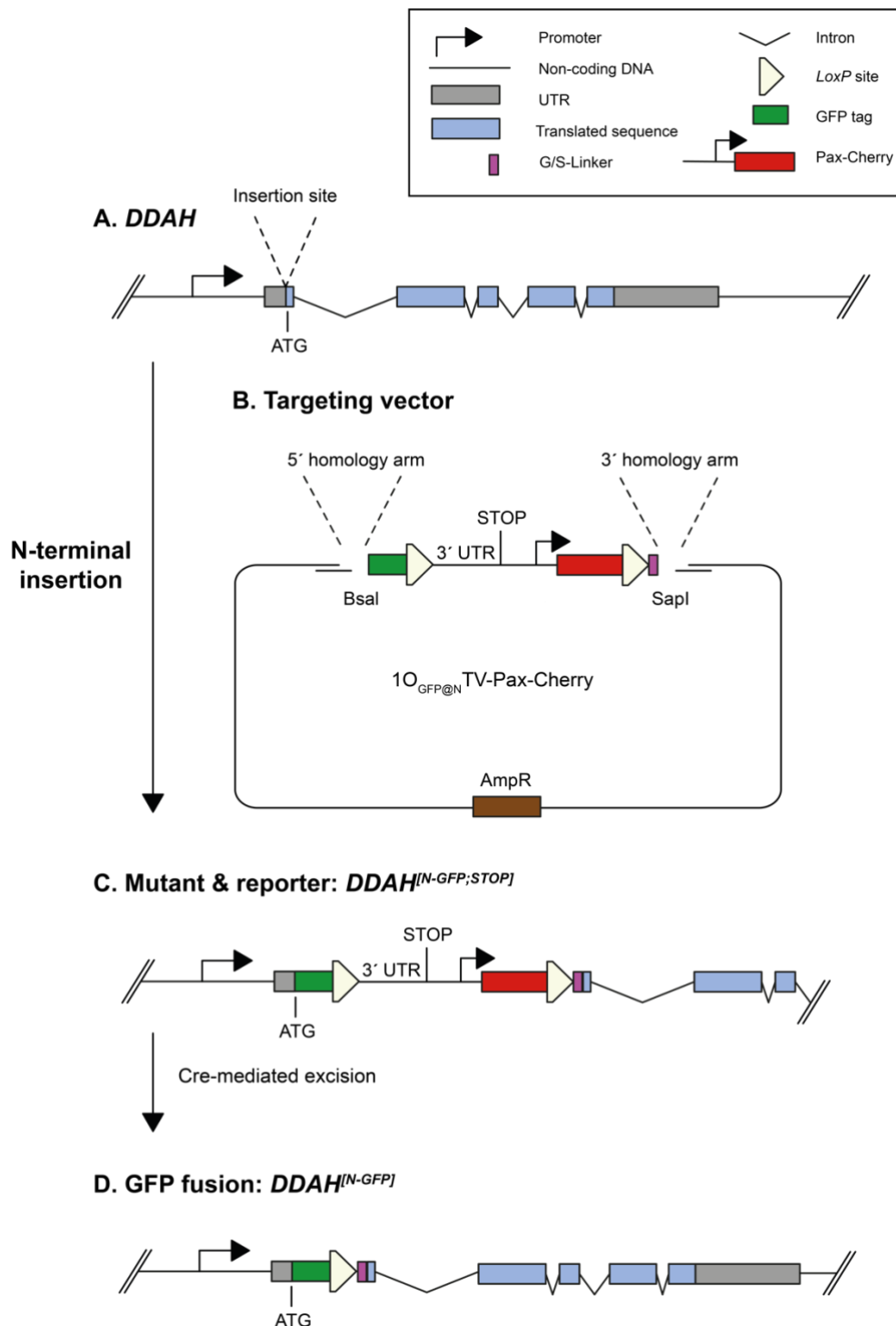


Figure 4.2 Schematic representation of the CRISPR/Cas strategy used to generate a LOF mutant combined with a transcriptional reporter and GFP-tagged protein from a single site-specific insertion

(A) A schematic representation of the *DDAH* locus, with coding exons in blue and untranslated sequences in grey. (B) Targeting vector for N-terminal insertion using 10GFP@NTV-Pax-Cherry (Poernbacher et al., 2019), where homology arms are inserted either side of the sequence introduced into the genome. (C) Following integration, the resulting allele harbours the Cre-excisable region comprising the termination codon and the *Pax-Cherry* cassette. This is expected to disrupt expression of the targeted gene. GFP will be expressed in the pattern of the targeted gene. (D) Cre-mediated excision fuses the reporter to the 5' end of the coding sequence, resulting in the expression of an N-terminal fusion protein with an intervening LoxP and G/S linker. Injections were carried out by Joachim Kurth at the Francis Crick Institute. Candidates were sequenced by A J. Vincent.

4.2.2. Generation of the *DDAH* loss-of-function and GFP transcriptional reporter allele, *DDAH*^[N-GFP;STOP]

4.2.2.1. gRNA expression vector

Suitable target sequences located 3 bp and 35 bp upstream of the *DDAH* translation initiation codon (Fig. 4.3A) were identified using flyCRISPR target finder (<https://flycrispr.org/>; Gratz et al., 2013) and checked for possible off-targets. The genomic region containing the target sites was sequenced in the *nos-Cas9* stock to verify that no polymorphisms were present in the target sequences. The target sequences actgggtgctcgtcctcgg (T1) and TGTACTTGGGCGACATggt (T2) were then cloned into the gRNA expression vector pCFD4_U6:1-gRNA-U6:3-gRNA (referred to as pCFD4; addgene, #49411) by Gibson Assembly (Fig. 4.3).

The plasmid pCFD4 contains two RNA polymerase III promoters (U6:1 and U6:3), each followed by a *BbsI* spacer and a gRNA scaffold (Fig. 4.3B,C). The spacers were replaced with the target sequences by PCR using the oligos pCFD4_T1_F_DDAH_N-GFP and pCFD4_T2_R_DDAH_N-GFP (Appendix 1.1) and using pCFD4 as a template (Fig. 4.3B-E). In parallel, pCFD4 was digested with *BbsI* and, after gel extraction of both the PCR product (the insert) and pCFD4 digested backbone, the fragments were assembled by Gibson cloning (Fig. 4.4). NEB-recommended concentrations of each fragment were followed for optimal assembly, except that a 3-fold molar excess was used for inserts. The following formula was used for calculations: pmols = (weight in ng) x 1,000 / (length in bp x 650 Da). The assembled plasmid was then transformed into NEB 5-alpha competent *E. coli* cells and initially screened by colony PCR before sequencing. Plasmid DNA was isolated (QIAGEN midiprep) and co-injected with the targeting vector into the germline of *nos-Cas9* animals. Injections were carried out by Joachim Kurth at the Francis Crick Institute. Following injection, candidates were screened for the presence of Pax-Cherry by fluorescence microscopy before being sequenced.

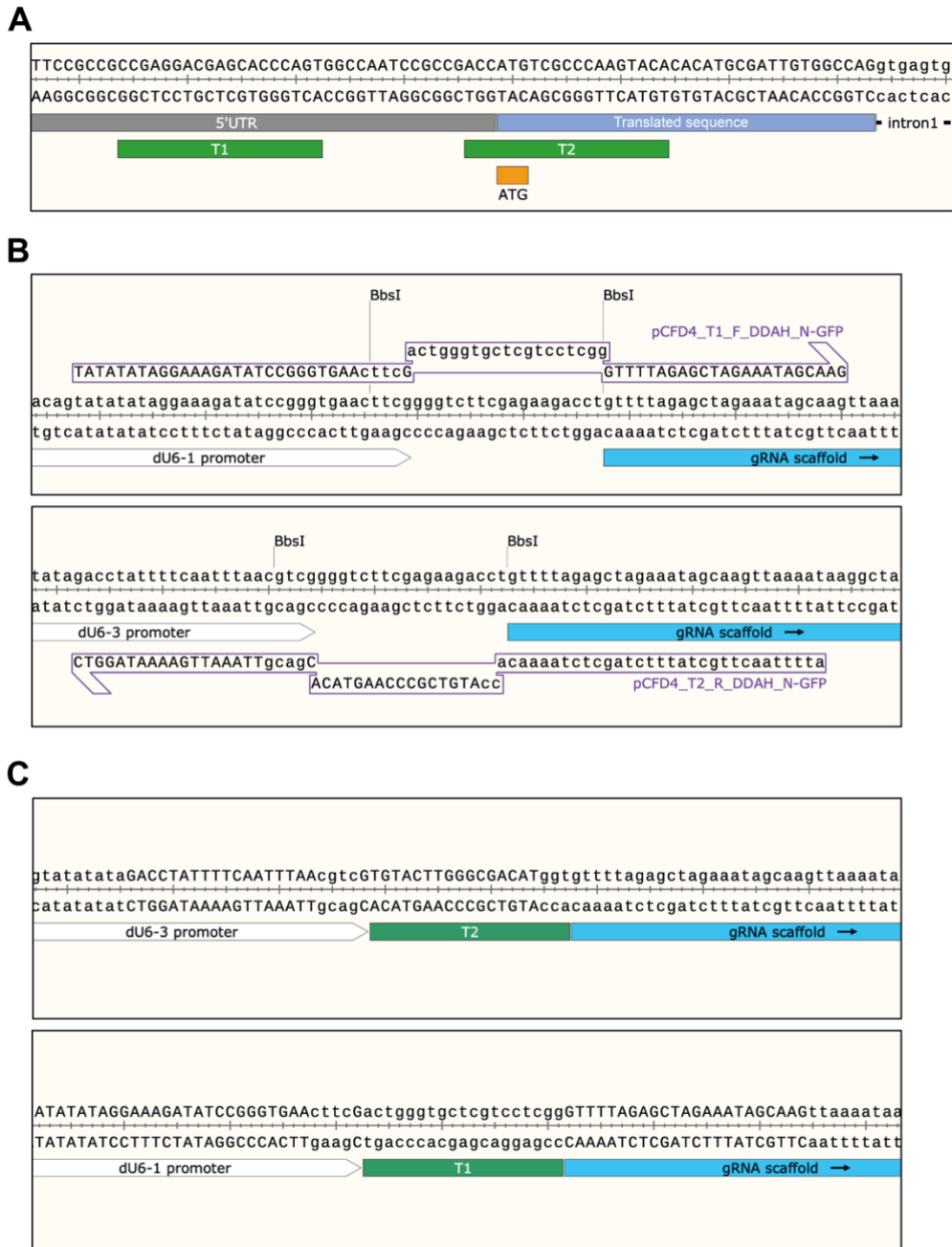


Figure 4.3 SnapeGene screenshots illustrating the generation of the pCFD4 insert containing T1 and T2

(A) T1 and T2 are located 3 bp and 35 bp upstream of the *DDAH* translation initiation codon. (B-E) pCFD4 contains two *BbsI* spacers that are replaced by T1 and T2. (B) T1 and T2 are included in primer design. (C) T1 and T2 are introduced by PCR using oligos pCFD4_T1_F_DDAH_N-GFP & pCFD4_T1_R_DDAH_N-GFP and pCFD4_T2_F_DDAH_N-GFP & pCFD4_T2_R_DDAH_N-GFP respectively to generate the insert for Gibson assembly.

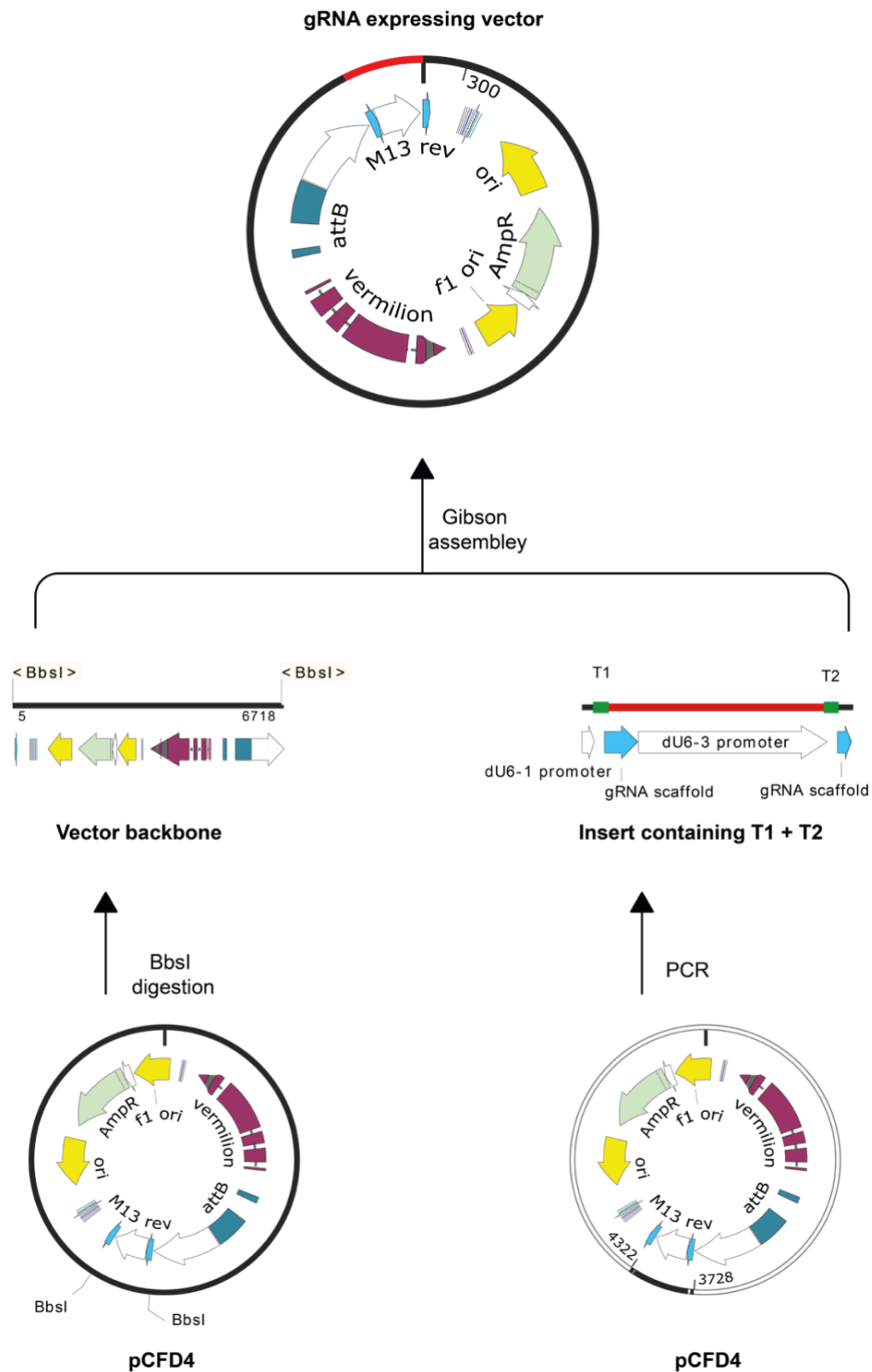


Figure 4.4 Schematic representation of gRNA expressing vector cloning using pCFD4

The vector backbone is generated by restriction digestion of pCFD4 with *BbsI*. The insert is generated by PCR using pCFD4 as a template. Fragments are assembled by Gibson assembly.

4.2.2.2. Targeting vector (donor template)

The 1OGFP@NTV-Pax-Cherry plasmid (Poernbacher et al., 2019) was used to generate the donor template by cloning ~1 kb homology arms either side of the sequence to be inserted. This sequence consisted of the GFP coding sequence, followed by a Cre-excisable Pax-Cherry genetic marker, and a glycine-rich linker (Fig. 4.2B). Homology arms were designed to insert the coding sequence of GFP directly downstream of the *DDAH* translation start codon, which is expected to result in the expression of GFP under control of the authentic *DDAH* promoter (Fig. 4.2C). Integration of this construct is also expected to inactivate *DDAH* gene function due to the presence of the transcription termination signal from the Pax-Cherry cassette (Fig. 4.2C). The presence of the Pax-Cherry genetic marker also facilitates screening and subsequent tracking of the allele.

Importantly, *DDAH*^[N-GFP;STOP] can be converted to an N-terminal DDAH-GFP fusion protein expressing allele by the excision of the LoxP-flanked Pax-Cherry cassette with Cre recombinase (expressed from a transgene) (Fig. 4.2C,D). Both the GFP coding sequence and the LoxP sites were designed to be in-frame with the DDAH translation start codon. An N-terminal tag was chosen for multiple reasons. Firstly, C-terminal tags are reported to disrupt protein folding of mammalian DDAH, while N-terminal GFP and GST tags have a minimal impact on the enzyme activity of DDAH1 (James Leiper, personal communication). Secondly, the C-terminus of *Drosophila* DDAH is predicted to form part of the active site (Fig. 3.3). Therefore, based on domain predictions and sequence homology, an N-terminal GFP tag was expected to be least disruptive for *Drosophila* DDAH. A glycine-rich linker was also included to minimise any potential interference of the GFP tag on DDAH protein localisation or function.

The homology arms were generated by PCR using primer pairs HR1F_DDAH_N-GFP & HR1R-4bp_DDAH_N-GFP and HR2F_DDAH_N-GFP & HR2R-4bp_DDAH_N-GFP (Appendix 1.1), using genomic DNA from the *nos-Cas9* strain as a template. These primers were designed to generate homology arms with the necessary overlap regions for Gibson assembly with *Bsa*I- and *Sap*I-digested 1OGFP@NTV-Pax-Cherry. Primers were designed to ensure overhang overlaps ≥16 bp, with a T_m ≥ 48°C and CG content of 40-60%. Gene-specific regions were between 18-25 nt with normal primer parameters (checked with bioinformatic.org). Primer design for assembly with restriction enzyme-digested vectors generally include overlap regions which line up

with the 3' end of the digested vectors or inserts. The T5 exonuclease used in Gibson assembly digests DNA from 5'-3', consequently 5' overhangs from restriction digested fragments are lost during assembly and are not present in the final product. In some instances (e.g. *BsaI*-digested GFP fragment), it is necessary to keep the 5' overhangs as they contain essential nucleotides (the first 4 nucleotides of GFP) (Fig. 4.5B). In this case, the 5' overhangs are reintroduced in the primer design (Fig. 4.5A,B). The digested 1OGFP@NTV-Pax-Cherry backbone, GFP-containing fragment and both homology arms were then assembled by Gibson Assembly (Fig. 4.6) using similar calculations as for pCFD4, with adjustments for the size and number of fragments used. Plasmids were screened by colony PCR before sequencing. Plasmid DNA was then isolated (QIAGEN midiprep) and co-injected with the gRNA expression vector into the germline of *nos-Cas9* animals. Injections were carried out by Joachim Kurth at the Francis Crick Institute. Following injection, candidates were screened for the presence of Pax-Cherry by fluorescence microscopy before being sequenced.

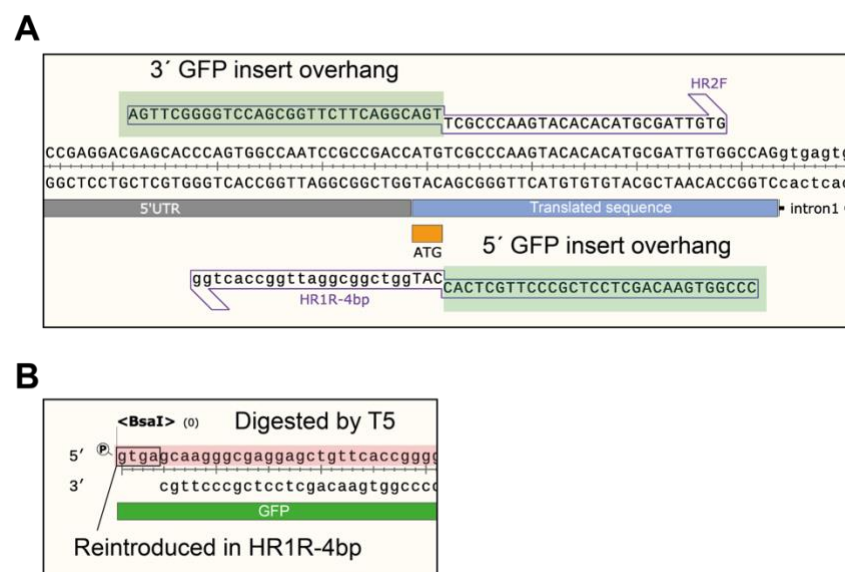


Figure 4.5 Schematic illustrating the generation of the GFP insert

(A) HR2F and HR1R-4bp contain overhangs necessary for assembly with the GFP (sequence) containing insert. Following Gibson assembly, the GFP (sequence) containing fragment will be inserted in between these overlap regions. The overhang of HR1R-4bp contains the first coding nucleotides of GFP. Overhangs are highlighted in green **(B)** During Gibson assembly, the 5' overhang of *BsaI*-digested GFP fragment is lost. The sequence digested during Gibson assembly by T5 is highlighted in pink. The first 4 coding nucleotides of GFP (gtga) are reintroduced in HR1R-4bp.

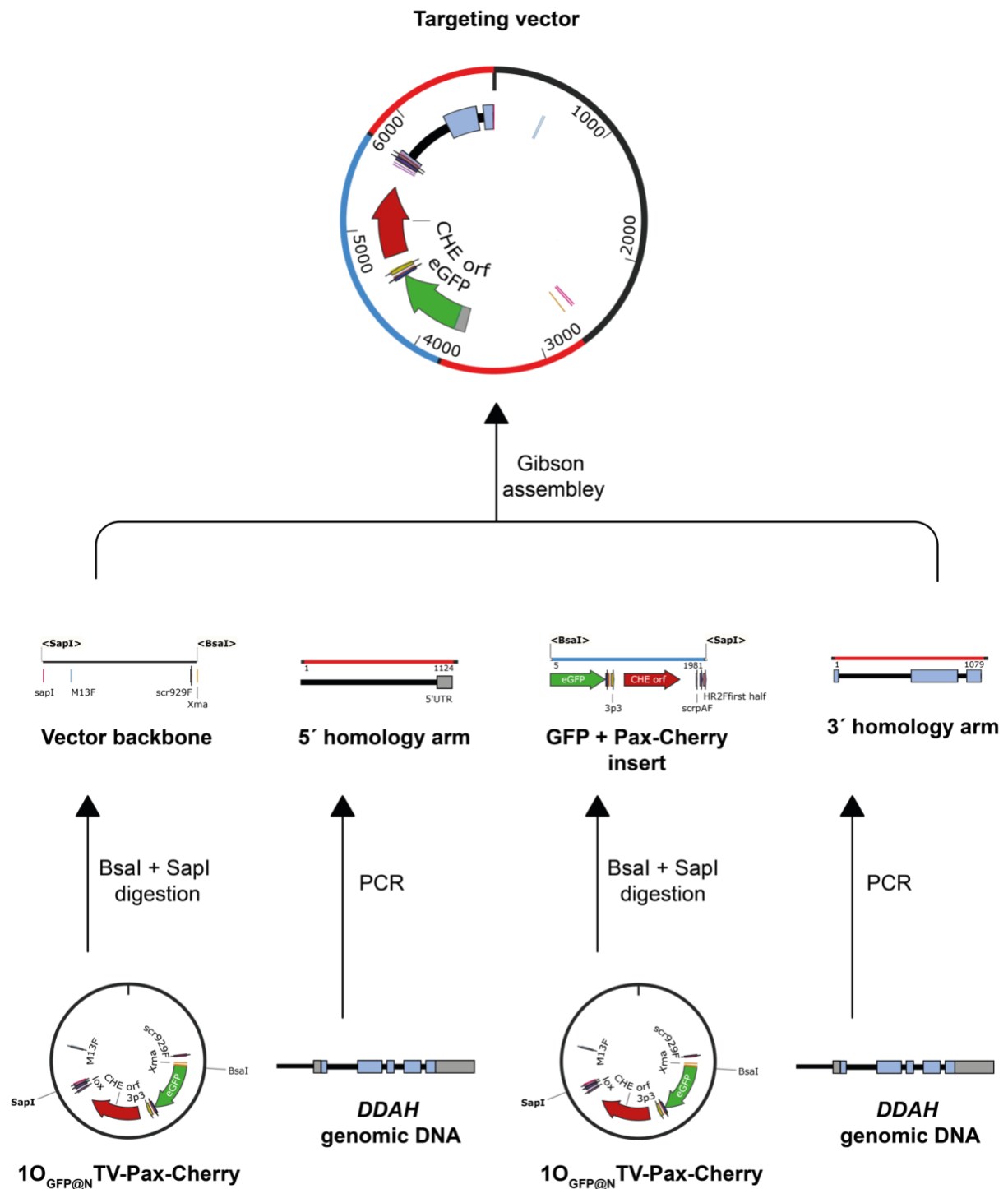


Figure 4.6 Schematic illustrating the generation of the targeting vector using 10GFP@NTV-Pax-Cherry

The vector backbone is generated by restriction digestion of 10GFP@NTV-Pax-Cherry with *BsaI* and *SapI*. Homology arms are generated by PCR using genomic DNA template. Homology arms contain overlap regions for vector backbone and GFP + Pax-Cherry insert. Fragments are assembled by Gibson assembly.

4.2.3. Generation of the *DDAH* N-terminal GFP fusion reporter, *DDAH*^[N-GFP]

DDAH^[N-GFP;STOP] was converted to an allele expressing an N-terminal GFP-*DDAH* fusion protein by the excision of the LoxP-flanked Pax-Cherry cassette. This was achieved by crossing the *DDAH*^[N-GFP;STOP] line with a heat shock inducible ubiquitous Cre recombinase expressing line (*Cre*^{hs}). The resulting *DDAH*^[N-GFP] allele expresses an N-terminal GFP fusion protein from the endogenous *DDAH* locus (Fig. 4.2D), allowing the monitoring of *DDAH* protein abundance and localisation. Crucially, *DDAH*^[N-GFP;STOP] was designed for the GFP coding sequence to be in-frame with the *DDAH* ORF following Cre-Lox recombination.

4.2.4. Validation and phenotyping of *DDAH*^[N-GFP;STOP]

DDAH^[N-GFP;STOP] was designed to serve as a LOF allele, with gene inactivation predicted to arise from premature transcription termination. *DDAH*^[N-GFP;STOP] was initially validated by qPCR to assess the expression of *DDAH*. A significant reduction the *DDAH* mRNA (~3 fold) (Fig. 4.7A) was observed suggesting that transcription is indeed prematurely terminated. However, residual expression (presumably of a truncated message) suggests some degree of alternative transcription initiation downstream of the *Pax-Cherry* cassette via an unknown (cryptic) TSS. Whether this truncated message is translated is unknown. The first possible in-frame translation start site is 89 amino acids into the ORF (268 total), therefore, if translated, the chances of a functional protein being produced are slim. Taken together, this allele is likely to represent a *DDAH* LOF mutant. The *DDAH*^[N-GFP;STOP] allele therefore constitutes an alternative to the independently generated *DDAH*^{XP} allele. This was considered important due to the proximity of the breakpoint of *DDAH*^{XP} to the neighbouring gene, *CG1622*. A significant decrease in whole animal TAG levels was observed in *DDAH*^[N-GFP;STOP] animals (Fig. 4.7B), although this was significantly milder than the reduction observed in *DDAH*^{XP} animals (Fig. 4.7C). Due to phenotypic differences, a third allele, *DDAH*^{Δe2-5}, was generated by the deletion of *DDAH* coding exons 2-5 and the concomitant insertion of an integrase site. The *DDAH*^{Δe2-5} KO allele was designed to distance the breakpoint from *CG1622* to minimise any potential effects on gene activity, while still removing the majority of the *DDAH* ORF guaranteeing *DDAH* gene inactivation. In addition to being a KO allele, *DDAH*^{Δe2-5} was

used as an intermediate line for the generation the *DDAH-GAL4* driver line (described below; Section 4.3.2.3).

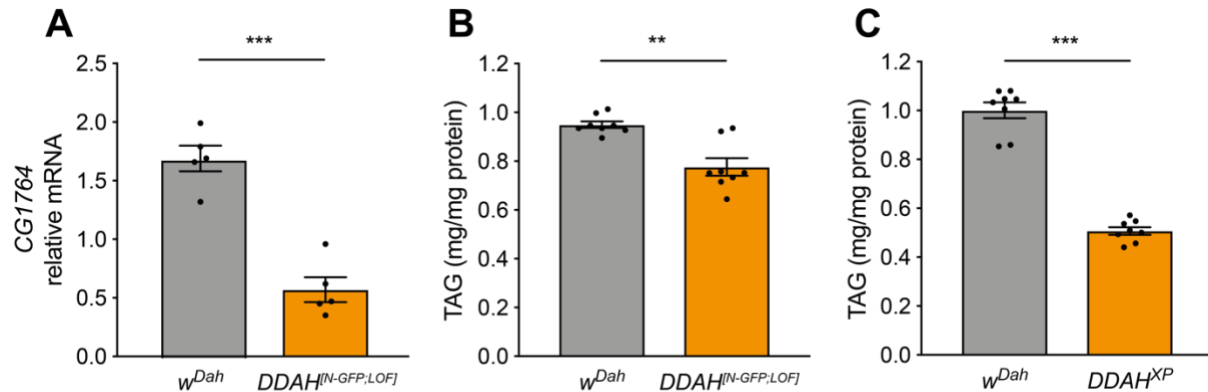


Figure 4.7 Validation and phenotyping of *DDAH^[N-GFP;STOP]*

(A) *CG1764* expression in *w^{Dah}* and *DDAH^[N-GFP;STOP]* whole adult males (day 7), as determined by qPCR. *n*=5 per condition. Samples were normalised to *alphaTub84B*. Results are shown as mean relative fold change $\pm$ SEM. Statistical analysis was carried out with an unpaired t-test. (B) TAG levels from *w^{Dah}* and *DDAH^[N-GFP;STOP]* whole adult males (day 7). *n*=8 per condition. TAG levels were normalised to total protein. Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with a Mann-Whitney test. (C) TAG levels from *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7). *n*=8 per condition. TAG levels were normalised to total protein. Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with an unpaired t-test. **, $P \leq 0.01$; ***, $P \leq 0.001$.

4.3.1. Generation of the ORF deletion allele with concomitant insertion of an integrase site, *DDAH^{Δe2-5}*

The ORF deletion allele was designed to ensure loss of gene function while distancing the deletion from a nearby adjacent gene, *CG1622*. Concomitant insertion of an integrase site (*attP*) allows for efficient subsequent insertions of genetic elements. Here, the *attP* site was used to insert the GAL4 transcriptional activator sequence at the locus to generate a *DDAH-GAL4* driver line. The *DDAH-GAL4* driver was primarily generated to be used as a transcriptional reporter by driving the expression of fluorescent proteins. This approach was considered necessary due to the low levels of *DDAH* expression and weak GFP signal from the *DDAH^[N-GFP;STOP]* and *DDAH^[N-GFP]* lines. GFP reporter lines have been engineered to contain between 1-40 UAS sites (Pfeiffer et al., 2010) or additional translational enhancers (Pfeiffer et al., 2012). Both types of modifications were reported to significantly amplify GFP signal (up to 20-fold). I therefore reasoned that the use of a *DDAH-GAL4* driver line in conjunction with one such modified GFP line would be a more sensitive reporter system for *DDAH*.

As with *DDAH*^[N-GFP;STOP], the deletion allele *DDAH*^{Δe2-5} was generated by Cas9-triggered homologous recombination. Homology arms were designed to recombine within the first intron and at the end of the last translated exon, resulting in the precise deletion of coding exons 2-5 (Fig. 4.8). Importantly, the first translated exon and half of the first intron were left in order to minimise impact of the deletion on the adjacent gene *CG1622*. The homology-directed repair replaced the deleted region with an insert containing an *attP* integrase site (Fig. 4.8). As before, the gRNA expression vector and donor template were co-injected into the germline of a Cas9 expression strain (Joachim Kurth, Francis Crick Institute). The *nos-Cas9* stock previously used for injections is relatively unhealthy. The *nos-Cas9-mSA* strain, which expresses Cas9 fused to a monomeric streptavidin, is healthier than the original *nos-Cas9* stock (Poernbacher et al., 2019), and was therefore chosen as the host strain.

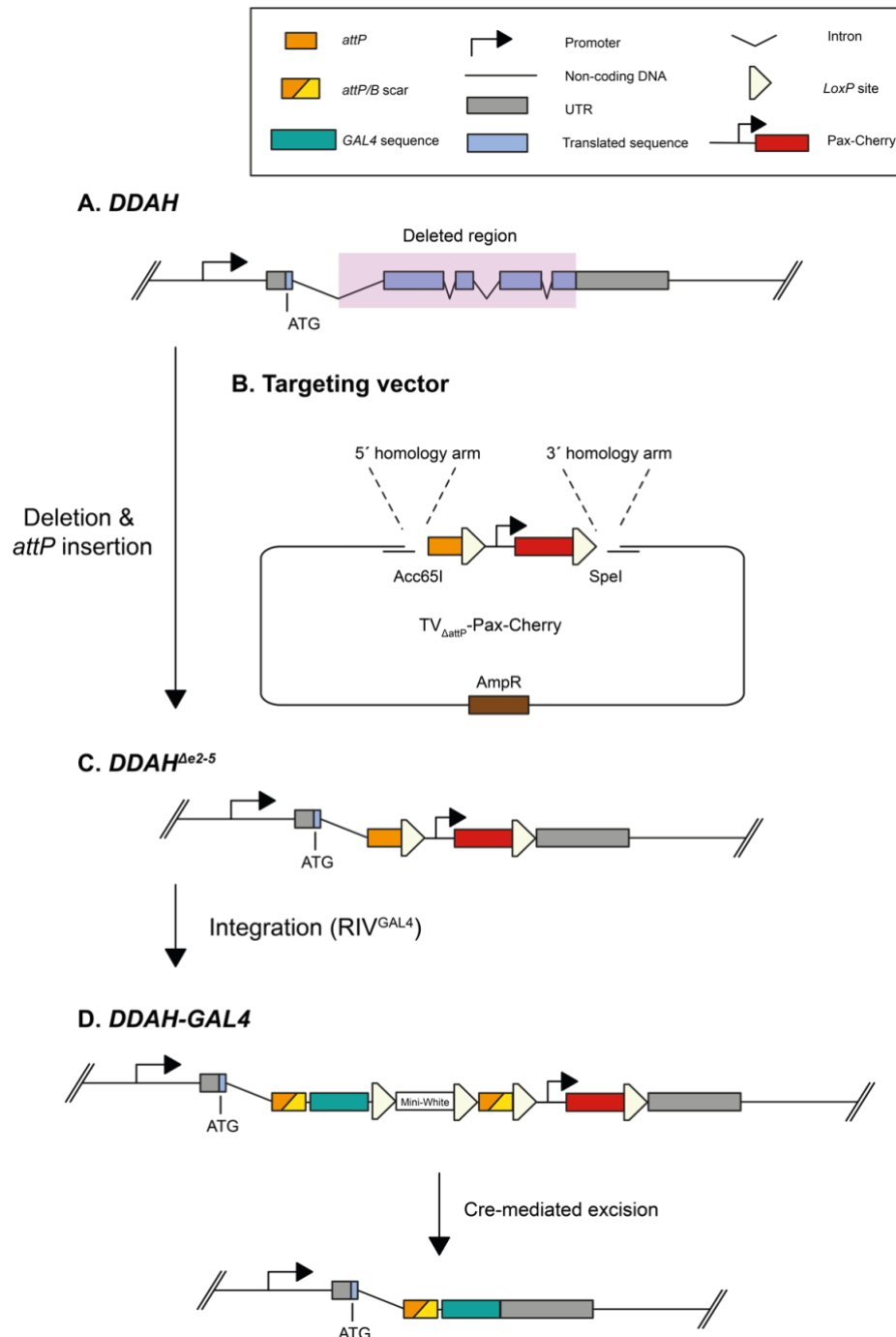


Figure 4.8 Schematic representation of the CRISPR/Cas strategy used to generate a clean exon deletion allele containing an *attP* site and subsequent the insertion of a GAL4 coding sequence

(A) A schematic representation of the *DDAH* locus, with coding exons in blue and untranslated sequences in grey. The deleted region is highlighted in pink (B) Targeting vector for the deletion of *DDAH* and insertion of an integrase site (*attP*) using TV_{ΔattP}-Pax-Cherry (Poernbacher et al., 2019). Homology arms are inserted either side of the sequence to be introduced into the genome. (C) Following integration, the resulting allele harbours the Cre-excisable *Pax-Cherry* cassette and an integrase site. (D) PhiC31 mediated integration using RIV^{GAL4}. The resulting allele harbours the *GAL4* coding sequence, the genetic marker *mini-white* in addition to the *Pax-Cherry* cassette. Both genetic markers (*mini-white* and *Pax-Cherry*) are Cre-excisable. (D) Cre-mediated excision of the genetic markers. Injections were carried out by Joachim Kurth at the Francis Crick Institute. Candidates were sequenced by A J. Vincent.

4.3.1.1. gRNA expression vector

Suitable target sequences and acttcaaaattggtatcctc (T1) and CTCGGCTCCCAGAGAGATT (T2) were identified and verified as for *DDAH*^[N-GFP;STOP], and cloned into the gRNA expressing vector pCFD4_U6:1-gRNA-U6:3-gRNA using the same protocol. The targets were chosen to induce double strand breaks within the first intron and directly downstream of the termination codon.

4.3.1.2. Donor template

TV Δ attP-Pax-Cherry (Poernbacher et al., 2019) was used to generate the donor template by cloning ~1 kb homology arms either side of the DNA to be inserted, which comprised an *attP* site, followed by a Cre-excisable genetic marker Pax-Cherry (Fig. 4.8). The homology arms were generated by PCR using primer pairs HR1F_DDAH_KO_attP & HR1R_DDAH_KO_attP and HR2F_DDAH_KO_attP & HR2R_DDAH_KO_attP (Appendix 1.1), using *nos-Cas9-mSA* genomic DNA as a template. These primers were used to generate homology arms with the overlap regions necessary for Gibson assembly with *Acc65I* and *SpeI*-digested TV Δ attP-Pax-Cherry. Primers were designed using the same parameters as for *DDAH*^[N-GFP;STOP] and fragments were assembled by Gibson Assembly as previously described (Section 4.2). After injection, candidates were screened for the presence of Pax-Cherry by fluorescence microscopy before being sequenced.

4.3.1.3. Generation of the driver line *DDAH-GAL4*

DDAH-GAL4 was generated by co-injection of a modified reintegration vector, RIV^{GAL4} (Baena-Lopez et al., 2013), and a plasmid encoding the PhiC31 integrase into the germ line of *DDAH* ^{Δ e2-5}. This resulted in the germline insertion of GAL4 cDNA within the first intron of *DDAH*. Thus, the expression of GAL4 is under control of the *DDAH* promoter at the endogenous locus. As the GAL4 cDNA is inserted within the first intron, the GAL4 protein is expected to bear the first 13 amino acids of the DDAH ORF at its N-terminus (Fig. 4.8). When designing *DDAH* ^{Δ e2-5}, the least invasive location for the insertion of the *attP* site was determined as an unconserved region in the middle to the first intron (Fig. 4.8) (sequence conservation not shown). This was to avoid disrupting the 5'UTR with the residual loxP site, and the attL and attR sequences after reintegration. Instead, artefacts within the first intron are expected to be removed following splicing. To avoid splicing issues, the missing genomic DNA comprising the

second half of intron 1 and the first 4 bp of exon 2 (containing the splice acceptor) were inserted into the RIV^{GAL4} upstream of the GAL4 cDNA. This was achieved by digesting RIV^{GAL4} and the genomic fragment with *EcoRI* and *SphI* (Promega) before ligating with NEB T4 DNA ligase. *EcoRI* and *SphI* restriction sites were introduced in the primers used to amplify the genomic fragment (second half on *DDAH* intron 1) using *nos-Cas9-mSA* genomic DNA as a template. *SphI* cuts 28 bp into the GAL4 ORF, therefore these bases were also included in the primer design. The modified RIV^{GAL4} was then transformed into NEB 5-alpha competent *E. coli* cells and screened by PCR before sequencing.

The *attP* site in *DDAH*^{Δe2-5} acts as a docking site for the *attB* site of RIV^{GAL4}. Recombination between *attB* and *attP* results in the integration of RIV^{GAL4} and therefore the insertion of the missing genomic sequence, followed by the GAL4 ORF, a *loxP* site and the genetic marker *mini-white*. *Mini-white* and the *Pax-Cherry* cassette can subsequently be excised via Cre-Lox recombination (Fig. 4.8D).

4.3.2. Validation and phenotyping of *DDAH*^{Δe2-5}

DDAH^{Δe2-5}, which lacks most of the *DDAH* ORF, was designed to provide an independent allele to *DDAH*^{XP} to investigate the role of *DDAH* in energy homeostasis. Surprisingly, no change in TAG levels were observed between *DDAH*^{Δe2-5} and control animals (Fig. 4.9A). No *DDAH* mRNA was detected by qPCR confirming the deletion of *DDAH* (Fig. 4.9B). *DDAH*^{Δe2-5} animals exhibited elevated ADMA levels (Fig. 4.9C) (although not statistically significant), consistent with the loss of *DDAH*. Similar changes in mean ADMA levels were observed between *DDAH*^{XP} and *DDAH*^{Δe2-5} animals and their respective controls (Fig. 4.9C). No significant difference in TAG levels were observed between transheterozygotes of the two alleles and *DDAH*^{XP} heterozygotes (Fig. 4.9D) suggesting the *DDAH*^{Δe2-5} allele does not contribute to the TAG phenotype observed in the transheterozygotes. No significant difference in TAG levels between *DDAH*^{XP} homozygotes and heterozygotes was observed (Fig. 4.9D) demonstrating the associated phenotype is dominant. Taken together, these results suggest that neither the loss of *DDAH* nor elevated ADMA levels are involved the TAG phenotypes observed in *DDAH*^{XP} animals. This raises the question of how the two alleles (*DDAH*^{XP} and *DDAH*^{Δe2-5}) differ, and how these differences are related to the phenotypic differences observed.

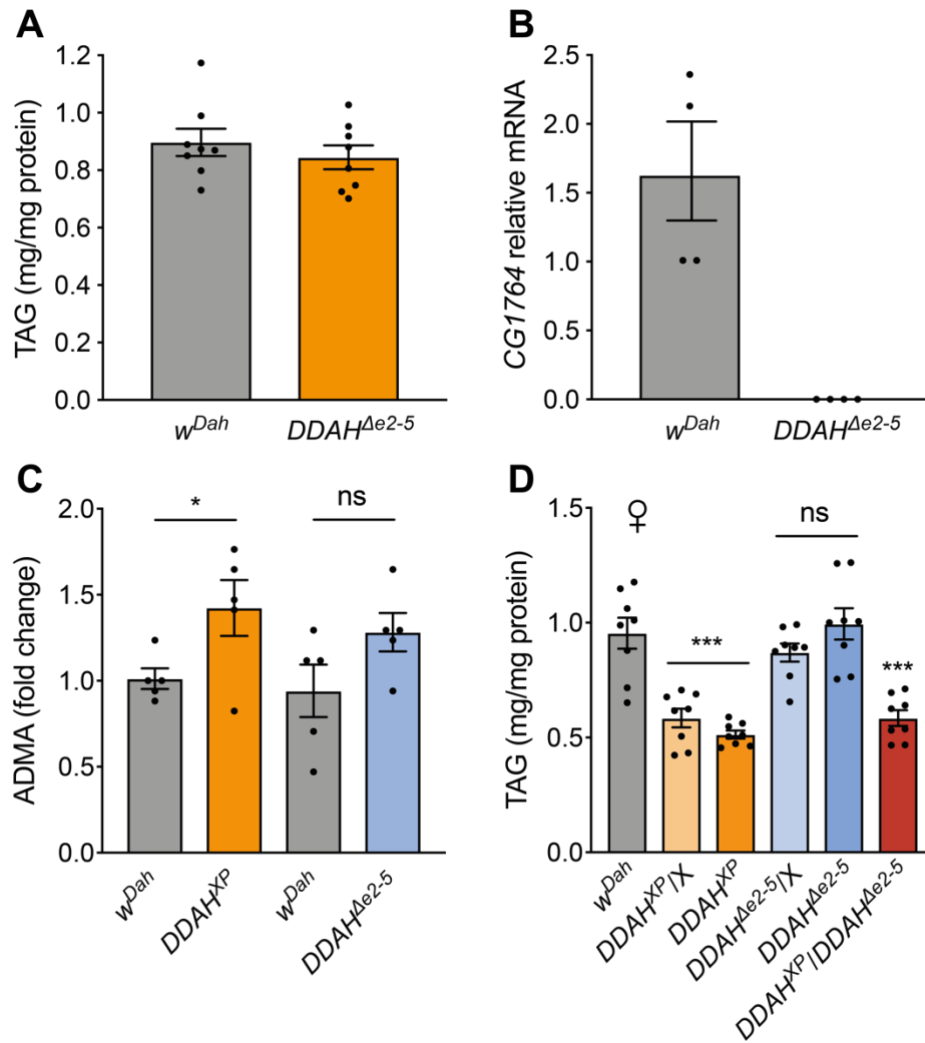


Figure 4.9 Validation and phenotyping of *DDAH^{Δe2-5} attP*

(A) TAG levels from *w^{Dah}* and *DDAH^{Δe2-5} attP* whole adult males (day 7). *n*=8 per condition. TAG levels were normalised to protein (mg/mg). Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with an unpaired t-test. **(B)** *CG1764* expression in *w^{Dah}* and *DDAH^{Δe2-5} attP* whole adult males (day 7), as determined by qPCR. *n*=4 per condition. **(C)** Targeted metabolomic analysis (ADMA) from whole adult males (day 7). *n*=5 per condition. All sample preparation for metabolomic data was performed by A J. Vincent. All metabolomic analyses were performed by the LMS Proteomics and Metabolomics core facility. **(D)** TAG levels from *w^{Dah}*, *DDAH^{XP}*, *DDAH^{Δe2-5} attP* (homozygotes, heterozygotes and trans-heterozygotes, day 7) *n*=8 per condition. TAG levels were normalised to protein (mg/mg). Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with a one-way ANOVA and a Dunnett's multiple comparisons test. ns, *P*>0.05; *, *P*≤0.05; ***, *P*≤0.001.

4.4. Discussion

In Chapter 4, I describe the generation of novel transgenic lines to explore the role of *DDAH* in the regulation of energy homeostasis. These include a number of reporter lines and two LOF alleles. The LOF alleles were deemed necessary due to (i) concerns over the proximity of the breakpoint of *DDAH*^{XP} to a neighbouring gene, *CG1622*, and (ii) the need for different, independently generated lines to ensure phenotypic consistency. Using a targeted approach, I was able to minimise any potential impact on the expression of *CG1622* by distancing the insertion/breakpoint from this gene.

To generate these lines, two different strategies were devised; both based on CRISPR/Cas triggered homologous recombination. The first being an insertion-only (i.e., no deletion) strategy, which allows the rapid simultaneous generation of a LOF mutant, a transcriptional reporter, and a GFP-tagged protein reporter from a single integration event. Generating these three alleles using traditional CRISPR based strategies would require three independent integration events (i.e., three rounds of injections) and the cloning of numerous gRNA expressing vectors and targeting vectors. Although this strategy generates a LOF allele in most cases (Pinheiro et al., 2017; Yamazaki et al., 2016; Yu et al., 2020), LOF cannot be guaranteed and may depend on the configuration of the target gene. In this design, LOF relies on the premature transcription termination resulting from the Pax-Cherry cassette. However, residual gene activity could arise from transcription re-initiation at cryptic TSSs downstream of the Pax-Cherry cassette (Poernbacher et al., 2019). Partial LOF is nevertheless expected, and incomplete gene inactivation still exhibits advantages over alternative methods of gene knockdown such as RNAi (avoiding off-target and insertion effects) and may suffice to infer gene function. Furthermore, a downstream in-frame translation initiation site would be required for the truncated mRNA to be translated. Therefore, even if downstream cryptic TSSs are present, the chances of a functional protein being translated are slim. In the case of *DDAH*, a heavily truncated protein would be expressed, as the first in-frame translation start site is 89 amino acids into the ORF (268aa total). Given that reporter lines of *DDAH* were required anyway, the LOF line would be generated at no extra cost. Therefore, I concluded that this strategy was the most efficient route to generate the necessary tools for the study of *DDAH*. In addition to being time and cost effective, the insertion only strategy has

another important advantage over deletion KOs – no DNA is deleted. This can minimise the disruption of neighbouring genes which was an important consideration for *CG1764* given the proximity of the adjacent gene *CG1622* (Fig. 4.1).

Initial phenotyping of *DDAH*^[N-GFP;STOP] (the allele resulting from the insertion only strategy) revealed a significant reduction in TAG levels, although this reduction was significantly milder than the one observed in *DDAH*^{XP} animals. Although *DDAH*^[N-GFP;STOP] likely constitutes a LOF allele, due to phenotypic differences between *DDAH*^{XP} and *DDAH*^[N-GFP;STOP] animals, a third *DDAH* null allele was generated, *DDAH*^{Δe2-5}. The deletion of *DDAH*^{Δe2-5} was more restricted than the larger *DDAH*^{XP} deletion, yet the majority of the ORF was still removed. This design was intended to minimise any potential impact of the deletion on the expression of *CG1622*, while still ensuring loss of *DDAH* gene function. Surprisingly, no change in TAG levels was observed between *DDAH*^{Δe2-5} and control animals suggesting that the deletion of *DDAH* is not responsible for the lean phenotype observed in *DDAH*^{XP} animals. *DDAH*^{XP} animals exhibit a dominant phenotype (Fig. 4.9B); this is somewhat surprising considering dominant LOF (haploinsufficiency) phenotypes are far less frequent than dominant gain of function (GOF) phenotypes. If the loss of *DDAH* was responsible for the lean phenotype, with a single WT *DDAH* allele in *DDAH*^{XP} heterozygotes, one might expect some reduction in the abundance of the enzyme and incomplete dominance at best. Nevertheless, one cannot exclude the possibility that a downstream signalling pathway could require an ADMA threshold to be met, generating a binary outcome. The elevated ADMA levels observed in *DDAH*^{Δe2-5} animals (to a similar degree as *DDAH*^{XP} animals), would contradict this idea. I therefore decided to consider the possibility that *DDAH*^{XP} could carry a GOF mutation affecting a different gene, which in turn would be responsible for the metabolic phenotypes observed. This will be discussed in Chapter 5.

Chapter 5. Characterisation of CG1622

5.1. Introduction

In Chapter 3, I showed that *DDAH^{XP}* animals exhibit a reduction in fat storage and have elevated levels of respiration. Following these findings, in Chapter 4, I described the generation of novel transgenic lines to gain mechanistic understanding into the role of *DDAH* in the regulation of energy homeostasis. Surprisingly, no lean phenotype was observed in the new *DDAH* ORF deletion line, *DDAH^{Δe2-5}*. I therefore concluded that the loss of *DDAH* is not responsible for the metabolic phenotypes observed in *DDAH^{XP}* animals. Both *DDAH^{XP}* and *DDAH^{Δe2-5}* are KO alleles lacking the *DDAH* ORF. Neither allele expresses *DDAH*, and both exhibit similar levels of ADMA, yet they differ phenotypically. This naturally raises the questions of how these two KO alleles differ, and how these differences are related to the phenotypic differences observed. In Chapter 5, I explore the genetic differences between the *DDAH^{XP}* and *DDAH^{Δe2-5}* KO alleles, and present evidence pointing towards the involvement of the adjacent gene, *CG1622*, in the metabolic phenotypes observed in *DDAH^{XP}* animals.

5.1.1. CG1622

CG1622 encodes a currently uncharacterised protein predicted to contain a pre-mRNA-splicing factor 38 (PRP38) domain. This domain was first identified in the PRP38 protein of yeast, which was reported to be required for pre-mRNA splicing (Blanton et al., 1992), and later for catalytic activation of the spliceosome (Xie et al., 1998). In addition to containing the predicted PRP38 domain, the protein encoded by *CG1622* was identified in proteomic analysis of *Drosophila* spliceosomal complexes (Herold et al., 2009), providing direct experimental evidence linking *CG1622* to splicing.

CG1622 is located directly adjacent to *CG1764* (*DDAH*), albeit in the opposite orientation (sequence location: X:13,297,082..13,299,910 [+]) (Fig. 5.1A). *CG1622* has three annotated transcripts producing three distinct polypeptides. The RA transcript is the most abundant, with the expression of RB and RC almost negligible (www.flyatlas2.org, Leader et al., 2018). High throughput studies show that *CG1622*

is expressed broadly in all tissues. In males, *CG1622* is most enriched in the testis and head. Expression was also found to be sexually dimorphic with expression being highest in females, which could be due to the large mass of the ovaries where *CG1622* is strongly enriched (modENCODE Tissue Expression Data, Brown et al., 2014; www.flyatlas2.org, Leader et al., 2018). High throughput temporal expression data (modENCODE, Graveley et al., 2011) suggest expression is highest during embryo development and also in adult females.

5.2. Results

5.2.1. Highly conserved intronic region that may represent *CG1622* *cis*-regulatory elements is lost in *DDAH^{XP}* but not *DDAH^{Δe2-5}*

DDAH^{XP} and *DDAH^{Δe2-5}* are both KO alleles lacking the *DDAH* ORF. However, they differ phenotypically: *DDAH^{XP}* animals exhibit a significant reduction in total TAG levels, which was not observed in *DDAH^{Δe2-5}* animals, suggesting that *DDAH* does not contribute to the metabolic phenotypes observed in *DDAH^{XP}*. Both alleles were backcrossed into the same outbred WT strain (*w^{Dah}*), excluding genetic background effects. To understand these phenotypic differences, I explored how the two alleles differ genetically, and more specifically whether the deletion in *DDAH^{XP}* animals could affect the expression of the adjacent gene, *CG1622*. The *DDAH^{XP}* deletion is larger, extending from the 5' UTR to the 3' UTR, while the *DDAH^{Δe2-5}* deletion is more restricted, spanning from the middle of the first intron to the end of the last translated exon (Fig. 5.1A). The aim was to remove as much of the *DDAH* coding sequence as possible, while minimising any potential effects on *CG1622*. Given that the proximity of the deletion in *DDAH^{XP}* to *CG1622* was a concern, I focused on the ~400 bp difference between the two alleles at the 5' end of *DDAH* (Fig. 5.1A, highlighted in green). The UCSC genome browser (<https://genome.ucsc.edu/>) was used to explore sequence conservation between insects within this region, which revealed a highly conserved intronic region (Fig. 5.1B). Given that conserved non-coding sequence are characteristic of *cis*-regulatory elements, I next used modENCODE ChIP-chip data (whole embryo) to search for transcription factor binding sites within the highlighted region. A number of sites were identified (Fig. 5.1C). Although from whole embryos (not adults), the ChIP-chip data indicate that the highlighted region could harbour *cis*-

regulatory elements. It is conceivable that the loss of these elements in *DDAH^{XP}* could be responsible for the metabolic phenotypes observed via the misexpression of the adjacent gene *CG1622*.

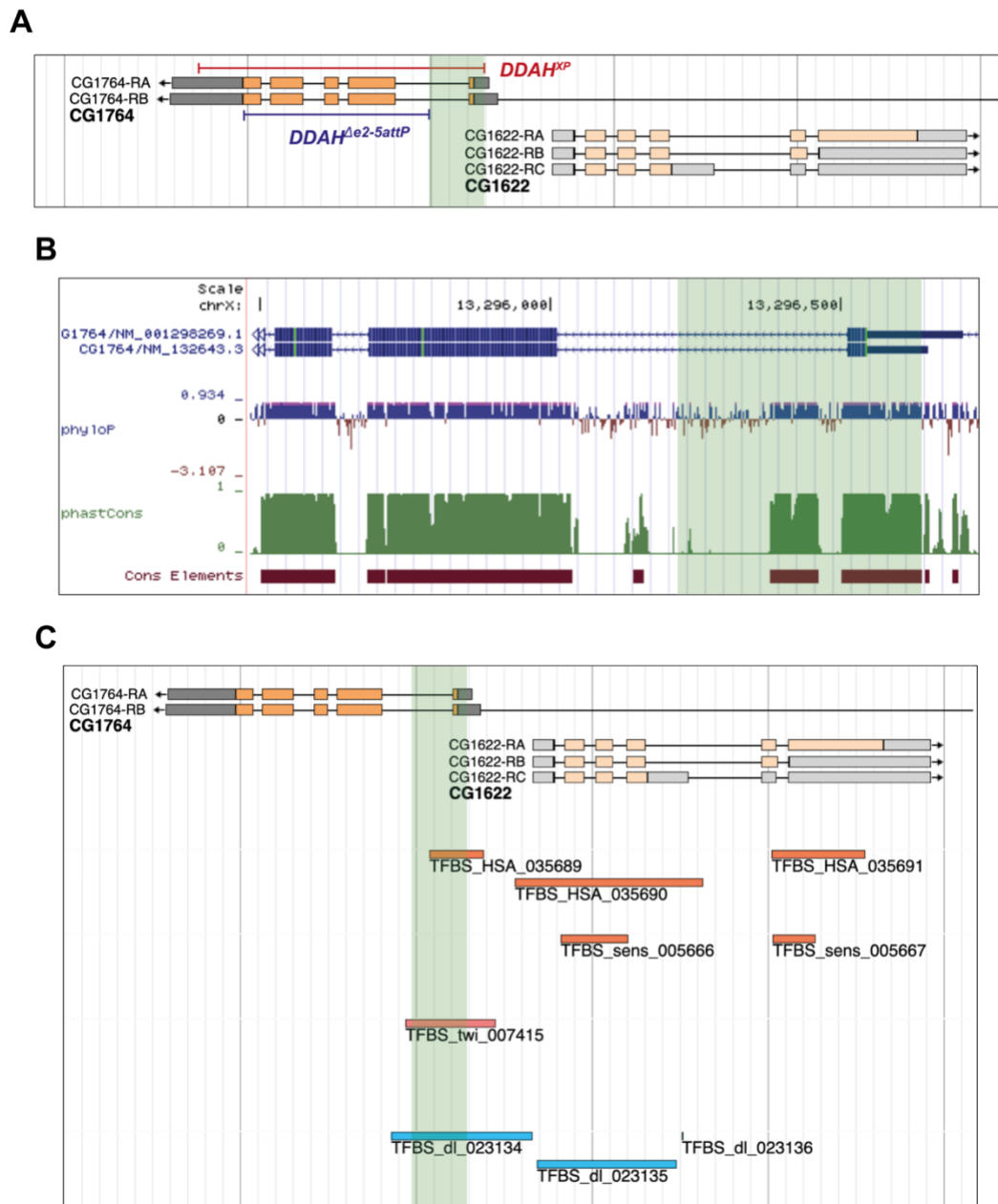


Figure 5.1 Exploring genomic differences between the *DDAH* KO alleles *DDAH^{XP}* and *DDAH^{Δe2-5 attP}*

(A) Schematic representation of the *DDAH* locus with the *DDAH^{XP}* deletion marked in red and the *DDAH^{Δe2-5 attP}* deletion in blue. A ~400 bp region at the 5' end of *DDAH* is deleted in *DDAH^{XP}* but not in *DDAH^{Δe2-5 attP}* (highlighted in light green). Adapted from J Browse (FlyBase). **(B)** A highly conserved intronic region is identified within the ~400 bp region of interest (highlighted in light green). Adapted from the UCSC Genome Browser. **(C)** A number of TF binding sites (modENCODE ChIP-chip) were identified within the ~400 bp region of interest (highlighted in light green). Adapted from J Browse (FlyBase).

5.2.2. *CG1622* is transcriptionally upregulated in *DDAH^{XP}* animals

To explore the hypothesis that the loss of *cis*-regulatory elements in *DDAH^{XP}* could result in the misexpression of the adjacent gene *CG1622*, I assessed the expression of *CG1622* in both *DDAH^{XP}* and *DDAH^{Δe2-5}* KO mutants by qPCR. *CG1622* was significantly upregulated (~2-fold) in *DDAH^{XP}* animals (Fig. 5.2A), suggesting that repressor elements may have been lost. These data lend support for a role of *CG1622* in the metabolic phenotypes observed in *DDAH^{XP}* animals. Upregulation of *CG1622*, as opposed to downregulation, also seems consistent with the dominant phenotype observed in *DDAH^{XP}* animals (Fig. 4.9B), as dominant GOF phenotypes are far more frequent than dominant LOF (haploinsufficiency) phenotypes.

CG1622 was also significantly upregulated in *DDAH^{Δe2-5}* animals, although upregulation was very mild compared to *DDAH^{XP}* (Fig. 5.2). It is possible that the deletion in *DDAH^{Δe2-5}* may still partially disrupt repressor binding, resulting in mild upregulation. This could also explain the intermediate phenotype observed in *DDAH^[N-GFP;STOP]* animals (Fig. 4.7B), which harbour an insertion between the breakpoints of *DDAH^{XP}* and *DDAH^{Δe2-5}*.

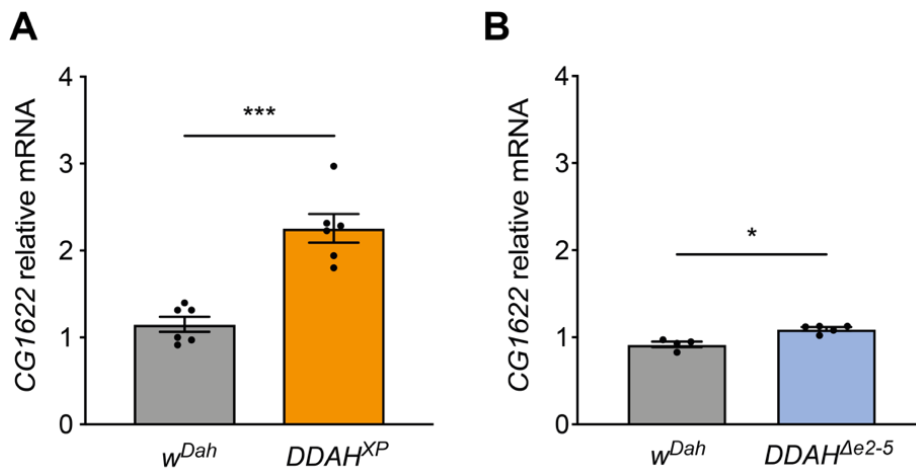


Figure 5.2 *CG1622* is transcriptionally upregulated in *DDAH^{XP}* animals

(A) *CG1622* expression in *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7), as determined by qPCR. n=6 per condition. (B) *CG1622* expression in *w^{Dah}* and *DDAH^{Δe2-5 attP}* whole adult males (day 7), as determined by qPCR. n=5 per condition. Samples are normalised to Rpl32 in both experiments. Results are shown as mean relative fold change $\pm$ SEM and analysed by unpaired t-test in both datasets. *, $P \leq 0.05$; ***, $P \leq 0.001$.

5.2.3. RNAi experiments implicate **CG1622** in energy homeostasis

Next, I explored whether *CG1622* could indeed play a role in energy homeostasis, and whether upregulation of *CG1622* in *DDAH^{XP}* animals could explain the metabolic phenotypes observed. I initially turned to the literature to search for any evidence linking *CG1622* to adiposity. Interestingly, *CG1622* was identified as an obesity-associated gene in two independent genome-wide RNAi screens (Baumbach et al., 2014; Pospisilik et al., 2010). To first confirm these results, I assessed total TAG levels in *CG1622* knockdown animals (adult day 7) using the same RNAi line used in the two screens (*CG1622^{RNAi GD}*). FB-GAL4>*CG1622^{RNAi GD}* males were assessed first and had significantly elevated TAG levels compared to all controls (Fig. 5.3A,B), consistent with the findings from the RNAi screens (Baumbach et al., 2014; Pospisilik et al., 2010). *CG1622^{RNAi GD}/+* (no driver control) animals also exhibited significantly elevated TAG levels compared to *w¹¹¹⁸* and FB-GAL4/+ controls, although the effect was milder than the one observed in FB-GAL4>*CG1622^{RNAi GD}* animals (Fig. 5.3A,B). It is possible that elevated TAG levels in *CG1622^{RNAi GD}/+* could be a result of 'leaky' expression (basal expression of the UAS line in the absence of GAL4). Alternatively, I cannot exclude the possibility that the random insertion of the GD RNAi construct by *P*-element-mediated transformation may have disrupted a locus that affects TAG levels. However, this seems less likely given that TAG levels increase in the presence of GAL4 (Fig. 5.3A,B).

Following the initial experiment with FB-GAL4>*CG1622^{RNAi GD}* males, I carried out more comprehensive investigations. TAG levels and starvation sensitivity were assessed in both males and females following knockdown of *CG1622* using a number of fat body drivers (*cg*-, FB-, *Lpp*- and *to*-GAL4) to drive the expression of *CG1622^{RNAi GD}*. In females, these experiments yielded mixed results with changes in TAG or starvation sensitivity observed using some drivers (*cg*- and *Lpp*-GAL4; Fig. 5.3C,D) but not others (FB- and *to*-GAL4; not shown). In contrast to my initial findings, no significant change in TAG levels or starvation sensitivity were observed from any condition in males including from FB-GAL4>*CG1622^{RNAi GD}* animals. The reason for this lack in reproducibility between experiments is unclear.

The effects of *CG1622* knockdown on TAG storage and starvation sensitivity differed significantly between fat body driver lines. To investigate the source of such

differences, I assessed the activity of these drivers by crossing them to a UAS-eGFP reporter line. The resulting expression patterns were surprisingly variable (Fig. 5.4), not only between the drivers but also between individuals within the same condition (Fig. 5.4C,D) and even patchy expression within a single animal (Fig. 5.4I). Oddly, the *to-GAL4* line cited in the literature as being a fat body driver was found to be exclusively active in the head in females (Fig. 5.4E). Such discrepancies could explain the phenotypic variability between conditions. This also serves as a reminder to be caution when choosing the most appropriate driver line for an experiment.

Taken together, these results appear to implicate *CG1622* in the regulation of energy homeostasis, however inconsistencies between repeats led me to assess the degree of knockdown in *CG1622* FB-GAL4>*CG1622*^{RNAi GD} animals. RNA was extracted from isolated abdomens to enrich for fat body tissue, and the expression of *CG1622* was determined by qPCR. No change in *CG1622* mRNA was observed in *CG1622* FB-GAL4>*CG1622*^{RNAi GD} animals (Fig. 5.3E). I reasoned that because endogenous expression of *CG1622* exhibits low tissue specificity, mRNA from other abdominal tissues could mask the knockdown of *CG1622* when using FB-GAL4. However, no change in *CG1622* mRNA was observed for RNA extracted from dissected fat body tissue (Fig. 5.3E). Without being able to reliably demonstrate knockdown of *CG1622* using *CG1622*^{RNAi GD}, I decided to stop using this line and instead turned towards other available RNAi lines targeting *CG1622*.

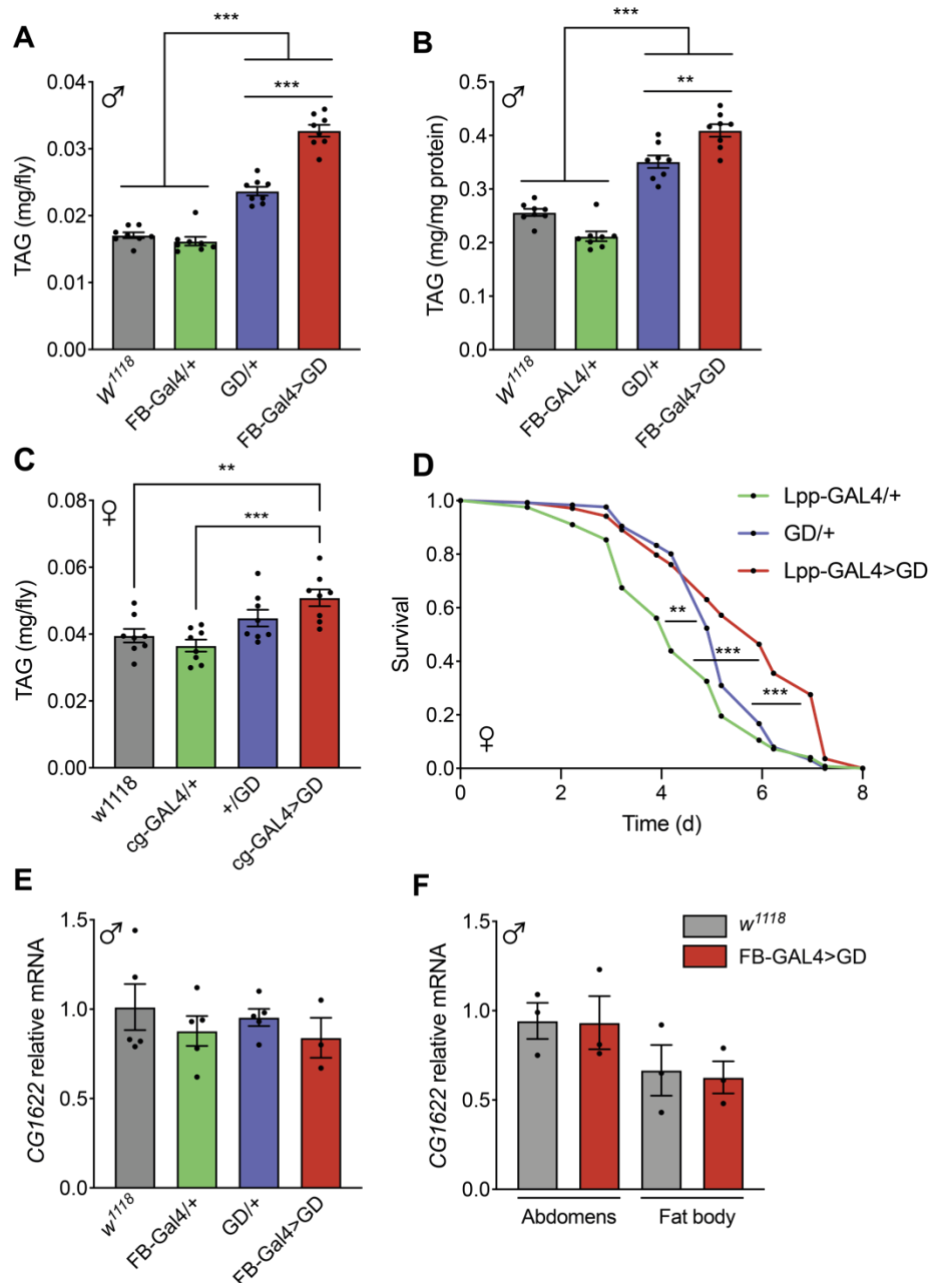


Figure 5.3 RNAi experiments implicate *CG1622* in energy homeostasis

(A, B) TAG levels from *FB-GAL4>CG1622^{RNAi GD}* and control (*w¹¹¹⁸*, *FB-GAL4/+*, *CG1622^{RNAi GD} /+*) whole adult males (day 7). *n*=8 per condition. Results are shown as mean $\pm$ SEM. Statistical analyses were carried out with one-way ANOVA and a Dunnett's multiple comparisons test. (A) TAG levels were normalised by the total number of animals (mg/fly) due to condition-dependent changes in protein levels. (B) TAG levels normalised to protein (mg/mg). (C) TAG levels from *cg-GAL4>CG1622^{RNAi GD}* and control (*w¹¹¹⁸*, *cg-GAL4/+*, *CG1622^{RNAi GD} /+*) whole adult females (day 7). *n*=8 per condition. Results are shown as mean $\pm$ SEM. Statistical analysis was carried out with one-way ANOVA and a Dunnett's multiple comparisons test. TAG levels were normalised by the total number of animals (mg/fly). (D) Survival of *Lpp-GAL4>CG1622^{RNAi GD}* and control (*Lpp-GAL4/+*, *CG1622^{RNAi GD} /+*) adult females. *n*=170-210 per condition. Statistical analysis was carried out with a Log-Rank test on survivorship data. (E) *CG1622* expression in *FB-GAL4>CG1622^{RNAi GD}* and control (*w¹¹¹⁸*, *FB-GAL4/+*, *CG1622^{RNAi GD} /+*) adult abdomens (day 7), as determined by qPCR. *n*=4-5 per condition. (F) *CG1622* expression in *FB-GAL4>CG1622^{RNAi GD}* and *w¹¹¹⁸* adult abdomens and dissected fat body tissue (day 7), as determined by qPCR. *n*=3 per condition. Samples are normalised to α Tub84B in both experiments. Results are shown as mean relative fold change $\pm$ SEM and analysed by unpaired t-test in both datasets. ns, *P*>0.05; *, *P*≤0.05; **, *P*≤0.01; ***, *P*≤0.001.

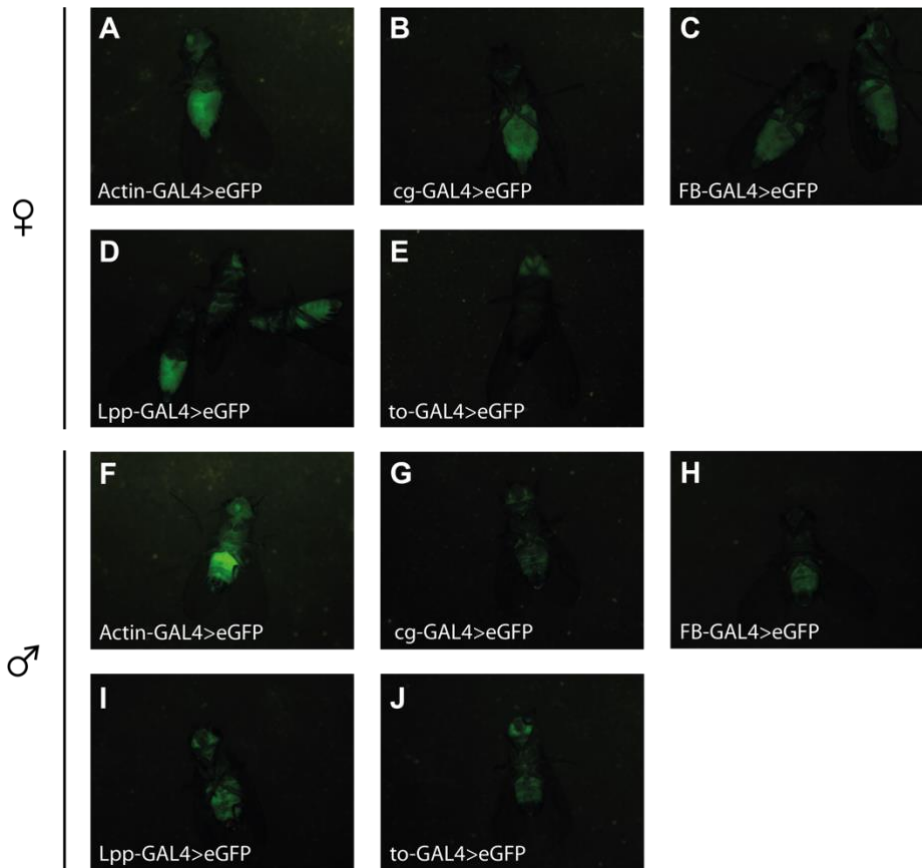


Figure 5.4 Assessing driver activity using UAS-eGFP

(A-J) UAS-eGFP expression is under the control of ubiquitous (*Actin-GAL4*) and fat body drivers (*cg-*, *FB-*, *Lpp-*, *to-GAL4*). (A-E) GFP expression in adult females. (F-J) GFP expression in adult males (~day 7).

5.2.3. Validation of *CG1622* RNAi lines

Having been unable to detect knockdown of *CG1622* using *CG1622*^{RNAi GD}, I next validated other publicly available RNAi lines. The ubiquitous driver *da-GAL4* was used to drive the expression of *CG1622*^{RNAi KK}, *CG1622*^{RNAi TRIP 60408} and *CG1622*^{RNAi TRIP 55648} before RNA was extracted from whole adults for qPCR analysis. Of the four RNAi lines, only *CG1622*^{RNAi KK} and *CG1622*^{RNAi TRIP 60408} exhibited knockdown and although significant, knockdown was mild (Fig. 5.5A). The strongest knockdown (~50%) was observed in *da-GAL4>CG1622*^{RNAi KK}, which also exhibited a semi-lethal phenotype, with escapers unable to inflate their wings (Fig. 5.5B). This phenotype is known to be associated with a second unintended insertion found in ~25% of KK RNAi lines. This second insertion site lies within the 5' UTR of the transcription factor *tiptop*, resulting in ectopic UAS-driven expression of *tiptop*. Ectopic expression of *tiptop* in turn modulates the Hippo pathway leading to wing inflation defects (Green et al., 2014;

Visser et al., 2016). Given that *CG1622*^{RNAi KK} animals exhibited the strongest knockdown and that the choice of effective RNAi lines available was limited, I determined whether an insertion within *tiptop* was indeed present in the *CG1622*^{RNAi KK} stock. KK insertion site occupancy was determined by multiplex PCR as described in the literature (Visser et al., 2016). Different primer combinations were used to determine the occupancy of the intended (referred to as @30) and unintended (referred to as @40) insertion sites. The multiplex PCR yields a smaller product (~450 bp for @40 and ~600 bp for @30) in the case of an insertion or a larger product (~1050 bp for @40 and ~1200 bp for @30) in the case of an empty landing site. Both the @30 and @40 landing sites were found to be occupied in *CG1622*^{RNAi KK} animals (Fig. 5.6A) consistent with the wing inflation defects observed. The two insertions were then separated by meiotic recombination to generate a clean *CG1622*^{RNAi KK} line containing only the @30 insertion (Fig. 5.6B). Although *CG1622* was identified as an obesity-associated gene during the primary screen from two independent studies (Baumbach et al., 2014; Pospisilik et al., 2010), it was dropped as a candidate following subsequent validation experiments with *CG1622*^{RNAi KK}. This raises the question of whether the @40 insertion in this KK stock could have masked a fat phenotype. Unfortunately, I was unable to phenotype these animals due to unexpected time restraints arising from lockdown restrictions resulting from Covid-19.

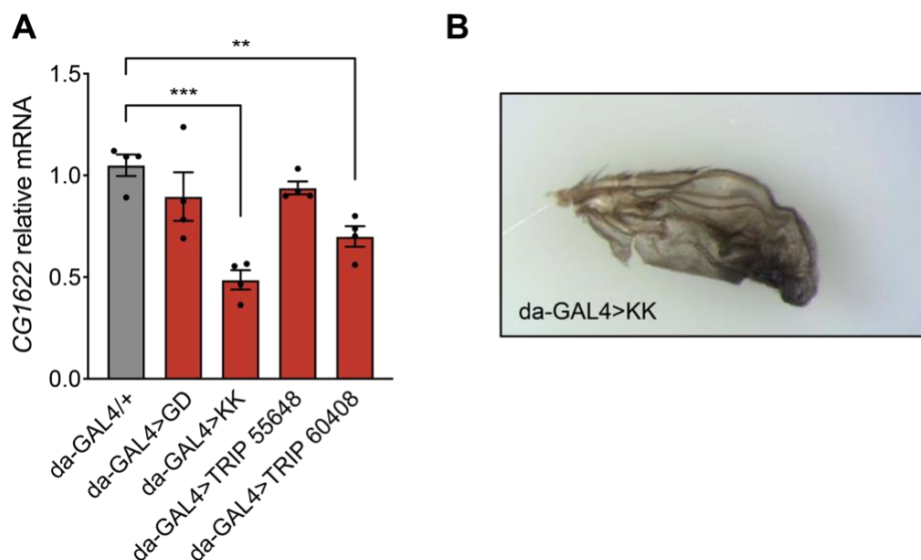


Figure 5.5 Validation of *CG1622* RNAi lines

(A) *CG1622* expression between RNAi lines targeting *CG1622* (*CG1622*^{RNAi GD}, *CG1622*^{RNAi KK}, *CG1622*^{RNAi TRIP 60408} and *CG1622*^{RNAi TRIP}) driven by *da-GAL4*, as determined by qPCR. RNA was extracted from whole adult males (day 7). *n*=4 per condition. Samples are normalised to Rpl32. Results are shown as mean relative fold change $\pm$ SEM and analysed with a one-way ANOVA and a Dunnett's multiple comparisons test. ns, *P*>0.05; **, *P*≤0.01; ***, *P*≤0.001. **(B)** *da-GAL4*> *CG1622*^{RNAi KK} animals exhibit a wing inflation defect. Image from (Visser et al., 2016) as an example of the wing inflation defect observed.

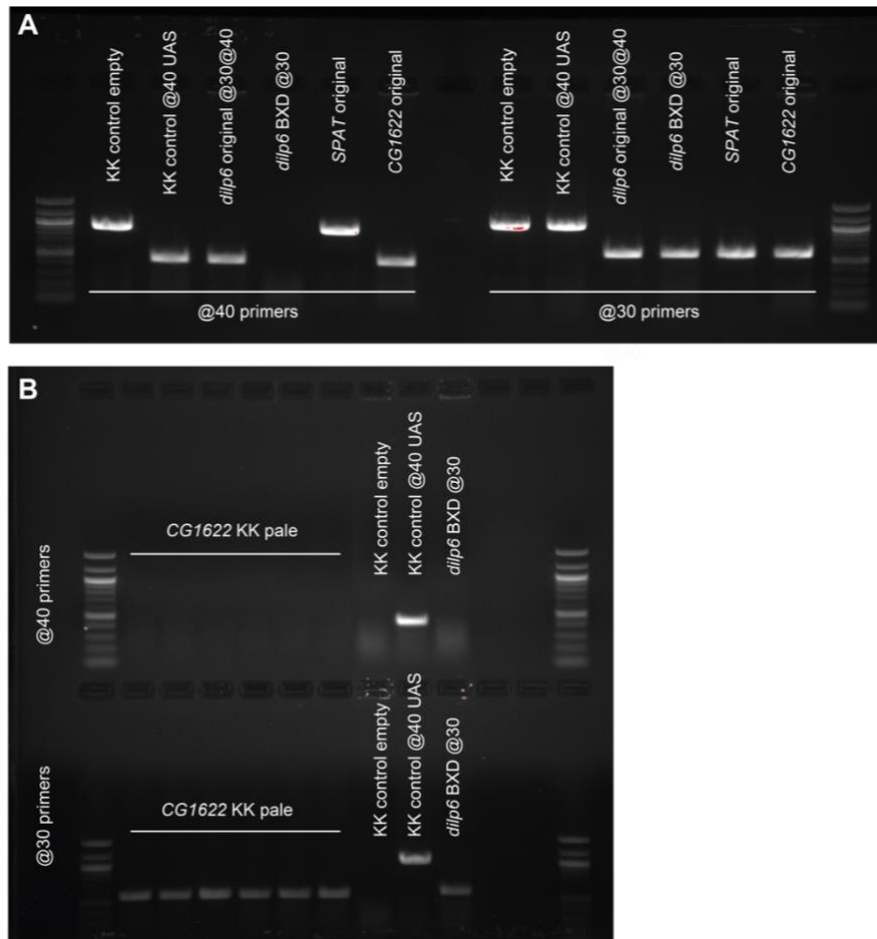


Figure 5.6 Both KK insertion sites are identified in *CG1622*^{RNAi KK} and are separated by meiotic recombination

(A) KK control empty, KK control @40, *dilp6* original@30@40, SPAT original serve as controls with known insertion site occupancies. Larger DNA fragments represent empty insertion sites. Smaller DNA fragments represent an occupied insertion site. Smaller fragments for CG1622 original using both @40 and @30 primer combinations demonstrates occupancy of both insertion sites. (B) The two insertions are separated by meiotic recombination to generate a clean *CG1622*^{RNAi KK} line containing only the @30 insertion.

5.2.4. *CG1622* knockdown in the fat body using *PBac{WH}CG1764*^{f07996} results in elevated TAG levels

As generating a clean *CG1622*^{RNAi KK} line required multiple generations, I searched for an alternative strategy to manipulate *CG1622* in parallel. I identified *PBac{WH}CG1764*^{f07996} (referred to as *CG1622*^[PBac] from here on) as a candidate line in the Gene Disruption Project collection, Exelixis (Bellen et al., 2004; Thibault et al., 2004). The *CG1622*^[PBac] allele was derived by transposable element mobilisation of the *PBac{WH}* construct resulting in its insertion 41 bp upstream of the *CG1622* TSS (Fig. 5.7A). The *PBac{WH}* construct carries a *mini-white* marker, a single long FRT

site, and a terminal UAS site for GAL4-driven misexpression. Although *CG1622*^[PBac] is described in the literature as a line to manipulate the expression of *CG1764* (*DDAH*), I found the construct to be positioned in the same orientation (+) as *CG1622* (Fig. 5.7A), and therefore predicted that it could instead be used for GAL4-dependent misexpression of *CG1622*. FB-GAL4>*CG1622*^[PBac] animals exhibited a significant reduction in *CG1622* mRNA (Fig. 5.7B) when compared to *CG1622*^[PBac]/+ controls. Knockdown of *CG1622* was GAL4-dependent suggesting that downregulation was not simply a result of the insertion itself. Since the UAS sites of *CG1622*^[PBac] are located upstream of the normal *CG1622* TSS, I suggest that the expression of an abnormal, long transcript negatively regulates *CG1622* mRNA levels. I initially reasoned that this could be a result of nonsense-mediated mRNA decay (NMD). Upstream translation start sites could produce an out-of-frame message containing premature stop codons, and potentially elicit an NMD response. I therefore checked for translation start sites in the extra 41 bp downstream of the PBac{WH} construct and upstream of original *CG1622* TSS, however none were identified within this region (Fig. 5.7E). Two translation start sites (Fig. 5.7E, orange) were identified within the original 5' UTR, but they are almost immediately followed by stop codons (Fig. 5.7E, star). Although no translation start sites were found, I cannot exclude the possibility that an unknown NMD signal could be present within the 41 bp. Alternatively, the altered 5' UTR arising from the expression of *CG1622*^[PBac] could impact mRNA stability leading to the reduction in *CG1622* mRNA observed. Although the exact mechanism is unclear, *CG1622* mRNA is significantly reduced allowing the study of gene function.

Knockdown of *CG1622* in FB-GAL4>*CG1622*^[Pbac] animals was associated with significantly elevated TAG (Fig. 5.7C) levels corroborating previous results (Section 5.2.3), and providing independent evidence for a role of *CG1622* in energy homeostasis. Of note, there is a distinct possibility that the insertion of the ~7.2 kb Pbac{WH} construct directly upstream of *CG1622* could disrupt its promoter region. If so, *CG1622* may also be downregulated in *CG1622*^[Pbac]/+ flies. More controls would be necessary (i.e., FB-GAL4/+ or *w*¹¹¹⁸) to determine the true extent of knockdown in FB-GAL4>*CG1622*^[Pbac] animals. It is also possible that greater changes in TAG would be observed when comparisons are made with more appropriate controls. *w*¹¹¹⁸ females generally have between 0.3-0.4 mg TAG/mg protein. *CG1622*^[PBac]/+ control

animals had ~0.6 mg TAG/mg protein, suggesting they may already exhibit elevated TAG levels.

Non-driven *CG1622^[PBac]* males (*CG1622^[PBac]/Y*) had significantly increased TAG levels compared to *w¹¹¹⁸* controls (Fig. 5.7D), supporting the notion that the insertion of PBac{WH} on its own reduces *CG1622* transcription. The PBac{WH} insertion is 360 bp upstream of the annotated TSS for *DDAH* (– orientation) (Fig. 5.7A), therefore I cannot exclude the possibility that the insertion may also disrupt *DDAH* cis-regulatory elements. However, this was not a major concern given previous results excluding a role for *DDAH* in TAG storage (Section 4.3.2).

5.2.5. *CG1622^[PBac]* animals may exhibit increased fecundity

The results above (Sections 5.2.3 and 5.2.4) lend support for a role of *CG1622* in energy homeostasis. Given this putative role in energy homeostasis and with *CG1622* mRNA being most enriched in the ovaries in females (FlyAtlas2) (Fig. 5.8A), I was interested in whether *CG1622* may also influence fecundity. Observations from experiments designed for another purpose did indeed provide a possible link. An increase in the number of wandering third instar larvae was observed in homozygous *CG1622^[PBac]* vials compared to controls following a 24 h laying period (Fig. 5.8B). Although egg laying was not directly assessed here, these data seem to suggest *CG1622^[PBac]* animals may lay more eggs, possibly implicating *CG1622* in fecundity. I cannot exclude the possibility that these effects arise from changes in mating behaviour and/or viability, although these seem unlikely given the similarity in the proportion of eggs hatching between conditions. Eclosion was synchronised also excluding accelerated development (observation, not shown). *CG1622^[PBac]* females weighed significantly more than controls and had significantly elevated levels of total protein, possibly representing increased egg production/retention. Of note, the elevated TAG levels in hemizygous *CG1622^[PBac]/Y* males (Fig. 5.7D) support the idea that PBac{WH} insertion reduces expression of *CG1622*. Homozygous *CG1622^[PBac]* females would therefore also be expected to exhibit reduced *CG1622* expression.

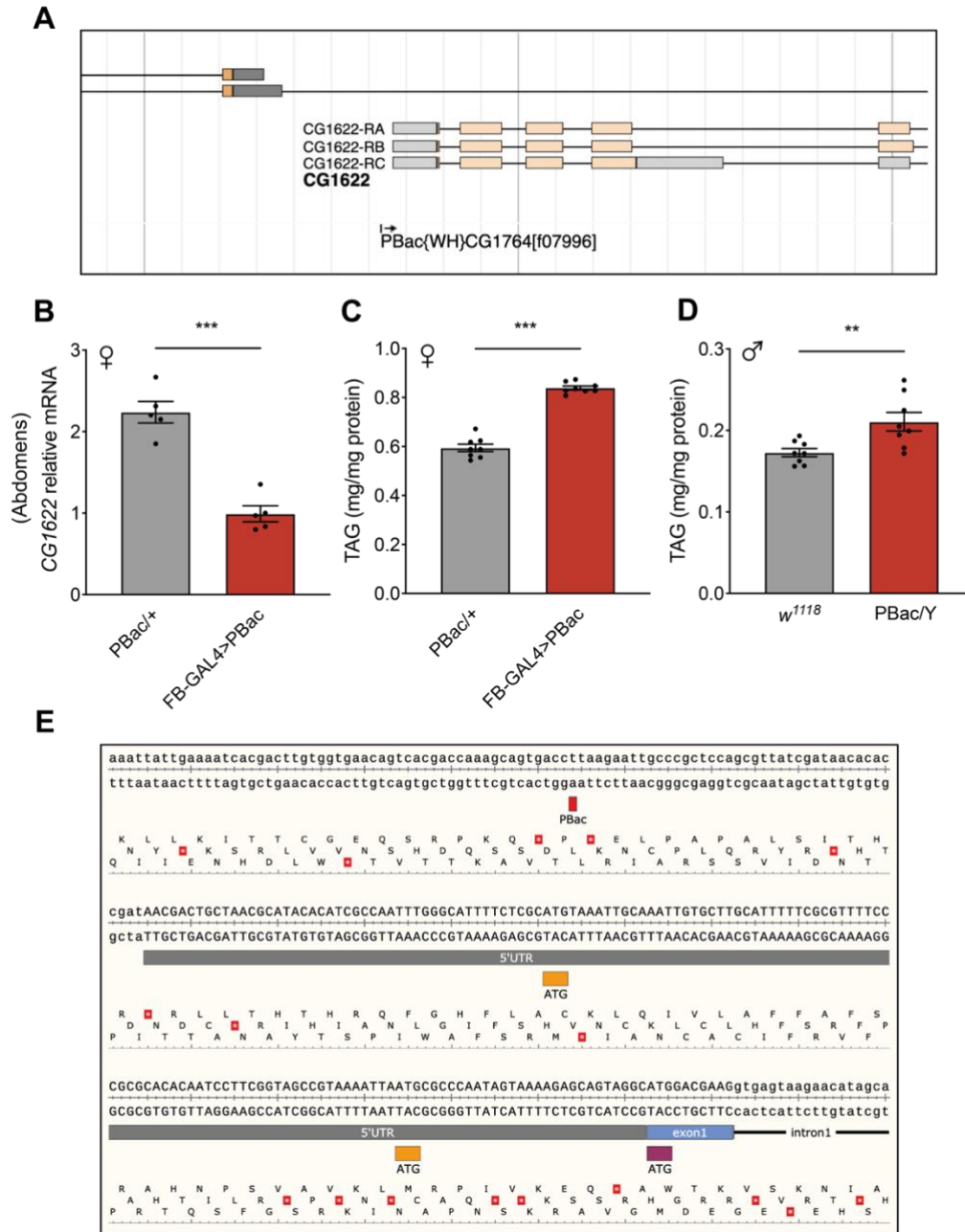


Figure 5.7 *CG1622* knockdown in the fat body using *PBac{WH}CG1764^{f07996}* results in elevated TAG levels

(A) A schematic representation of the *CG1622* locus showing *PBac{WH}CG1764^{f07996}* (*PBac^{CG1622}*). (B) *CG1622* expression in FB-GAL4>*PBac^{CG1622}* and control (*PBac^{CG1622}/+*) adult female abdomens (day 7), as determined by qPCR. n=5 per condition. Samples are normalised to RpL32. Results are shown as mean relative fold change $\pm$ SEM and analysed by unpaired t-test. (C) TAG levels from FB-GAL4>*PBac^{CG1622}* and control (*PBac^{CG1622}/+*) whole adult females (day 7). n=8 per condition. (D) TAG levels from *PBac^{CG1622}* and *W¹¹¹⁸* whole adult males (day 7). n=8 per condition. (C, D) TAG levels were normalised to protein (mg/mg). Results are shown as mean $\pm$ SEM. Statistical analyses were carried out with an unpaired t-test. (E) A schematic representation of the genomic region around the PBac insertion. The annotated translation start site is marked in purple. Other upstream codons specifying methionine are marked in orange. Stop codons are marked with a star. Annotations were made in SnapGene. **, $P \leq 0.01$; ***, $P \leq 0.001$.

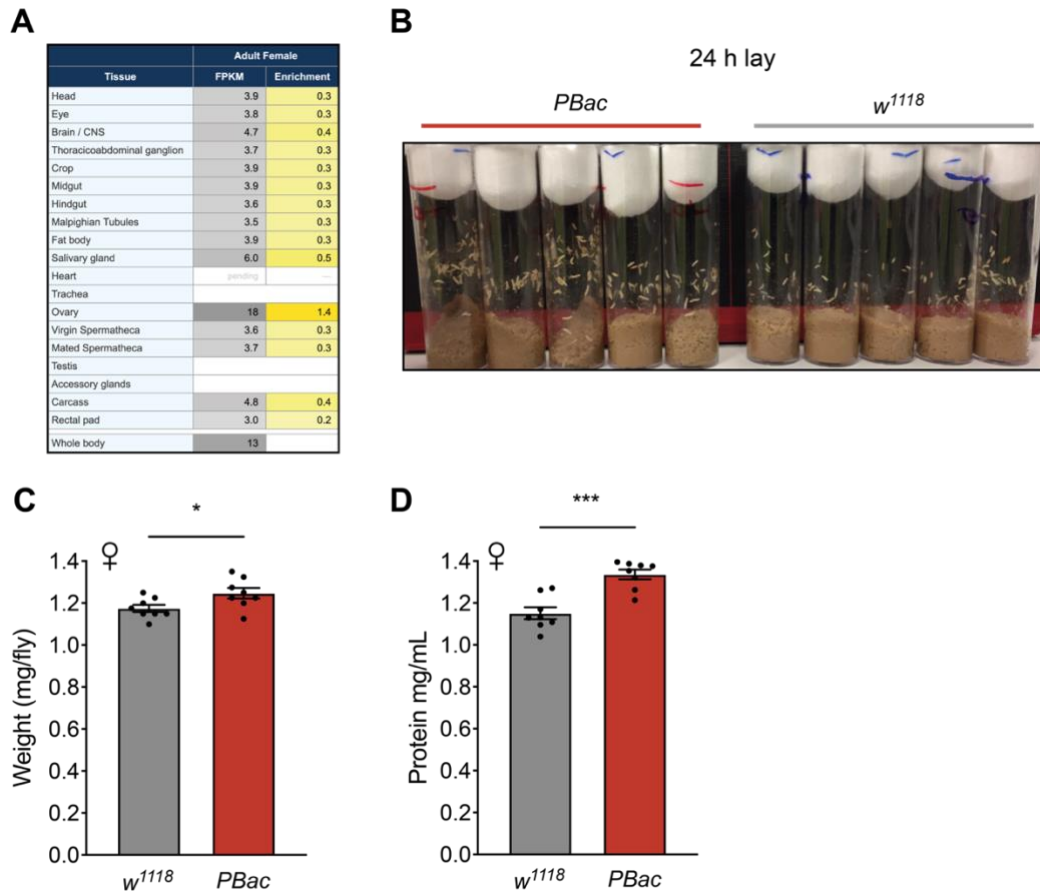


Figure 5.8 *PBac*[CG1622] animals may exhibit increased fecundity

(A) CG1622 expression is enriched in the ovaries (www.flyatlas2.org). (B) Wandering third instar larvae of *PBac*^[CG1622] and *w¹¹¹⁸* following a 24 h lay. Not quantified (C) Weight (mg/fly) of *PBac*^[CG1622] and *w¹¹¹⁸* adult females (day 7). (D) Protein (mg/mL) of *PBac*^[CG1622] and *w¹¹¹⁸* adult females (day 7). (C, D) n=8 per condition. Results are shown as mean ± SEM. Statistical analyses were carried out with an unpaired t-test. *, P≤0.05; ***, P≤0.001.

5.2.6. The expression of *dAGXT* correlates with *CG1622*

Much of the work in chapter stems from the initial observation that a highly conserved intronic region believed to represent *CG1622* cis-regulatory elements is lost in *DDAH^{XP}* but not *DDAH^{Δe2-5}*. This hypothesis was supported by qPCR data demonstrating significant upregulation of *CG1622* in *DDAH^{XP}* animals while only a small change was observed in *DDAH^{Δe2-5}* (Fig. 5.9B,D). The expression of *dAGXT*, which was found to be upregulated in *DDAH^{XP}*, was unchanged in *DDAH^{Δe2-5}* animals (Fig. 5.9C,E), correlating with the expression of *CG1622* in these two mutant backgrounds. These results suggest that upregulation of *dAGXT* in *DDAH^{XP}* is not due to compensation for the loss of *DDAH* as previously assumed (Section 3.2.4). Given that these two alleles are genetically identical aside from a ~700 bp difference around

the *DDAH* locus, these results instead raise the question whether *CG1622* may directly or indirectly regulate the expression of *dAGXT*. *AGXT* is a liver-specific aminotransferase involved in both gluconeogenesis and glyoxylate detoxification. Functional deficiency of *AGXT* is associated with primary hyperoxaluria type 1 (PH1), a rare autosomal recessive disorder of glyoxylate metabolism (discussed in detail in Chapter 6). Given the known metabolic functions of *AGXT* and relevance to human disease, I further investigated the relationship between *CG1622* and *dAGXT*.

A

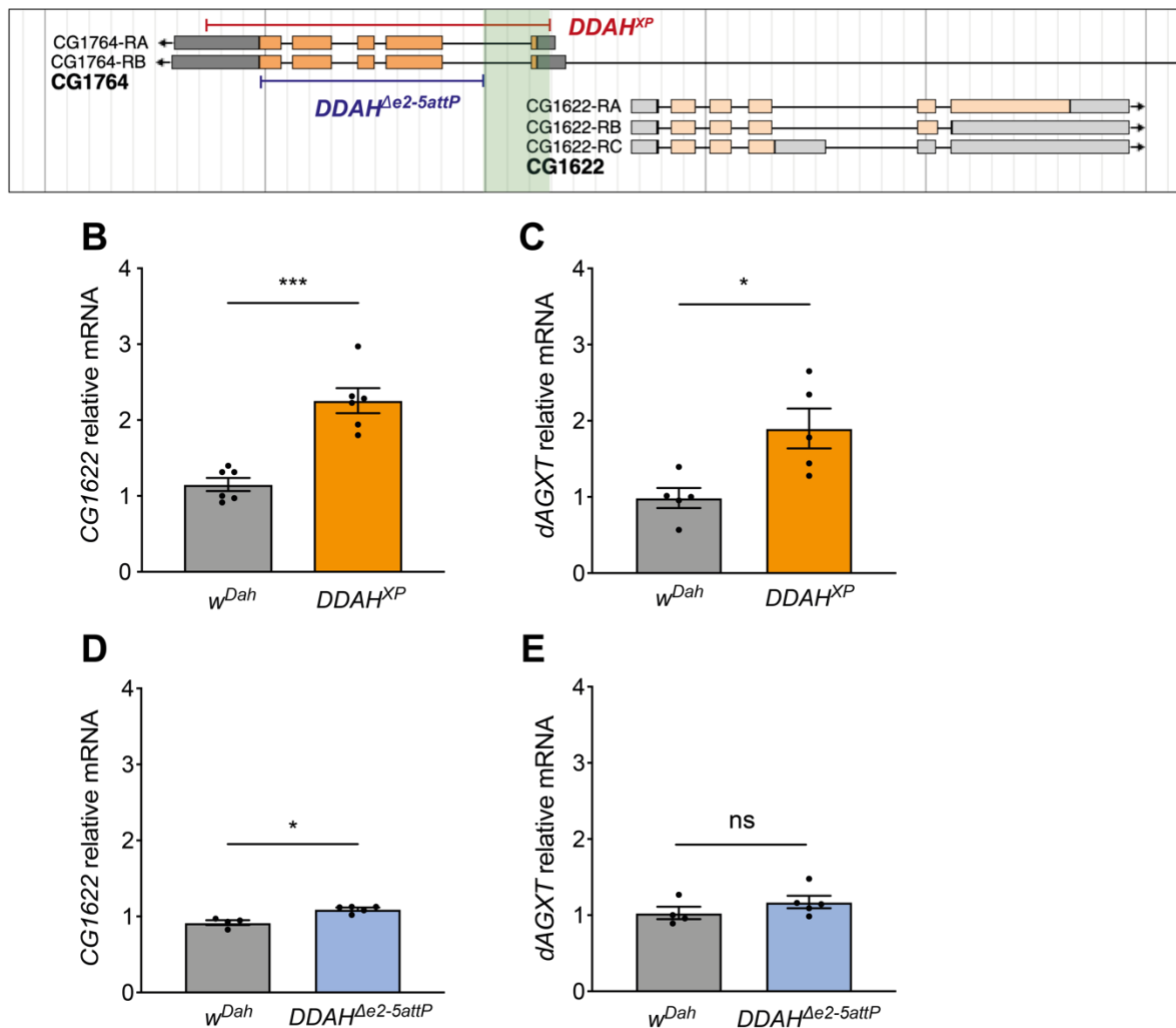


Figure 5.9 The expression of *dAGXT* correlates with *CG1622*

(A) Schematic representation of the *DDAH* locus showing the *DDAH^{XP}* and *DDAH^{Δe2-5 attP}* deletions. Copy of Fig. 5.1A. **(B)** *CG1622* expression in *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7). n=6 per condition. **(C)** *dAGXT* expression in *w^{Dah}* and *DDAH^{XP}* whole adult males (day 7). n=5 per condition. **(D)** *CG1622* expression in *w^{Dah}* and *DDAH^{Δe2-5 attP}* whole adult males (day 7). n=5 per condition. **(E)** *dAGXT* expression in *w^{Dah}* and *DDAH^{Δe2-5 attP}* whole adult males (day 7). n=5 per condition. **(B-E)** Expression determined by qPCR. All samples are normalised to Rpl32. Results are shown as mean relative fold change ± SEM and analysed by unpaired t-test in all datasets. ns, P>0.05; *, P≤0.05; ***, P≤0.001.

5.2.7. Reduced *dAGXT* exon junction 1-2 probe binding but not 2-3 in *CG1622* knockdown animals

To explore whether *CG1622* may regulate the expression of *dAGXT*, mRNA levels were assessed in FB-GAL4>*CG1622*^[PBac] animals by qPCR using a probe targeting *dAGXT* exon junction 1-2. A striking reduction in *dAGXT* mRNA levels was observed in FB-GAL4>*CG1622*^[PBac] animals compared to controls (Fig. 5.10A). Similar results were obtained for FB-GAL4>*CG1622*^{RNAi GD} animals (Fig. 5.10B). Consistent results between independent lines targeting *CG1622* exclude the possibility of line-specific effects. To confirm the above results, qPCRs were repeated using an additional probe targeting a different exon boundary (2-3). Interestingly, a significant reduction in *dAGXT* exon junction 1-2 probe binding but not 2-3 was observed in FB-GAL4>*CG1622*^[PBac] animals (Fig. 5.10C-E). qPCR products produced bands of expected sizes when analysed by gel electrophoresis confirming probe specificity (Fig. 5.11A). Together, these results suggest that (i) a short *dAGXT* variant may exist, and (ii) *CG1622* may differentially regulate the expression of *dAGXT* transcript variants, either directly or indirectly.

Given that *CG1622* is a putative splicing factor, I next sought to investigate whether the reduced signal from the probe targeting the junction of exon 1 and 2 of *dAGXT* could result from RNA splicing. To do so, RT PCR was carried out using a combination of primers spanning the known *dAGXT* transcript (Fig. 5.11B) however no smaller PCR products were identified. I suggest a short variant may instead be generated via other mechanisms such as alternative transcription initiation and/or RNA cleavage (addressed in detail in Section 6.2.5).

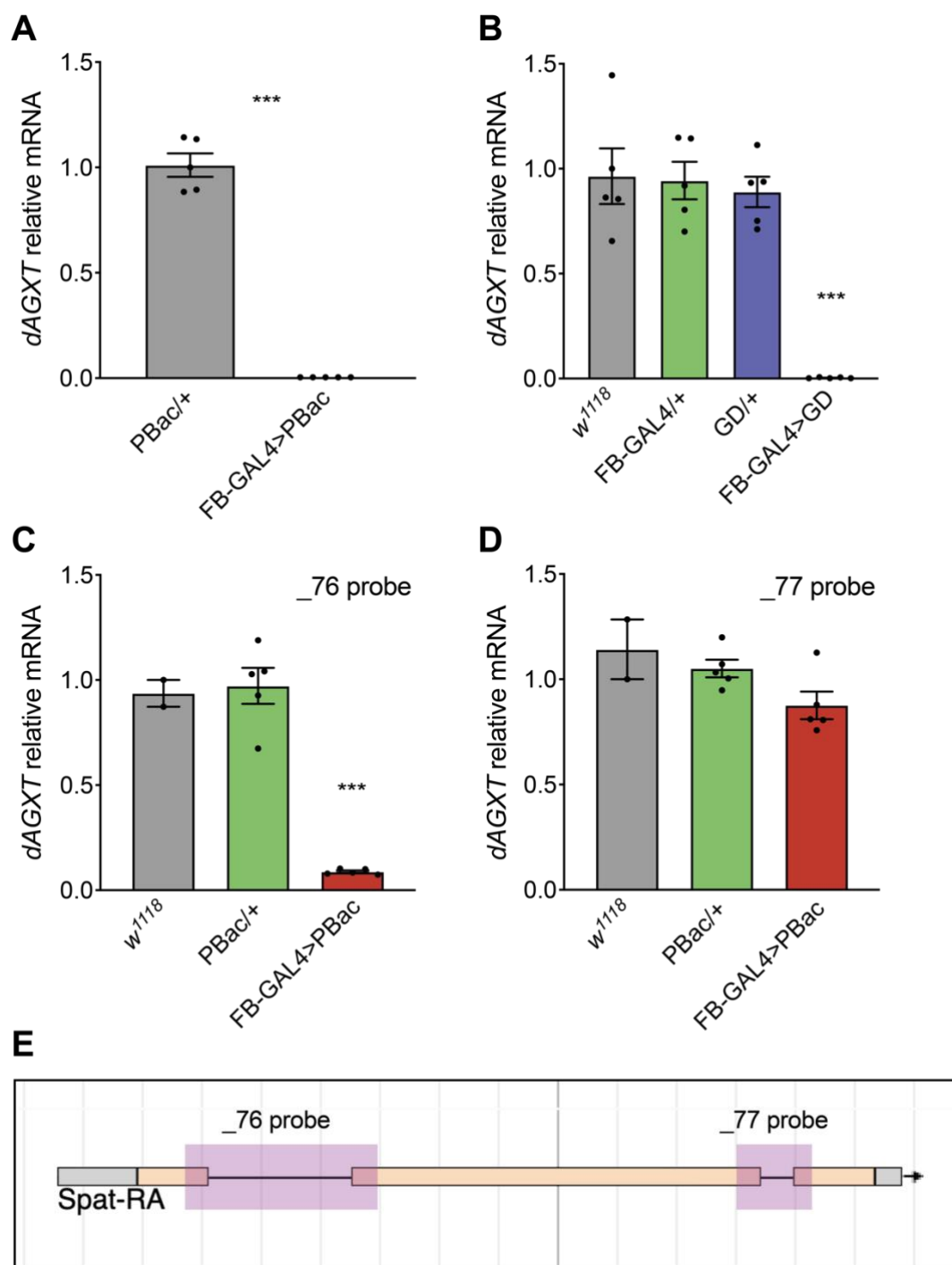


Figure 5.10 Reduced *dAGXT* exon junction 1-2 probe binding but not 2-3 in *CG1622* knockdown animals

(A) *dAGXT* expression in FB-GAL4>PBac^[CG1622] and control (PBac^[CG1622]/+) adult female abdomens (day 7). n=5 per condition. (B) *dAGXT* expression in FB-GAL4>CG1622^{RNAi GD} and control (W¹¹¹⁸, FB-GAL4/+, CG1622^{RNAi GD} /+) adult male abdomens (day 7). n=5 per condition. (A, B) Input cDNA was outside the range for which linearity was observed, therefore relative changes in mRNA may not be fully accurate. Input cDNA was adjusted to fall within the dynamic range subsequent qPCR (C, D). (C, D) *dAGXT* expression in FB-GAL4>PBac^[CG1622] and control (W¹¹¹⁸, PBac^[CG1622]/+) adult female abdomens (day 7). n=2-5 per condition. (C) Amplification with _76 probe spanning exon junction 1-2. (D) Amplification with _77 probe spanning exon junction 2-3. (A-E) Expression determined by qPCR. All samples are normalised to Rpl32. Results are shown as mean relative fold change ± SEM and analysed by (A) unpaired t-test or (B-D) one-way ANOVA and a Dunnett's multiple comparisons test. ns, P>0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001.

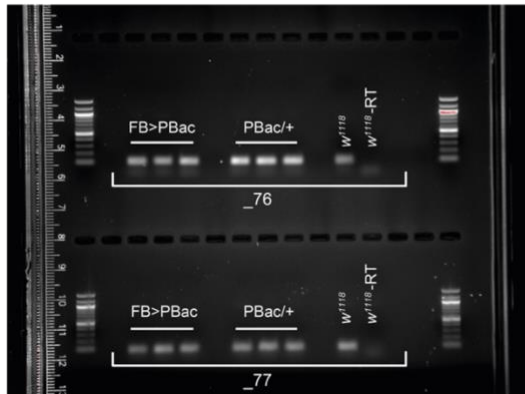
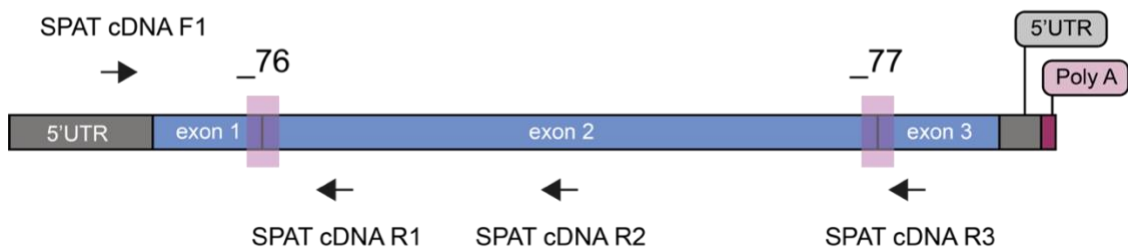
A**B**

Figure 5.11 The reduced signal from the probe targeting the junction of exon 1 and 2 of *dAGXT* may not arise from RNA splicing

(A) qPCR products from Fig. 5.10C,D analysed by gel electrophoresis. (B) Schematic representation of *dAGXT* cDNA showing primer combinations used to test for a splicing event in FB-GAL4>*PBac*^[CG1622] by RT-PCR.

5.3. Discussion

DDAH^{XP} and *DDAH^{Δe2-5}* are both KO alleles lacking the *DDAH* ORF, yet only *DDAH^{XP}* animals exhibit a lean phenotype. In Chapter 5, I explored the genetic differences between these two *DDAH* KO alleles in order to understand the cause of these phenotypic differences. *DDAH^{XP}*, generated by the imprecise excision of a *P*-element, has the larger deletion of the two alleles. Within this larger deletion, I identified a highly conserved intronic region that likely represents *cis*-regulatory elements leading me to speculate that the loss of these elements in *DDAH^{XP}* could be responsible for the metabolic phenotypes observed via the misexpression of the adjacent gene, *CG1622*. Indeed, *CG1622* was found to be upregulated in *DDAH^{XP}* animals suggesting repressor elements may have been lost. *CG1622* had previously been identified as an obesity-associated gene in two independent genome-wide RNAi screens (Baumbach et al., 2014; Pospisilik et al., 2010) (Fig. 5.12) supporting the idea that *CG1622* may be responsible for the metabolic phenotypes of *DDAH^{XP}* animals. Using the same RNAi line (GD) as in the published RNAi screens (Baumbach et al., 2014; Pospisilik et al., 2010), I found that knockdown of *CG1622* results in an obesity-like phenotype. However, due to variability between experiments and the inability to demonstrate significant knockdown, I stopped using the GD line. The choice of effective RNAi lines targeting *CG1622* is limited; only two of the four RNAi lines available produce significant knockdown. The strongest knockdown (~50%) was observed using the KK line. However, this line contained a second unintended insertion (present in ~25% of KK lines) within the 5' UTR of the transcription factor *tiptop*, resulting in ectopic UAS-driven expression of *tiptop* and developmental defects. It is possible that the developmental defects arising from the presence of the unintended insertion could have masked a fat phenotype resulting *CG1622* being dropped as a candidate after the second round of screening from the previous studies (Baumbach et al., 2014; Pospisilik et al., 2010). Finding an effective and clean RNAi line proved difficult and I therefore took advantage of my observation that driving *CG1622^[PBac]* in the fat body resulted in *CG1622* knock down. These animals exhibit high levels of fat storage, providing independent evidence supporting a role for *CG1622* in energy homeostasis.

The results presented in Chapters 4 and 5 of this thesis appear to rule out *DDAH* from having a role in the regulation of adiposity in *Drosophila*. This contrasts with previous

work from the Leiper lab demonstrating increased lipid storage in adipocyte-specific *DDAH* KO mice. It is unclear why *DDAH* deficiency has no effect on adiposity in *Drosophila*. Possibly there is redundancy in the enzymes that metabolise ADMA in *Drosophila* and that the loss of *DDAH* does not sufficiently elevate ADMA levels. The aminotransferase AGXT may play a more significant role in the metabolism of ADMA in *Drosophila* than mammals. Although initially it may seem surprising that its neighbouring gene, *CG1622*, could be involved in a similar biological process as mammalian *DDAH* (i.e., energy metabolism), this may not be unexpected from an evolutionary perspective. Clusters of functionally related genes have been positively selected for and are commonly found in eukaryotic genomes. Having functionally related genes co-inhabiting a chromatin domain or “genomic neighbourhood” allows co-regulation of genes involved in the same biological process.

dAGXT was not upregulated in *DDAH* ^{$\Delta e2-5$} animals suggesting that the upregulation observed in *DDAH*^{XP} animals is not due to compensation for the loss of *DDAH* as originally assumed. Instead, the expression of *AGXT* appears to correlate with the expression of *CG1622* in these two mutant backgrounds. Given that the two KO alleles are genetically identical aside from a ~700 bp difference around the *DDAH* locus, it seemed conceivable that *CG1622* may directly or indirectly regulate the expression of *dAGXT* in *DDAH*^{XP} animals. Given the known metabolic functions of AGXT (gluconeogenesis and glyoxylate metabolism) and its role in human metabolic disease (PH1), I began to explore the relationship between *dAGXT* and *CG1622*/adiposity. Intriguingly, I found a strong reduction in *dAGXT* exon junction 1-2 probe binding but not exon junction 2-3 in *CG1622* animals suggesting: (i) a short *dAGXT* variant may exist, and (ii) *CG1622* may differentially regulate the expression of *dAGXT* transcript variants, either directly or indirectly. More specifically, knockdown of *CG1622* leads to downregulation of the long *dAGXT* variant.

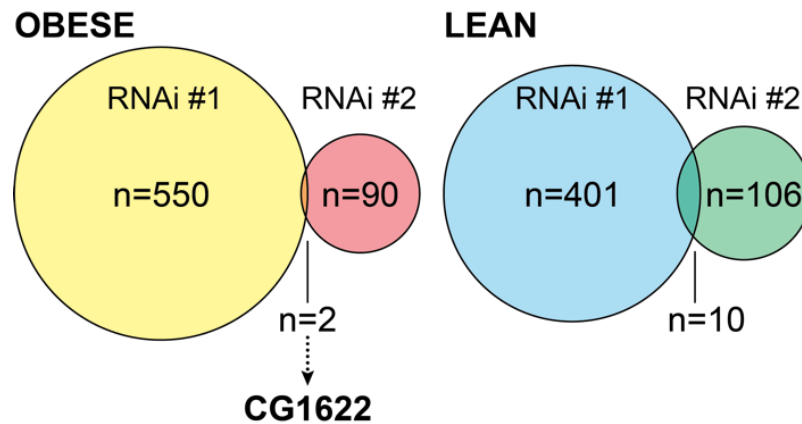


Figure 5.12 *CG1622* was identified as an obesity-associated gene in two independent genome-wide RNAi screens

Representation of data from the 1st round of screening published in Pospisilik et al., 2010 (#1) and Baumbach et al., 2014 (#2), showing *CG1622* as one of only two genes identified as being associated with adiposity in both studies.

In summary, data from Chapter 5 suggest the following key conclusions:

- (i) *DDAH* deficiency has no effect on adiposity in *Drosophila*
- (ii) *CG1622* is identified as a novel putative regulator of adiposity
- (iii) A short, uncharacterised *dAGXT* variant may exist
- (iv) *CG1622* may directly or indirectly differentially regulate the expression of *dAGXT* transcript variants

Chapter 6. Characterisation of *Drosophila* *AGXT*

6.1. Introduction

In Chapter 5, I showed that upregulation of *CG1622* is associated with upregulation of the aminotransferase *dAGXT* in *DDAH^{XP}* animals, which exhibit a lean phenotype. To explore this association, I assessed the expression of *dAGXT* in *CG1622* knockdown animals, which exhibit a fat phenotype. This revealed a significant loss of signal from a probe targeting the junction of exon 1 and 2 of *dAGXT*. These findings suggest that a short, uncharacterised *dAGXT* variant may exist and that *CG1622* may directly or indirectly differentially regulate the expression of *dAGXT* transcript variants. More specifically, knockdown of *CG1622* appears to reduce the expression of the full length *dAGXT* transcript, while presumable favouring an unknown shorter transcript (no significant change in probe binding at the 3' end). *dAGXT* was not identified as an obesity-associated gene in the genome-wide RNAi screens (Baumbach et al., 2014; Pospisilik et al., 2010). Therefore, although there is a genetic interaction between *CG1622* and *dAGXT*, the effects of *CG1622* on adiposity are likely to be independent of *dAGXT*. Interestingly, *AGXT* has been found to be nutritionally regulated in a number of high throughput studies in *Drosophila* - transcriptionally upregulated in response to starvation (Grönke et al., 2005; Sonn et al., 2018; Zinke et al., 2002) and downregulated in response to a HSD (Zinke et al., 2002). These data suggest that the transcriptional changes of *dAGXT* following knockdown of *CG1622* may be in response to the changes in nutritional status.

AGXT is a liver-specific aminotransferase with dual metabolic functions; gluconeogenesis and glyoxylate detoxification. Given its essential role in glyoxylate detoxification, functional deficiency of *AGXT* is associated with PH1, a rare autosomal recessive disorder of glyoxylate metabolism characterised by the formation CaOx renal stones. In spite of its relevance to human disease, *AGXT* is almost completely unstudied in *Drosophila*; there is currently one publication specifically focusing on *dAGXT* (Yang et al., 2018). The observation that knockdown of *CG1622*, a novel regulator of adiposity, leads to downregulation of *dAGXT* provides a link between

glyoxylate metabolism and adiposity which could be relevant to understanding the aetiology of obesity-associated renal stones. Currently, the relationship between adiposity and AGXT has not been explored in the literature. In this final results chapter, I aim to characterise *dAGXT* and explore its role in nephrolithiasis in *Drosophila*. Finally, I describe how I used *Drosophila* to model obesity-associated renal stones and explore their aetiology.

6.1.1. Metabolic functions of AGXT

AGXT, also known as serine-pyruvate aminotransferase (SPAT) is a liver-specific aminotransferase (also called transaminase) containing the evolutionary conserved aminotransferase class-V domain. Aminotransferases catalyse the transfer of an amino group to an acceptor - usually an α -ketoacid (InterPro). They are required for breakdown of amino acids during gluconeogenesis and for *de novo* synthesis of non-essential amino acids. As such, aminotransferases play a key role in amino acid metabolism. Like all aminotransferases, AGXT requires the cofactor pyridoxal 5'-phosphate (PLP) – the active form of vitamin B₆ (InterPro). AGXT is found in both mitochondria and peroxisomes (Birdsey & Danpure, 1998; Danpure, 2006; Huber et al., 2005; Oda et al., 2000; Takada et al., 1990). Subcellular localisation reflects the dual metabolic role of AGXT. In the mitochondria, AGXT is mainly involved in gluconeogenesis, primarily from the transamination of serine (Danpure, 1997; Just et al., 2020). In the peroxisomes, AGXT plays an essential role in the clearance of the metabolic intermediate glyoxylate. It is worth noting that the primary site of glyoxylate metabolism varies between organisms depending on the nature of the glyoxylate precursor (discussed in Section 6.1.4). Glyoxylate is cleared as AGXT catalyses the transamination of L-alanine and glyoxylate to pyruvate and glycine via a classical 'ping pong' mechanism. This is considered a detoxification reaction as excess glyoxylate is oxidised to oxalate, a metabolic end-product. Oxalate accumulation can result in the formation and deposition of calcium oxalate (CaOx) crystals causing urolithiasis and nephrocalcinosis (Danpure & Jennings, 1986, 1988; Danpure, 2006; Oppici et al., 2015). While the primary function of AGXT is glyoxylate metabolism, it exhibits a range of substrates, most notably L-serine and pyruvate. AGXT2 also displays ADMA-glyoxylate aminotransferase activity and is therefore considered to have a role in ADMA clearance, although to a lesser degree than DDAH (Dowsett et al., 2020).

dAGXT was also found to regulate ADMA levels in *Drosophila* (Leiper lab, unpublished), which prompted my initial interest in AGXT.

6.1.2. AGXT in Primary Hyperoxaluria Type 1

Reduced AGXT activity and/or loss of peroxisomal targeting was demonstrated in the 1980s to be associated with PH1, a rare autosomal recessive disorder of glyoxylate metabolism (Danpure & Jennings, 1986). Functional deficiency of AGXT in PH1 causes excessive glyoxylate accumulation in the peroxisomes, the major site of glyoxylate metabolism in humans. Excess glyoxylate escapes to the cytosol where it is oxidised to oxalate. Elevated oxalate synthesis and urinary excretions lead to the progressive deposition of CaOx in the kidney and urinary tract, which can ultimately lead to renal failure. Once progressed to renal failure, systemic oxalosis can occur, a potentially fatal condition where CaOx crystals accumulate in other tissues including blood vessels, heart, retina and central nervous system (Danpure, 2006; Oppici et al., 2015). Most PH1 patients reach end-stage renal disease (ESRD) during the third to fifth decade and approximately 50% patients presenting in childhood reach end-stage renal failure by the age of 15 (Milliner et al., 2017; Williams et al., 2009). Currently, the only curative treatment for PH1 is a liver transplant (effectivity amounting to enzyme replacement therapy). Combined liver and kidney transplantation can be necessary if the patient has reached ESRD. Treatment with vitamin B₆, a precursor of the AGXT cofactor PLP, has been shown to reduce oxalate excretion in a subset (~30%) of PH1 patients (Cochat et al., 2012; Fargue et al., 2013b; Hoppe & Langman, 2003). Pathogenic variants associated with mitochondrial mistargeting of AGXT including Gly170Arg or Phe152Ile mutations are most likely to respond to vitamin B₆ treatment. PLP is believed to exert a chaperone role, increasing peroxisomal localisation and of such variants, thus improving glyoxylate clearance and reducing oxalate excretion (Cellini et al., 2014). High fluid intake and alkalinisation of urine (via potassium citrate) are also recommended treatments to reduce stone formation or nephrocalcinosis in PH1 patients (Cochat et al., 2012).

6.1.3. AGXT mutations associated with PH1

To date, >200 PH1 associated AGXT mutations have been reported (mostly point mutations) (Dindo et al., 2020; Lu et al., 2019), many of which are covered in a

comprehensive review of AGXT mutations (Williams et al., 2009). AGXT also has two common polymorphic variants - the “major” and “minor” alleles. Significant synergy between allelic variants and mutations exists (Fargue et al., 2013a; Lumb & Danpure, 2000). Many of the most common mutations co-segregate and functionally interact with the Pro11Leu polymorphism of the minor allele, only becoming pathological in this context. As such, the minor allele occurs at a high frequency in PH1 patients. As well as a Pro11Leu replacement in exon 1, the minor allele is characterised by an imperfect duplication of 74 bp in intron 1, tandem repeats in intron 4 and an Ile340Met substitution in exon 10 (Danpure, 2006; Oppici et al., 2015; Williams et al., 2009). Of the invariant changes, the Pro11Leu replacement is most functionally significant, affecting AGXT stability/dimerization, activity and subcellular localisation (Danpure, 2006). The most common mutations (most notably Gly170Arg) associated with PH1 cause mitochondrial mistargeting by effectively strengthening a cryptic N-terminal mitochondrial targeting sequence (MTS) associated with the minor allele (Danpure et al., 1993; Fargue et al., 2013a; Lumb & Danpure, 2000; Purdue et al., 1990). The Gly170Arg mutation in the minor allele background has been shown to delay AGXT dimerization (Leiper et al., 1996), a process that is believed to favour mitochondrial targeting. Delayed dimerization could affect the kinetic partitioning of AGXT since peroxisomes can import fully folded oligomeric proteins whereas mitochondria will only import unfolded/loosely folded monomeric polypeptides (Danpure, 2006; Djordjevic et al., 2010). Delayed dimerisation is likely therefore to favour mitochondrial targeting. Although mitochondrial AGXT remains catalytically active, it is unlikely to contribute to the metabolic clearance of glyoxylate, as glyoxylate is primarily synthesised in peroxisomes in humans - efficient clearance requires AGXT to be at the primary site of synthesis. Of note, PLP (from vitamin B₆ treatment) is believed to act on the kinetic partitioning of AGXT by improving folding and/or dimerisation, thus increasing peroxisomal localisation and glyoxylate clearance in pathogenic variants associated with mitochondrial mistargeting.

6.1.4. Subcellular localisation of AGXT

As mentioned, AGXT localises to both peroxisomes and mitochondria. The degree of peroxisomal vs mitochondrial targeting can vary greatly between organisms. A correlation between diet and organelle targeting suggests that subcellular localisation

of AGXT may have been influenced by dietary selection pressure throughout evolutionary history (Danpure et al., 1990; Danpure, 2006). Accordingly, the appropriate subcellular distribution would allow optimal AGXT activity in response to the metabolic demands of an organism. The primary site of glyoxylate synthesis is predicted to differ between organisms depending on the major precursor and is believed to influence the subcellular localisation of AGXT (efficient detoxification of glyoxylate requires AGXT to localise at the major site of synthesis). The major precursor of glyoxylate in herbivores is believed to be glycolate. Glycolate is converted to glyoxylate by glycolate oxidase in peroxisomes. Therefore, it may be beneficial for AGXT to localise to peroxisomes in herbivores. By contrast, in carnivores, the major precursor is thought to be hydroxyproline. Hydroxy-2-ketoglutarate derived from hydroxyproline is converted to glyoxylate by 4-hydroxy-2-ketoglutarate aldolase (HKA) in the mitochondria. This would explain why mitochondrial AGXT may have been favoured in carnivores (Danpure, 2006). In carnivores, AGXT may also have a more significant role in gluconeogenesis due to high protein and low carbohydrate diets, a feature that could also favour mitochondrial targeting. Although this hypothesis is attractive, one must bear in mind that there are exceptions to the correlation between diet the subcellular localisation of AGXT, suggesting that other factors must be at play.

6.1.5. AGXT contains both an N-terminal MTS and C-terminal peroxisomal targeting sequence

AGXT is a homodimeric protein with each subunit composed of three distinguishable structural/functional domains: an N-terminal extension containing an MTS, a large central domain - containing most of the active site residues, the dimerization interface and a PLP binding site - and a small C-terminal domain containing an atypical peroxisomal targeting sequence (PTS1) (Danpure, 2006; Oppici et al., 2015). The presence of both an MTS and a PTS1 results in a “tug of war” with the outcome determining subcellular localisation. The strength of the MTS varies greatly between organisms. Human AGXT for example has a weak/cryptic MTS and primarily target peroxisomes, whereas many other mammals (e.g., rats) make use of second, upstream translation start site resulting in an extra 22 amino acids at the N-terminal extension forming a strong MTS. Together, the strengths of the MTS vs PTS1, in

addition to the rate of dimerisation will determine hierarchical dominance dictating the subcellular distribution.

6.2. Results

6.2.1. *dAGXT* knockdown causes renal stones

AGXT is essential for the metabolic clearance of glyoxylate (Sections 6.1.1 and 6.1.2). Functional deficiency of AGXT in PH1 patients results in excess glyoxylate accumulation leading to overproduction of endogenous oxalate. Elevated urinary excretion of oxalate ultimately leads to the progressive deposition of CaOx (stones) in the kidney and urinary tract. AGXT is also predicted to be essential for glyoxylate detoxification in *Drosophila*. Therefore, knockdown of *dAGXT* is expected to cause the accumulation of CaOx renal stones. The renal system of *Drosophila* includes two discrete organs anatomically and functionally: the Malpighian tubules and nephrocytes (Fig. 6.1D). Together, they carry out the functions analogous to those of the mammalian nephron – the basic structural and functional unit of the mammalian kidney (Fig. 6.1C). Nephrocytes filter the hemolymph much like podocytes in the human glomerulus. The Malpighian tubules are involved in osmotic and ionic regulation as well as primary urine secretion – functions assigned to the renal tubule of the human nephron (Dow & Romero, 2010). Primary urine carries excretory products of metabolism and toxic compounds to the hindgut. As such, the Malpighian tubules are a major site of calcium oxalate (CaOx) crystal deposition. Ubiquitous and constitutive knockdown of *dAGXT* (*act-GAL4>dAGXT^{RNAi KK}*) resulted in significant stone accumulation in the Malpighian tubules (Fig. 6.3A) consistent with a previous study (Yang et al., 2018), to date, the only publication focusing specifically on *dAGXT*. Dissected tubules were scored based on the severity of stone accumulation (Fig. 6.1B). Spontaneous precipitation of renal stones demonstrates that knockdown of *dAGXT* recapitulates a major pathological feature of PH1 making it a promising disease model. Although the composition of the renal stones were not assessed here, Yang and colleagues confirmed that the stones in *dAGXT* knockdown flies consist of CaOx (Yang et al., 2018). Nevertheless, oxalate measurement would be necessary to demonstrate that *act-GAL4>dAGXT^{RNAi KK}* animals are hyperoxaluric.

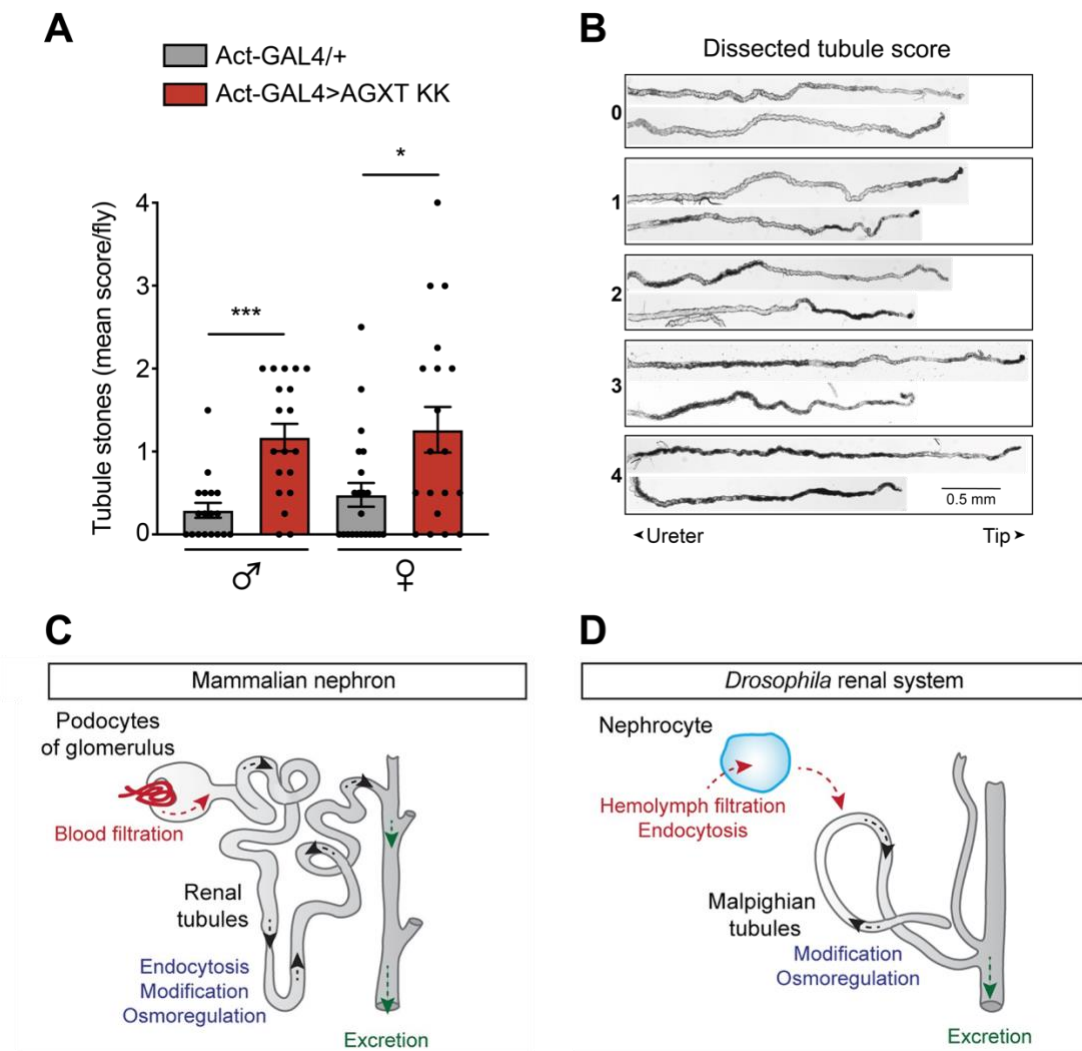


Figure 6.1 *dAGXT* knockdown causes renal stones

(A) Tubule stones scored based on severity in act-GAL4>*dAGXT*^{RNAi KK} and control (act-GAL4/+) adult male and females age day 28. n=18-22 per condition. Results are shown as mean score $\pm$ SEM. Male dataset analysed by unpaired t-test. Female dataset analysed by Mann-Whitney test. **(B)** Scoring system based on light microscopy imaging of dissected Malpighian tubules to quantify the tubule deposits, arranged from the ureter (left) to the distal tip (right). Scale bar = 500 μ m. Scoring: 0=clear (~0%), 1=mild (<25%), 2=moderate (25-50%), 3=strong (50-75%), 4=severe (>75%). **(C)** Schematic representation of the mammalian nephron, the basic structural and functional unit of the mammalian kidney. **(D)** Schematic representation of the *Drosophila* renal system. Adapted from (Lubojska et al., 2021). *, $P \leq 0.05$; ***, $P \leq 0.001$.

6.2.2. Generation of novel transgenic lines for the study of *Drosophila* AGXT

To date, AGXT is almost completely unstudied in *Drosophila*. There is currently only a single publication specifically focusing on *dAGXT* (Yang et al., 2018). The accumulation of renal stones in act-GAL4>*dAGXT*^{RNAi KK} animals (Fig. 6.1A) makes *Drosophila* a promising model for the study of PH1 and with *Drosophila* being an

emerging translational model of human nephrolithiasis (Miller et al., 2013), there is scope for novel and impactful research.

Given the direct relevance to human disease and the broad scope for novel findings, transgenic lines were generated for the study of *Drosophila* AGXT. KO alleles as well as a number of reporter lines were designed to investigate dAGXT function, tissue distribution, subcellular localisation and response to dietary manipulations. The reporter lines designed include an N-terminal GFP fusion, a C-terminal GFP fusion lacking PTS1, a C-terminal GFP fusion with the PTS1 intact and a transcriptional reporter (discussed in Section 7.2.4). Three GFP fusion lines were necessary to control for the likely disruption of putative targeting peptides (N-terminal MTS and C-terminal PTS1). The parental line used to generate the N-terminal GFP fusion - *AGXT*^[N-GFP;STOP] (Fig. 6.2) – acts as a transcriptional reporter due to GFP expression being under the control of the authentic promoter. The presence of a transcriptional stop sequence within the Pax-Cherry cassette (Fig. 6.2) terminates transcription initiated from the canonical TSS. Since *dAGXT* has a single annotated transcript, *AGXT*^[N-GFP;STOP] is predicted to be a LOF allele. However, alternative transcription initiation downstream of the Pax-Cherry cassette cannot be excluded and could generate a truncated message and a correspondingly truncated protein. A clean *dAGXT* exon deletion allele was also designed (discussed in Section 7.2.4). Of the lines designed, *AGXT*^[N-GFP;STOP] and *AGXT*^[N-GFP] were generated during this thesis.

6.2.2.1. Generation of the AGXT GFP reporter and N-terminal STOP allele: *AGXT*^[N-GFP;STOP]

AGXT^[N-GFP;STOP] was generated by cas9-triggered homologous recombination and following a similar design as for *DDAH*^[N-GFP;STOP] (described in Section 4.2.1). *AGXT*^[N-GFP;STOP] was then used as a parental line for the generating the N-terminal GFP fusion. The former is expected to be a LOF allele as well as a transcriptional reporter. The presence of the Pax-Cherry genetic marker facilitates screening and subsequent tracking of the allele. Due to lockdown restrictions resulting from Covid-19, the cloning of this line was done by a collaborator Cyrille Alexandre (Francis Crick Institute).

6.2.2.2. gRNA expression vector

Suitable target sequences aaataggggaacaaagacc (T1) and GGAGGTACCACCGCCACT (T2) were identified and verified (as described in Section 4.2.1 for *DDAH*^[N-GFP;STOP]) and cloned into the gRNA expressing vector pCFD4_U6:1-gRNA-U6:3-gRNA using the same protocol. The targets were chosen to induce double strand breaks directly adjacent to the translation start site of *dAGXT*.

6.2.2.3. Targeting vector

An improved 1OGFP@NTV-Pax-Cherry vector was used to generate the donor template (targeting vector), which differs from the previous version (described in Section 4.2.1) by the presence of a p10 3' UTR included to improve mRNA stability and maximise expression (Pfeiffer et al., 2012; Poernbacher et al., 2019). The donor template was generated by cloning ~1 kb homology arms either side of the sequence to be inserted. Homology arms were designed to insert the coding sequence of GFP at the *AGXT* translation start. The homology arms were generated by PCR using primer pairs HR1F_SPAT_N-GFP & HR1R_SPAT_N-GFP and HR2F_SPAT_N-GFP & HR2R_SPAT_N-GFP (Appendix 1.1), using a *nos-Cas9-mSA* genomic DNA template. These primers were used to introduce the necessary overlap regions needed for Gibson assembly with *BsaI* and *SapI*-digested 1OGFP@NTV-Pax-Cherry into the homology arms. Primers were designed using the same parameters described in Section 4.2.1 and fragments were assembled by Gibson Assembly as previously described in Section 4.2.1. The gRNA expression vector and donor template were co-injected into the germ line the *nos-Cas9-mSA* strain (injections by Joachim Kurth, Francis Crick Institute). Following injection, candidates were screened for Pax-Cherry before being sequenced.

6.2.2.4. Generation *AGXT*^[N-GFP]

AGXT^[N-GFP] was generated by the excision of the LoxP flanked Pax-Cherry cassette from *AGXT*^[N-GFP;STOP] (Fig. 6.2) as described for *DDAH*^[N-GFP] (Section 4.2.3).

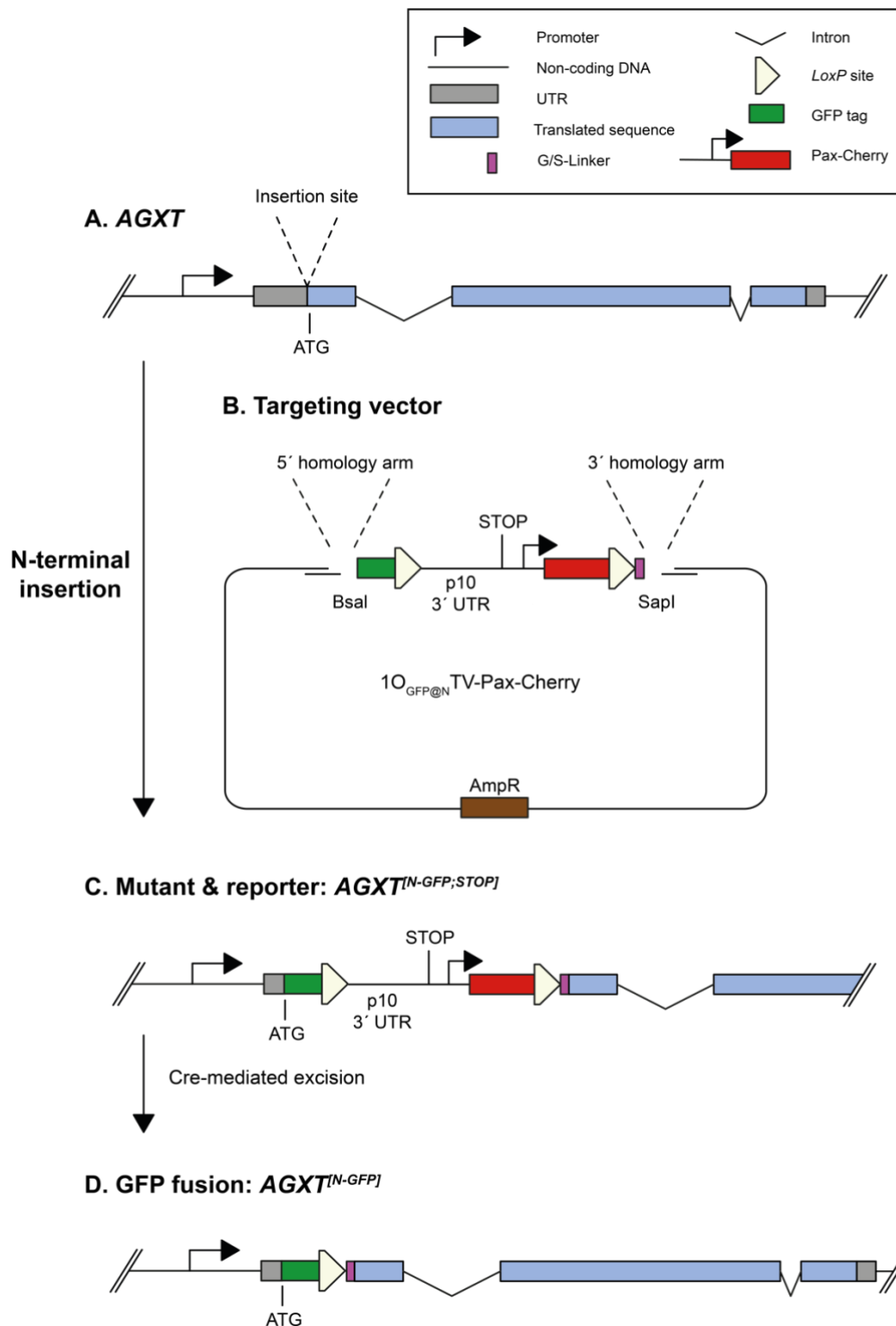


Figure 6.2 Schematic representations of the CRISPR/Cas strategy used to generate novel transgenic lines for the study of *dAGXT*

(A) A schematic representation of the *dAGXT* locus, with coding exons in blue and untranslated sequences in grey. (B) Targeting vector for N-terminal insertion using 10GFP@NTV-Pax-Cherry (Poembacher et al., 2019) where homology arms are inserted either side of the sequence introduced into the genome. (C) Following integration, the resulting allele harbours the Cre-excisable region comprising the termination codon, p10 3'UTR and the *Pax-Cherry* cassette. This is expected to disrupt expression of the targeted gene. GFP is expressed in the pattern of the targeted gene. (D) Cre-mediated excision fuses the reporter to the 5' end of the coding sequence, resulting in the expression of a N-terminal fusion protein with an intervening LoxP and G/S linker. Injections were carried out by Joachim Kurth at the Francis Crick Institute. Candidates were sequenced by A J. Vincent.

6.2.3. *Drosophila* AGXT is expressed in the fat body and may localise to peroxisomes

The AGXT^[N-GFP] fusion protein was expressed almost exclusively in the abdominal fat body, an organ functionally analogous to the human liver (Fig. 6.3A), consistent with human AGXT being a liver-specific enzyme. These data support the notion that AGXT detoxification of glyoxylate occurs in the fat body in *Drosophila*. AGXT^[N-GFP] was also expressed in the Malpighian tubules, although to a lesser degree (not shown).

Subcellular localisation of dAGXT is of interest given its significance for metabolic function. Yet the subcellular localisation of dAGXT is currently unknown. PTS1 prediction tools (Neuberger et al., 2003b, 2003a) yield a low score for dAGXT, which is classified as being in the 'twilight zone' for peroxisomal targeting. Proteins that lie in this zone score low on the PTS1 accessibility criteria or contain unusual residues within the C-terminal tripeptide. Indeed, the putative PTS1 of dAGXT is atypical with a C-terminal tripeptide consisting of SKI rather than the typical SKL. It is worth noting that the PTS1 of AGXT is atypical in other species, even in organisms where AGXT is predominantly peroxisomal (Table 6.1). Therefore, predicting peroxisomal targeting of AGXT is not readily achieved. Since GFP is appended at the N-terminus of dAGXT, the C-terminal (putative PTS1 containing) domain is unaltered in AGXT^[N-GFP] animals allowing targeting to be investigated. The GFP-AGXT fusion appears to localise to organelles that resemble peroxisomes (Fig. 6.3B). Co-localisation with a peroxisomal marker (e.g., catalase antibody) would be necessary to confirm this inference.

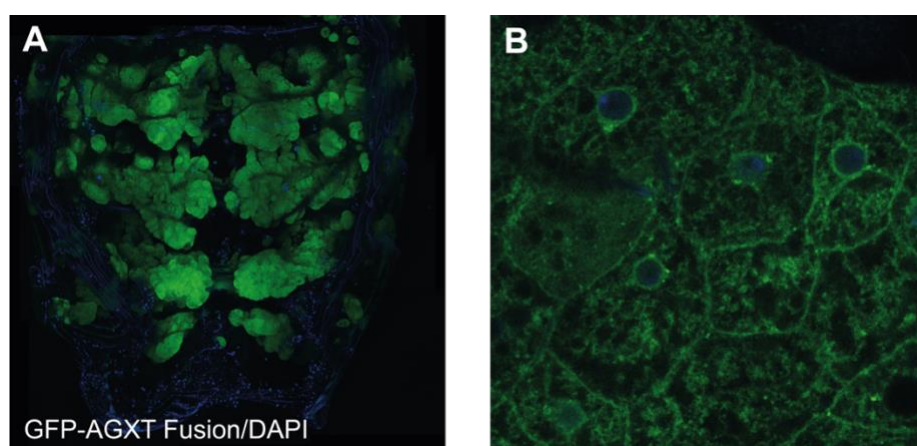


Figure 6.3 *Drosophila* AGXT is expressed in the fat body and may localise to peroxisomes

Confocal imaging of dissected abdomens (A) and adipocytes (B) of AGXT^[N-GFP] adult males (day 7), immunostaining with GFP (green) and DAPI (blue).

6.2.4. *dAGXT* knockdown in the fat body causes renal stone accumulation

AGXT^[N-GFP] was expressed almost exclusively in the fat body supporting the notion that AGXT detoxification of glyoxylate occurs in the fat body in *Drosophila*. *dAGXT* knockdown specifically in the fat body (FB-GAL4>*dAGXT*^{RNAi KK} and FB-GAL4>*dAGXT*^{RNAi GD}) was sufficient to cause significant renal stone accumulation (Fig. 6.4). Phenotypic consistency between independent RNAi lines targeting *dAGXT* exclude the possibility of RNAi-specific effects. These data confirm that AGXT detoxification of glyoxylate takes place in the fat body.

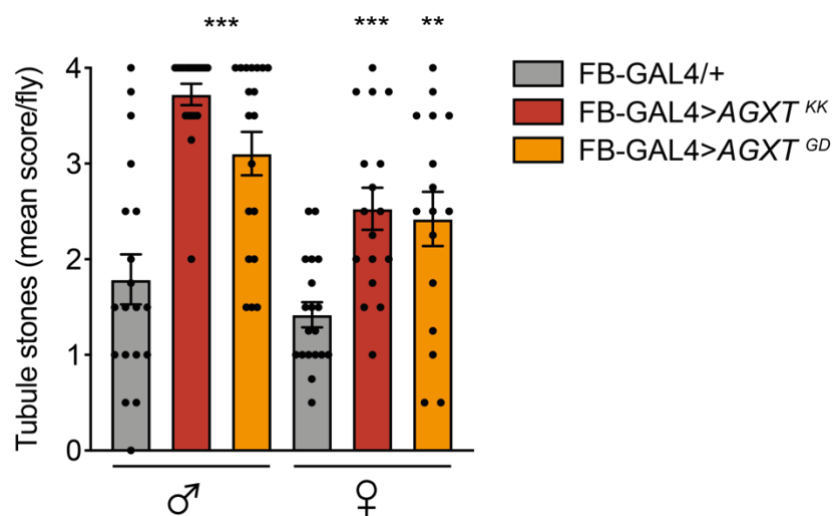


Figure 6.4 *dAGXT* knockdown in the fat body is sufficient for renal stone accumulation

Tubule stones scored based on severity in FB-GAL4>*dAGXT*^{RNAi KK}, FB-GAL4>*dAGXT*^{RNAi GD} and control (FB-GAL4/+) adult male and females age day 35-38 with 21-25 days on HFD. n=16-19 per condition. Results are shown as mean score $\pm$ SEM. Datasets analysed with a one-way ANOVA and a Dunnett's multiple comparisons test. Scoring as described in Fig. 6.1B. **, $P \leq 0.01$; ***, $P \leq 0.001$.

6.2.5. 5' RACE reveals multiple *AGXT* transcripts exists in *Drosophila*

qPCR data presented in the third results chapter (Section 5.2.7) revealed a significant loss of signal from a probe targeting the junction of exons 1-2 of *dAGXT* after knockdown of *CG1622* (a putative regulator of adiposity). Signal from the probe spanning the junction of exons 2-3 remained unchanged, indicating that a short uncharacterised *dAGXT* transcript may continue to be expressed. With *CG1622* being a putative splicing factor, I initially investigated whether a short variant could result

from a splicing event. To do so, RT PCR was carried out using a combination of primers spanning the known *dAGXT* transcript (Fig. 5.11B) however, no smaller PCR products were identified (data not shown). It is known that the archetypal mammalian *AGXT* generates two mRNAs via the use of alternative TSSs. Therefore, there is a distinct possibility that a short variant may arise from alternative transcription initiation in *Drosophila*. The use of alternative TSSs would generate mRNAs with different 5' ends. 5' RACE (rapid amplification of cDNA ends) allows the identification of transcripts with unknown 5' ends and was therefore used to identify all *dAGXT* transcripts.

Total RNA was extracted from the isolated abdomens of FB-GAL4>*CG1622*^{PBac} and *CG1622*^{PBac/+} (control) animals. Dissected abdomens were used for RNA extractions to enrich for fat body tissue. A significant loss of signal from the probe targeting the junction of exons 1-2 of *dAGXT* was observed in FB-GAL4>*CG1622*^{PBac} animals (Fig. 5.10), therefore, it was deemed a suitable condition for the identification of a short variant by 5' RACE. Generating 5' RACE ready cDNA required first strand cDNA synthesis using oligo dT and a reverse transcriptase with terminal transferase activity. This reverse transcriptase adds non-templated nucleotides to the 3' end of the new cDNA strand (Fig. 6.5A). The non-templated residues act as an anchoring site for a TS (template switching) oligo. Switching from the RNA template to the TS oligo generates cDNAs containing a universal sequence at the 3' end (5' end of the transcript) necessary for RACE (Fig. 6.5A).

A gene specific reverse primer (GPS2) was designed to target a 3' region located near the junction of exons 2-3. This region is expected to be present in any short variant as the signal from the probe spanning *dAGXT* exon 2-3 junction remained unchanged in FB-GAL4>*CG1622*^{PBac} animals. The GPS2 primer was checked for specificity to avoid non-specific RACE products. A homology region (15 bp overlap with vector at the 5' end) was included in the primer design to facilitate future cloning of RACE products. RACE products were amplified from the RACE ready cDNA using the universal and GPS2 primers (Fig. 6.5B).

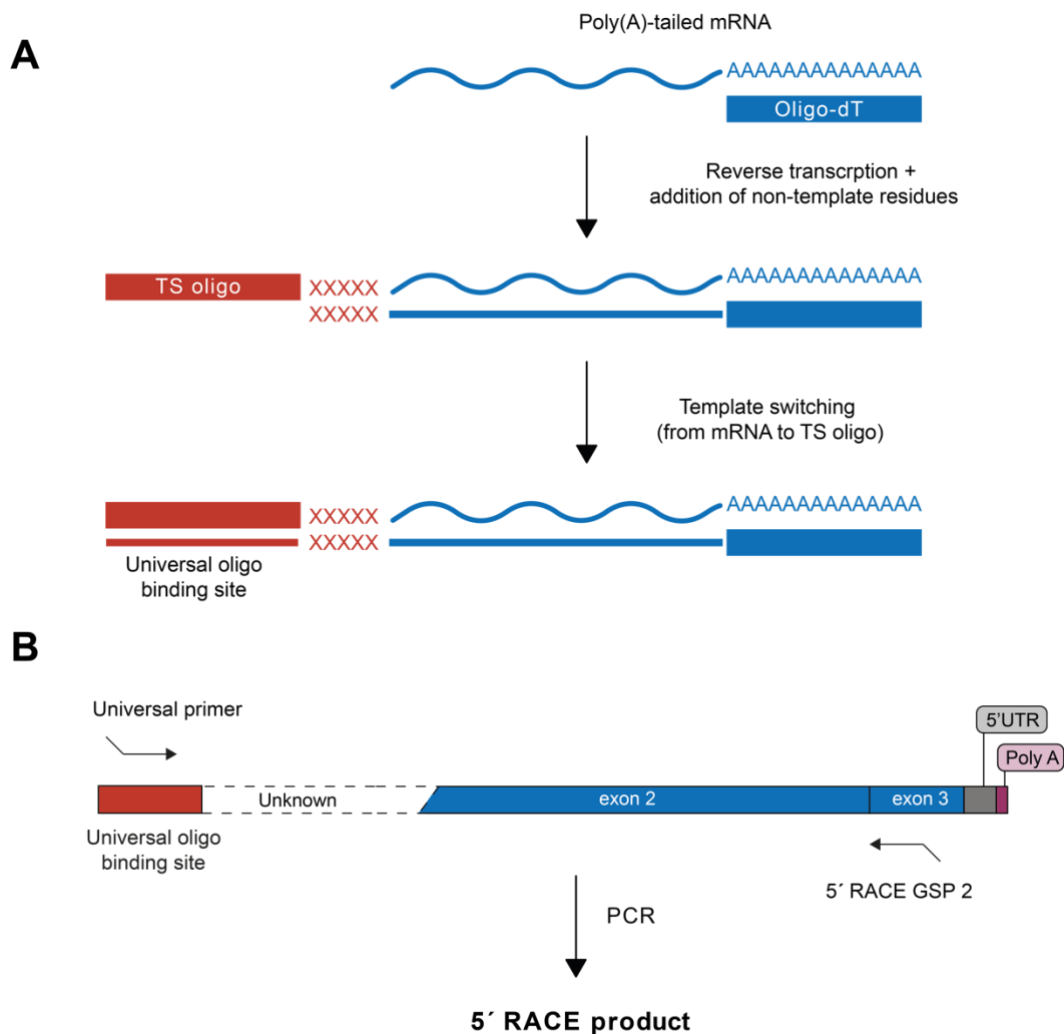


Figure 6.5 Schematic representation of 1st strand cDNA synthesis and 5' RACE

(A) A schematic representation of first strand cDNA synthesis using oligo dT and a reverse transcriptase with terminal transferase activity. Non-templated residues act as an anchoring site for a TS oligo (template switching). Switching from the RNA template to the TS oligo generates cDNAs containing a universal sequence at the 3' end. **(B)** 5' RACE using the universal and GSP2 primers.

Following amplification, 5' RACE products were run on an agarose gel. Two distinct bands were produced (Fig. 6.6) suggesting that multiple *dAGXT* transcripts exist. The large band at ~1.4 kb is of the expected size for the 5' end (using GSP2) of the currently annotated *dAGXT* transcript. The small band represents a shorter unannotated transcript, and is seen in both conditions indicating that a short transcript is also present under unchallenged conditions. Of note, 5' RACE is not quantitative and therefore the abundance of each product cannot be compared between conditions.

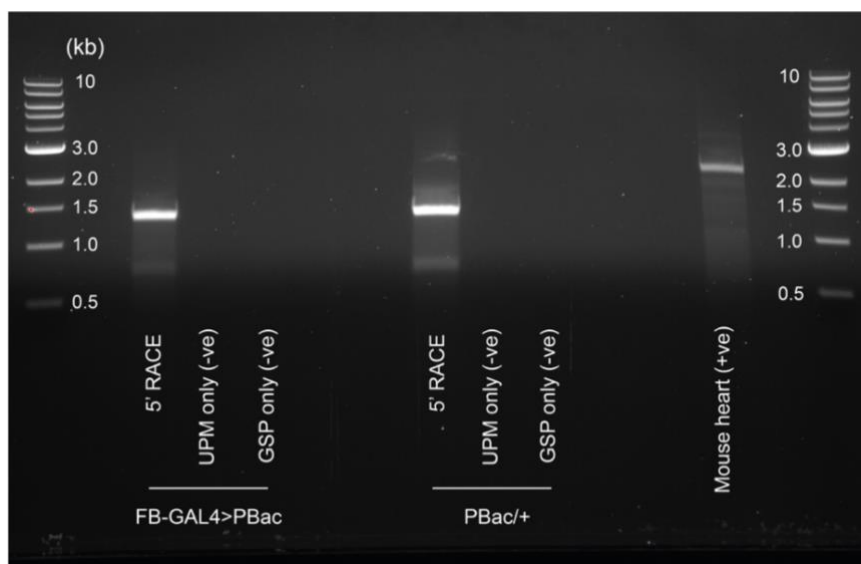


Figure 6.6 Identification of multiple 5' RACE products in FB GAL4>CG1622^{PBac} and CG1622^{PBac/+} (control) animals

5' RACE products from FB-GAL4>CG1622^{PBac} and CG1622^{PBac/+} (control) animals analysed by gel electrophoresis. Negative 5' RACE controls UPM (universal primer mix) only and GSP (gene-specific primers) only. Mouse heart positive 5' RACE control (using mouse specific GSP).

6.2.6. Characterisation of 5' RACE products

5' RACE products were cloned into a suitable vector before being transformed into NEB 5-alpha competent *E. coli* cells. Ten colonies were picked and grown before the plasmids were isolated (QIAGEN miniprep) for sequencing. Products were sequenced using vector-specific primers (M13F and M13R) targeting the flanking regions of the RACE products (facing inwards). Gene-specific primers targeting known 3' regions were also used for sequencing to ensure full coverage. Overlapping sequencing reads were assembled in SnapGene (genetic engineering software) before 5' RACE product boundaries were defined by annotating the Universal primer and GSP2 binding sequences. Features were then manually annotated using the reference sequence (<https://flybase.org>). A number of polymorphisms were identified (Fig. 6.7, indicated by red lines), all of which were synonymous mutations. Low quality bases were verified manually.

Characterisation of RACE products

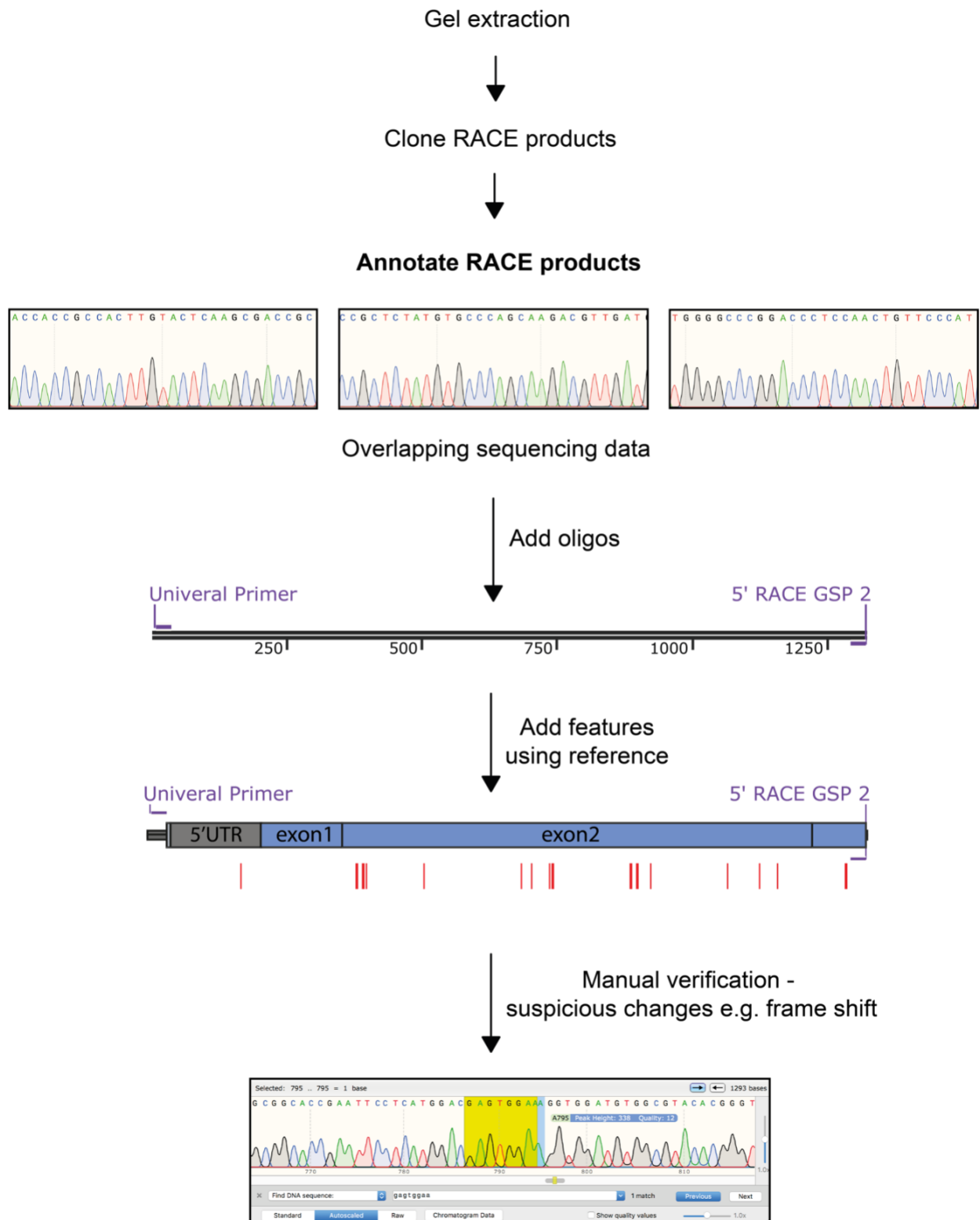


Figure 6.7 Characterisation of 5' RACE products

Schematic representation 5' RACE characterisation workflow. Sequence trace files are viewed, and peak quality checked using 4Peaks software. Trace files are imported and annotated using SnapGene software. Reference genome from FlyBase, Release 6.

5' RACE product sequences were aligned against the first annotated long 5' RACE product for comparison (Fig. 6.8). Each alignment represents the sequencing of an individual colony. With the exception of polymorphisms, all long 5' RACE products sequenced were identical and matched the current reference sequence. Interestingly, a number of short 5' RACE products were identified. These appear to be true messages - artifacts were not observed within the long 5' RACE products and are therefore unlikely to be present within the short products. Degraded RNA can also be excluded as only capped messages are amplified by RACE (the transcriptase used for synthesising the cDNA only adds non-templated nucleotides to capped messages). Therefore, the numerous short 5' RACE products are likely to result from either differential transcription initiation and/or post-transcriptional RNA processing (i.e., RNA cleavage) as discussed further in Section 6.3.1. The short 5' RACE products identified are also consistent with SAGE (serial analysis of gene expression) data from MachiBase (Ahsan et al., 2009, <http://machibase.gi.k.u-tokyo.ac.jp/>), where a number of 5' end tags were identified within the second exon of *dAGXT* (Fig. 6.11). The short 5' RACE products also share strong similarities with the annotated short variant of the archetypal mammalian *AGXT*, most notably with respect to the location of the downstream alternative translation start site relative to the PLP binding site (Fig. 6.12).

All RACE products appear to result from either differential transcription initiation or post-transcriptional RNA cleavage, with the exception of one product found exclusively in FB-GAL4>*CG1622*^{PBac} animals (Fig. 6.8; arrow). This RACE product is instead characteristic of a splice variant with the splice site being within the 5'UTR. The presence of splice sites within the 5' UTR is fairly common in many organisms including humans and *Drosophila* (Nagasaki et al., 2005). No predicted splice sites (GT-AG termini) for the major spliceosome were found at the expected location. There are however AT-AC termini - the consensus sequence recognised by the minor spliceosome (Otake et al., 2002). The fact that this possible splice variant appears exclusivity in FB-GAL4>*CG1622*^{PBac} animals raises the question of whether *CG1622* – a putative splicing factor – may directly regulate *dAGXT* (discussed in Section 7.2.3). All the short 5' RACE products identified would lack the exons 1-2 junction and could contribute to the loss of probe (┐76) binding observed in FB-GAL4>*CG1622*^{PBac} animals.

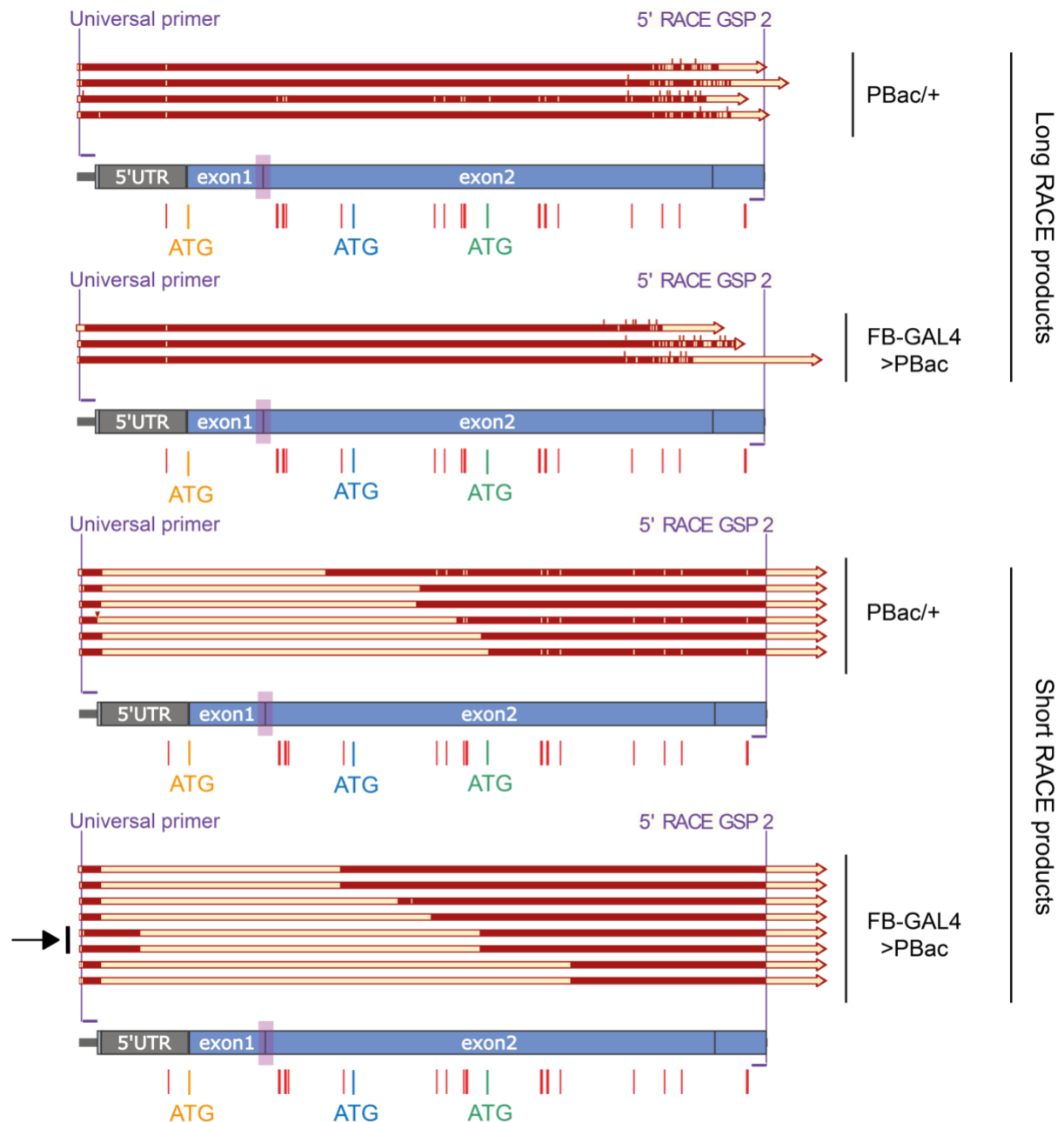


Figure 6.8 5' RACE product sequence alignments

5' RACE product sequences alignments from FB-GAL4>*CG1622*^{PBac} and *CG1622*^{PBac/+} (control) animals. Schematic representation of *dAGXT* mRNA with features below for reference. The currently annotated translation initiation codon (ATG orange) and potential alternative downstream in frame translation start site (ATG green). The longest transcript identified could potentially use another in frame translation start site (ATG blue). Numerous synonymous polymorphisms were identified (red lines).

Although multiple short *dAGXT* mRNAs exist, it is likely that that all produce the same polypeptide. With the exception of one transcript (longest), all short mRNAs identified would use the same downstream translation start site (ATG in green) (Fig. 6.8), although it is possible that not all messages are translated. This downstream alternative translation start site is in frame with the currently annotated start site

(Fig. 6.8; ATG in orange). Although all short mRNAs may encode the same protein, their 5' UTRs will differ in their sequence and length. Such differences could have a significant impact on translational efficiency, mRNA processing, stability and export. For the remainder of this thesis, I will refer to the protein encoded by the short mRNAs as the short dAGXT variant to distinguish it from the annotated long product.

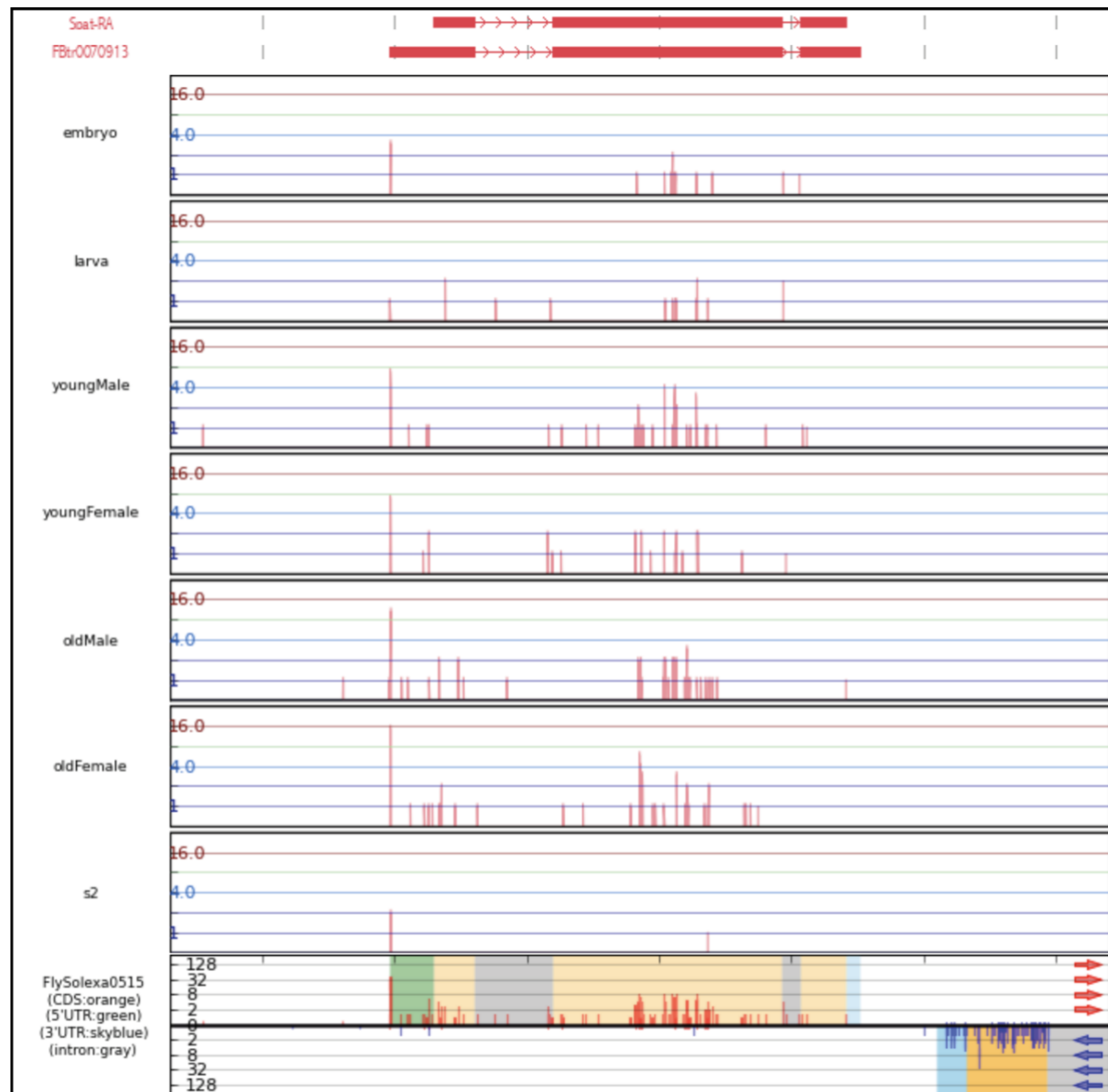


Figure 6.9 Numerous 5' end tags in SAGE data from MachiBase consistent with 5' RACE products

Top, CDS and reference mRNA from FlyBase. Peaks (red) represent 5' end tags. Bottom, merge of 5' end tags. Key features: 5'UTR (green), CDS (orange), intron (grey) and 3'UTR (sky blue).

The identification of short *dAGXT* transcripts and the likelihood that downstream TSSs exist suggest that *AGXT*^[N-GFP;STOP] animals may express a short variant, and only a short variant. The presence of a transcriptional stop sequence within the Pax-Cherry cassette (Fig. 6.2) would terminate transcription initiated from the TSS of the canonical transcript. This could stimulate the use of downstream TSSs which would generate shortened mRNAs similar to those identified by 5' RACE (Fig. 6.8). Thus it is likely that *AGXT*^[N-GFP;STOP] produces only the short polypeptide. Crucially, this variant would lack the N-terminal putative MTS and would be expected to exclusively target peroxisomes. As such, *AGXT*^[N-GFP;STOP] could in theory be used as a peroxisomal-only line to study the effects of AGXT mistargeting *in vivo*.

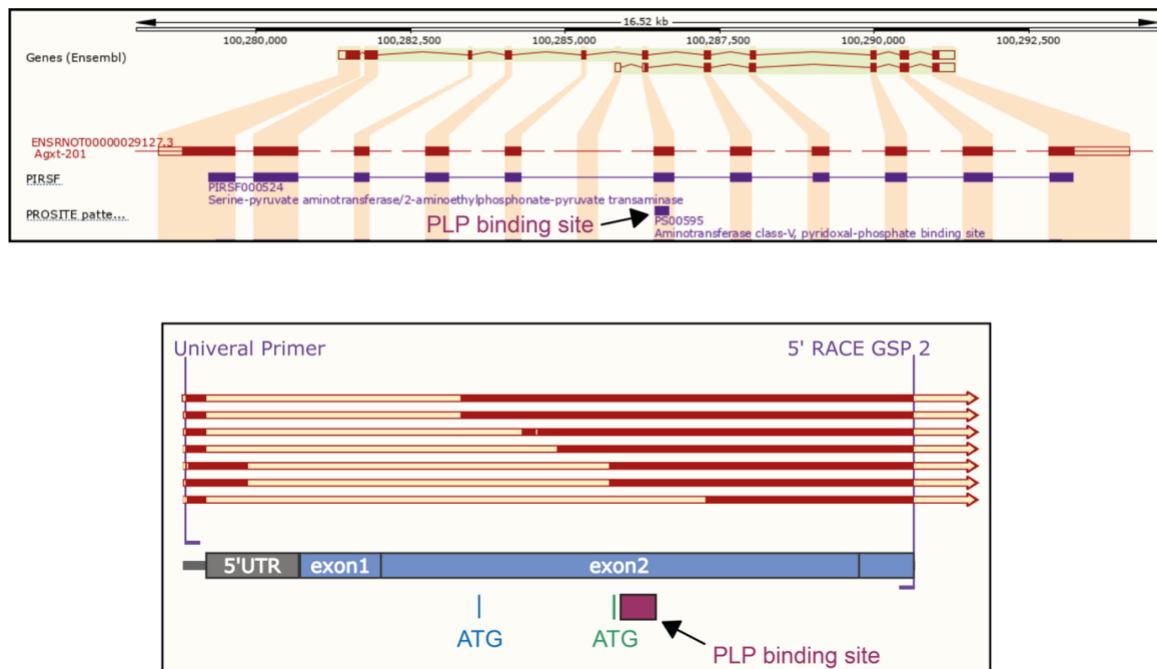


Figure 6.10 Short 5' RACE products share similarities with the annotated short variant of the archetypal mammalian *AGXT*

Top, rat annotated *AGXT* reference sequence (Ensembl, genome browser) with PLP binding site (arrow). Bottom, short 5' RACE products sequence alignments with annotated reference below and PLP binding site (arrow).

6.2.7. GFP-AGXT fusion is upregulated in response to starvation

The nutritional regulation of AGXT has not been fully explored in the literature. Although *dAGXT* has been found to be transcriptionally upregulated in response to starvation in a number of high throughput studies (Grönke et al., 2005; Sonn et al., 2018; Zinke et al., 2002), these results have not been followed up or validated. *AGXT*^[N-GFP] animals were used to investigate changes in protein expression in

response to starvation. Fat bodies of *AGXT^{IN-GFP}* animals were dissected and imaged following 24 h starvation. GFP intensity was enhanced following starvation demonstrating upregulation of dAGXT protein expression (Fig. 6.11). The upregulation of AGXT could be understood in light of its role in gluconeogenesis via transamination of serine. The gluconeogenic role of dAGXT is predicted to take place in the mitochondria. Therefore, starvation could be expected to particularly increase the mitochondrial pool of dAGXT. The identification of long and short *dAGXT*, which encode polypeptides with or without the putative MTS suggests a possible mode of regulation, whereby organelle targeting could be regulated via inclusion/exclusion of the putative MTS in response to nutritional cues. A similar mechanism has been described in mammals where specifically the long MTS-containing variant is upregulated in response to gluconeogenic stimuli (Miyajima et al., 1989; Oda et al., 1982; Uchida et al., 1994).

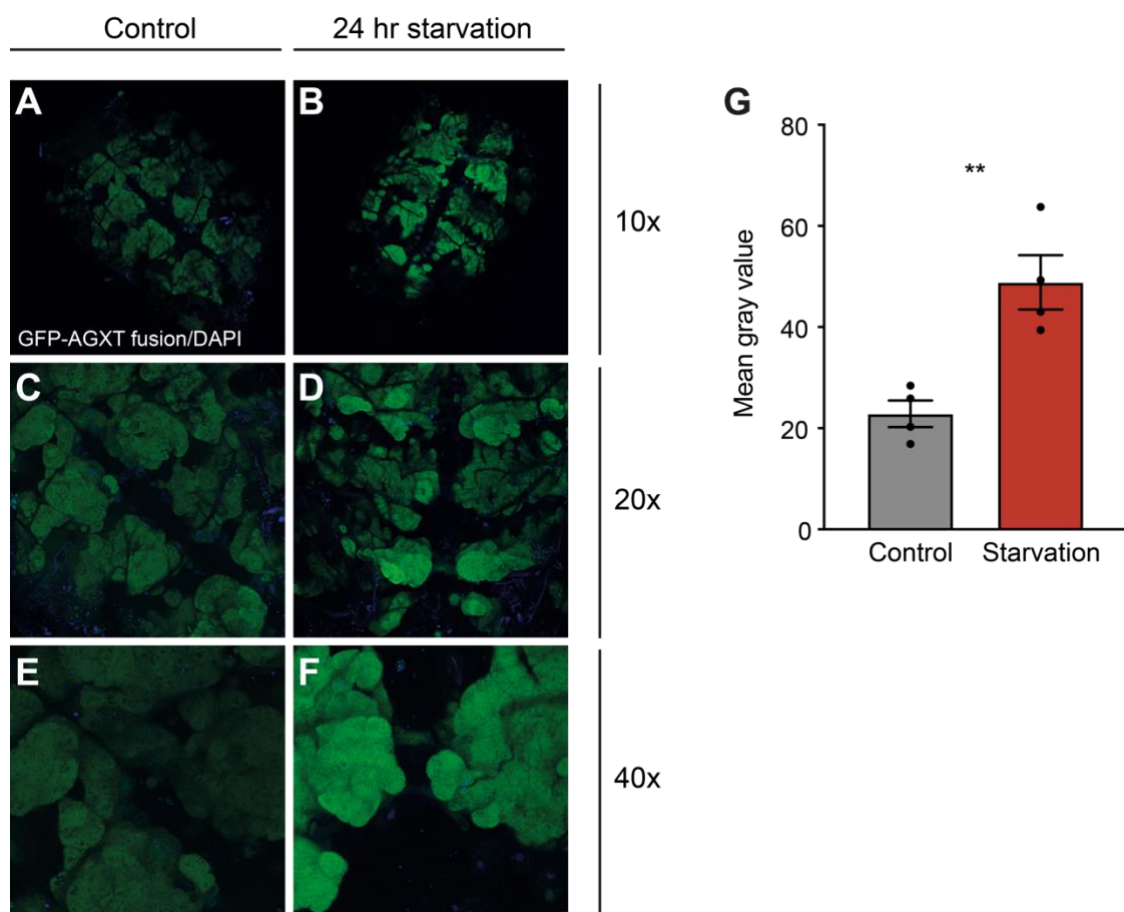


Figure 6.11 GFP-AGXT fusion is upregulated in response to starvation

Confocal imaging of dissected fat body tissue from *AGXT^{IN-GFP}* adult males (day 7) immunostained for GFP (green) and DAPI (blue) at (A, B) 10x, (C, D) 20x and (E, F) 40x magnification. (A, C, E) SYA control and (B, D, F) 24 h starvation. (G) GFP intensity was estimated by the quantification of gray levels across a selected region. The area of selected region was consistent between samples. Z-stacking and quantification were performed in ImageJ. Results are shown as mean ± SEM and analysed by unpaired t-test. **, P ≤ 0.01.

6.2.8. *AGXT*^[N-GFP;STOP] animals are not sensitive to starvation suggesting serine catabolism through dAGXT is not a major contributor to gluconeogenesis

dAGXT protein expression was upregulated in response to starvation (Fig. 6.11), consistent with results from mammalian studies demonstrating transcriptional upregulation of AGXT in response to starvation as well as other gluconeogenic stimuli (Miyajima et al., 1989; Toshiaki Oda et al., 1982; Uchidas et al., 1994). Upregulation of AGXT is believed to reflect its role in gluconeogenesis via the transamination of serine which takes place in the mitochondria. *AGXT*^[N-GFP;STOP] animals would at best express a short variant that exclusively targets peroxisomes (as previously discussed in Section 6.2.6). Such a protein would be unable to contribute to gluconeogenesis via transamination of serine. *AGXT*^[N-GFP;STOP] animals were not sensitive to starvation (Fig. 6.12) suggesting serine catabolism through dAGXT is not a major contributor to gluconeogenesis in *Drosophila*. No changes in glucose metabolism have been reported in PH1 patients (lacking functional AGXT) suggesting the same could be true for humans. This is further supported by similar findings in mice where blood glucose levels were similar in *AGXT*^{-/-} and control animals following fasting (Salido et al., 2006). L-serine may instead enter the gluconeogenesis pathway primarily through serine dehydratase.

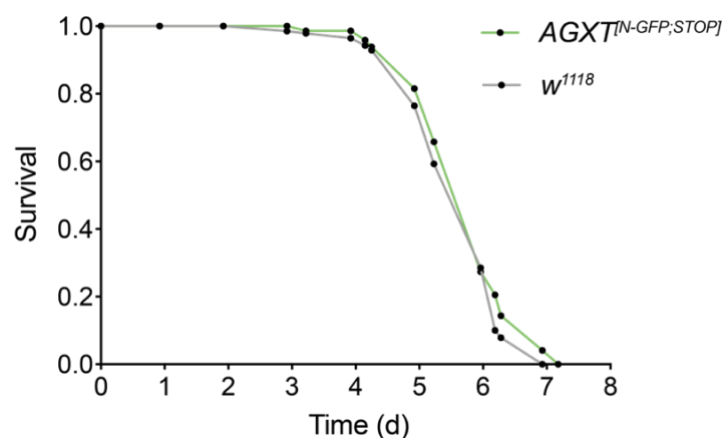


Figure 6.12 *AGXT*^[N-GFP;STOP] animals are not sensitive to starvation

Survival of *w*¹¹¹⁸ and *AGXT*^[N-GFP;STOP] adult males (aged to day 7 before treatment). n=140-146 per condition. Statistical analysis was carried out with a Log-Rank test on survivorship data. ns, P>0.05.

6.2.9. HFD causes renal stones in *Drosophila*

In the following sections, I will focus on renal stones in the context of HFD. Given the known nutritional regulation of AGXT, I also explored the relationship between dAGXT and HFD-associated renal stones.

The role of AGXT in CaOx nephrolithiasis was covered in detail (Section 6.1). Briefly, functional deficiency of AGXT in PH1 results in excessive glyoxylate accumulation which leads to increased endogenous synthesis of oxalate. Elevated urinary oxalate excretion is associated with an increased risk of recurrent CaOx nephrolithiasis and nephrocalcinosis which can lead to end-stage renal disease. Although PH1 is rare, nephrolithiasis itself is very common and currently affects around 10% of the world's population. The incidence and prevalence of nephrolithiasis is increasing globally and with high morbidity and recurrence, it represents a significant global disease burden. In humans, CaOx is the primary component of nephrolithiasis accounting for ~80% of all stones (Lemann et al., 1996; Sakhaee, 2018).

With obesity being a well-recognised risk factor of nephrolithiasis, I investigate whether a HFD could induce renal stone formation in *Drosophila*. Work from our group recently demonstrated that a HSD promotes uric acid stone formation as a result of water imbalance and altered purine metabolism (van Dam et al., 2020). I was next interested in exploring whether a HFD could also promote renal stone formation. Control animals (*w¹¹¹⁸*) were raised on a HFD (15% lard) for 24 days before the Malpighian tubules were dissected and scored. Animals raised on a HFD accumulated significantly more renal stones compared to controls (Fig. 6.13). These data indicate that *Drosophila* could be a promising translational model for studying the impact of HFD on renal stone formation.

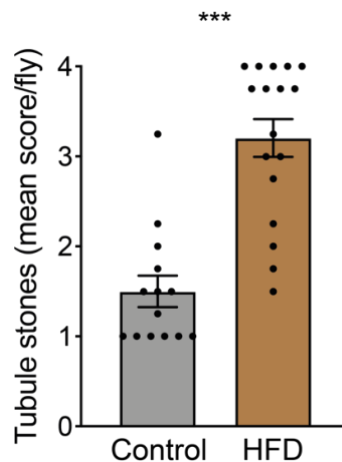


Figure 6.13 HFD causes renal stones in *Drosophila*

Tubule stones scored based on severity in *w¹¹¹⁸* adult males raised on control media (SYA) and HFD age 26 days with 24 days on HFD. n=14-17. Results are shown as mean score $\pm$ SEM and analysed by unpaired t-test. Scoring as described in Fig. 6.1B. ***, $P \leq 0.001$.

6.2.10. *AGXT^[N-GFP;STOP]* animals are protected from renal stone accumulation on HFD suggesting endogenous synthesis of oxalate contributes to HFD associated renal stones

Hyperoxaluria in obese patients is a major risk factor for CaOx nephrolithiasis (Poore et al., 2020; Sakhaee, 2018) with the degree of hyperoxaluria being related to the incidence of renal stones (Kleinman, 2007). Although the molecular mechanisms of obesity associated hyperoxaluria are unknown, endogenous hepatic oxalate synthesis is speculated to be a contributing factor. Having demonstrated that *Drosophila* accumulate renal stones on HFD, I used this model to explore the role of endogenous oxalate synthesis in HFD-associated renal stones.

If endogenous oxalate synthesis contributes to the formation of renal stones on a HFD, increased clearance of the oxalate precursor, glyoxylate, would be expected to reduce renal stone accumulation. To test this hypothesis, *AGXT^[N-GFP;STOP]* animals were raised on a HFD. *AGXT^[N-GFP;STOP]* (lacking the putative MTS) is expected to exclusively target the peroxisomes - the major site of glyoxylate detoxification by AGXT. Re-directing catalytically active AGXT away from the peroxisomes to the mitochondria in PH1 is known to reduce glyoxylate clearance and leads to CaOx renal stone formation. Conversely, increasing peroxisomal targeting of AGXT via vitamin B₆ treatment improves glyoxylate clearance and reduce oxalate synthesis in PH1 patients (Cellini et al., 2014). I therefore reasoned that re-directing AGXT exclusively to the

peroxisomes could improve glyoxylate clearance and reduce endogenous oxalate synthesis this model. With this assumption in mind, *AGXT*^[N-GFP;STOP] animals could be used to test this initial hypothesis since they are expected to exhibit improved glyoxylate clearance. *AGXT*^[N-GFP;STOP] animals have significantly reduced renal stone accumulation on a HFD compared to controls (Fig. 6.16B). These results support the notion that endogenous oxalate synthesis contributes to the formation of renal stones on a HFD in *Drosophila*. These data also further support the idea *AGXT*^[N-GFP;STOP] constitutes a GOF allele that boosts peroxisomal targeting and hence glyoxylate clearance. Further investigation into organelle targeting of AGXT in addition to oxalate measurements would be necessary to draw definitive conclusions (discussed further detail in Chapter 7).

I investigated the effects of increasing endogenous oxalate synthesis on renal stone formation in the context of a HFD by knocking down *dAGXT*. To do this, *act-GAL4>dAGXT*^{RNAi KK} animals were raised on a HFD (15% lard). Within 14 days on a HFD, these animals developed severe renal stones (Fig. 6.14). These results demonstrate that impaired glyoxylate detoxification via the knockdown of AGXT exacerbates HFD-associated renal stones.

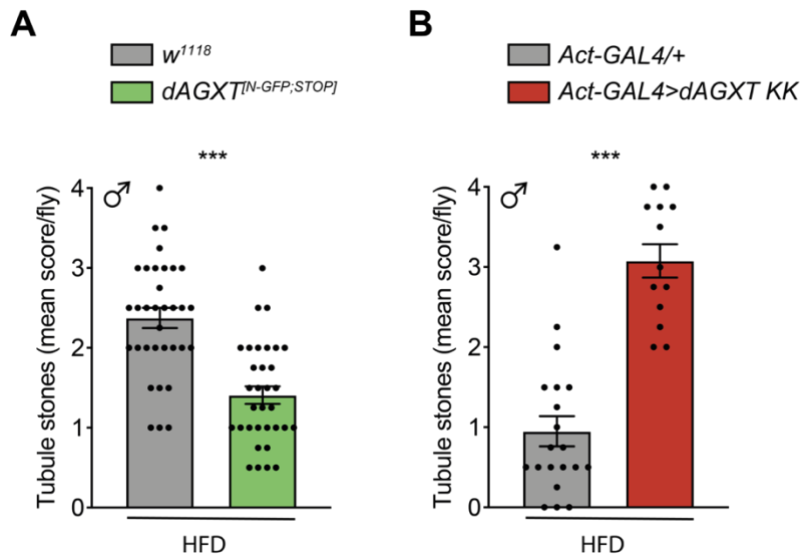


Figure 6.14 *AGXT*^[N-GFP;STOP] animals are protected from renal stone accumulation on a HFD

Tubule stones scored based on severity in (A) *w*¹¹¹⁸ and *AGXT*^[N-GFP;STOP] and (B) *act-GAL4>dAGXT*^{RNAi KK} and *act-GAL4/+* (control) adult males raised on HFD. (A) Age day 30 with 28 days on HFD. n=33-34. (B) Age day 28 with 14 days on HFD. n=13-20. Results are shown as mean score $\pm$ SEM and analysed by unpaired t-test in both datasets. Scoring as described in Fig. 6.1B. Of note *AGXT*^[N-GFP;STOP] animals were dissected after 28 days on HFD vs 14 days for *act-GAL4>dAGXT*^{RNAi KK}. Several deaths were observed throughout from *act-GAL4>dAGXT*^{RNAi KK} animals. ***, $P \leq 0.001$.

6.2.11. $AGXT^{[N-GFP;STOP]}$ males are long-lived

Lifespans were assessed as part of the phenotypic characterisation of $AGXT^{[N-GFP;STOP]}$ animals (Fig. 6.15). Intriguingly, $AGXT^{[N-GFP;STOP]}$ males were significantly longer lived than w^{1118} controls. $AGXT^{[N-GFP;STOP]}$ females were not significantly long-lived although control animals exhibit a somewhat unusual lifespan curve (tailing off from ~day 55). Female lifespan experiments would need repeating for more conclusive interpretations. $AGXT^{[N-GFP;STOP]}$ animals could conceivably be long-lived as a result of reduced renal stone deposition, assuming that renal stones contribute to mortality on the SYA diet. Alternatively, changes in mitochondrial or peroxisomal metabolism could contribute to increased longevity independently of renal stones. AGXT responds to nutrient-sensing pathways (including insulin), which are well known for their roles in ageing and longevity (Partridge et al., 2011).

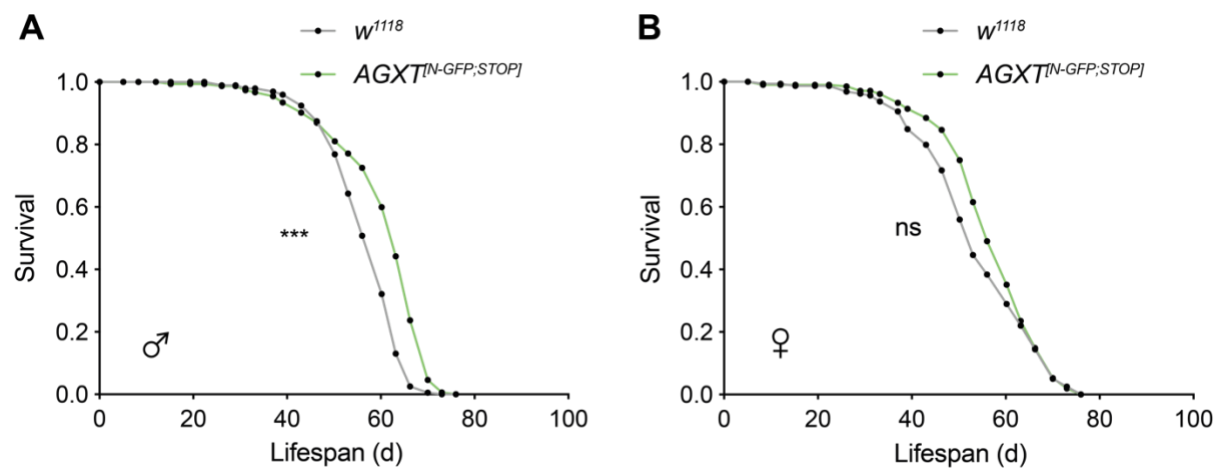


Figure 6.15 $AGXT^{[N-GFP;STOP]}$ males are long lived

Lifespans of w^{1118} and $AGXT^{[N-GFP;STOP]}$ male and females. n=210 per condition. Statistical analysis was carried out with a Log-Rank test on survivorship data. ns, $P>0.05$; ***, $P\leq 0.001$.

6.3. Discussion

AGXT is a liver-specific aminotransferase with dual metabolic functions; gluconeogenesis - primarily via the transamination of serine - and glyoxylate detoxification. These two distinct metabolic functions take place in different intracellular organelles; glyoxylate detoxification in peroxisomes, and gluconeogenesis in mitochondria. Functional deficiency of AGXT is associated with PH1, a rare autosomal recessive disorder of glyoxylate metabolism. Despite its relevance to human disease, AGXT is almost completely unstudied in *Drosophila*. Given its known metabolic functions, relevance to human disease and broad scope for novel findings, in Chapter 6 I set out to characterise *Drosophila* AGXT and explore its role in renal pathology.

Knockdown of *dAGXT* resulted in spontaneous precipitation of renal stones, recapitulating a major pathological feature of PH1 making it a promising translational model to explore new treatments. This is particularly important given that, currently, the only curative treatment for PH1 is a liver transplant. Following these initial findings, I generated novel genetic tools for the study of *dAGXT* including a knock-in mutant and GFP reporters. Using these tools, I was able to demonstrate that dAGXT protein localises to the fat body, consistent with human AGXT being liver-specific. Knockdown of *dAGXT* in the fat body was sufficient to cause renal stones confirming that glyoxylate detoxification by dAGXT takes place in the fat body. At the intracellular level, dAGXT (N-terminal GFP fusion) appears to localise to peroxisomes, consistent with the prediction that glyoxylate detoxification takes place in peroxisomes, although co-localisation experiments would be required to confirm this observation. The use of an N-terminal GFP fusion allows peroxisomal targeting to be assessed; the putative PTS1 is C-terminal and unaffected. However, with GFP appended to the N-terminus, the N-terminal MTS is likely to be disrupted. Therefore, a C-terminal GFP fusion would be required to investigate mitochondrial targeting (covered further in Chapter 7).

The identification of short *dAGXT* transcripts by 5' RACE was particularly intriguing given that these variants lack the putative N-terminal MTS. This raises the question of whether dAGXT organelle targeting, and therefore metabolic function, could be controlled by the differential regulation of transcripts. Indeed, a similar mode of control has been described in mammalian models, where AXGT targeting was demonstrated

to be transcriptionally regulated via the inclusion/exclusion of an MTS through the use of alternative TSSs in response to nutritional cues (Miyajima et al., 1989; Oda et al., 1982; Uchidas et al., 1994).

Following reports of *AGXT* being transcriptionally upregulated in response to starvation in a number of high throughput studies (Grönke et al., 2005; Sonn et al., 2018; Zinke et al., 2002), I used the N-terminal GFP fusion allele to assess changes at the protein level. Consistent with these studies, I found elevated levels dAGXT protein based on the novel GFP fusion reporter line in response to starvation, likely reflecting its role in gluconeogenesis.

Towards the end of Chapter 6, I describe a fly model for the study of HFD-associated renal pathology and use novel genetic tools to explore the underlying aetiology. With this model, I find evidence supporting the notion that endogenous oxalate synthesis contributes to HFD-associated renal stones in *Drosophila* (Figs. 6.16 and 6.17).

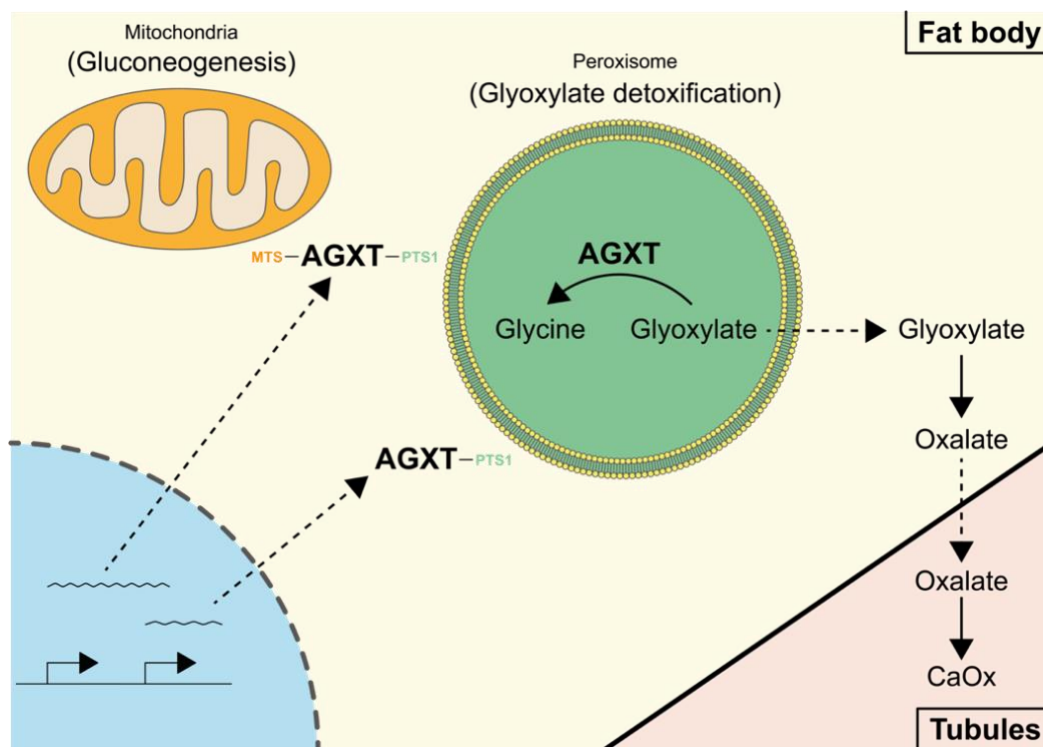


Figure 6.16 Working model: differential transcript choice, i.e., inclusion/exclusion of the MTS and hence organelle targeting, could underlie an important regulatory mechanism of metabolic activity

(A) A schematic depicting differential regulation of *dAGXT* transcripts. The long (currently annotated) variant encodes a protein containing a putative N-terminal MTS and C-terminal PTS1. The short variant lacks the putative MTS. Differential regulation of transcripts could allow an organism to respond to changing metabolic demands by influencing organelle targeting via the inclusion/exclusion of the MTS. AGXT organelle targeting is important in human disease; peroxisomal AGXT deficiency (mitochondrial mistargeting) is associated with PH1.

6.3.1. The identification of short *dAGXT* transcripts

The identification of short *dAGXT* transcripts hints towards a mechanism whereby organelle targeting, and ultimately metabolic activity, could be regulated by the use of alternate transcripts. A question that remains is how are these short messages generated? To understand the origin of these short 5' RACE products, it is important to consider the processes that generate transcript diversity. Differential transcription initiation, termination, alternative splicing and other forms of post-transcriptional modifications including RNA cleavage all contribute to the diversity of the transcriptome. Initially, the use of alternative TSSs seemed to be the most likely explanation for the short 5' RACE products. Alternative transcription initiation is common and a major contributor to transcriptional diversity. CAGE (cap analysis of gene expression), a technique used to tag the position of 5' caps of mRNAs using a similar approach to 5' RACE, was designed to assist in the identification of TSSs. The assumption was that 5' ends would primarily map TSSs. Although alternative promotor usage makes up a large proportion of CAGE tags, there is strong evidence that CAGE (and 5' RACE) will also identify other capped messages (Georgakilas et al., 2020; Mercer et al., 2010), including processed mRNAs. The 5' ends of the RACE products identified in this chapter all map within exonic coding sequences. Such intraexonic 5' tags were previously thought to derive solely from transcripts initiated from internal promoters. More recently, post-transcriptional RNA cleavage is believed to be important in generating such products. Although historically cleaved RNA transcripts were viewed as transitional intermediates in the degradation of mRNA, they are now viewed as stable and functional products of longer transcripts (Mercer et al., 2010). Previously, RNA capping was believed to only take place co-transcriptionally in the nucleus, but it is now recognised that cleaved mRNAs can undergo re-capping - also known as secondary capping - in the cytoplasm (Ramanathan et al., 2016). These messages can be stable and retain the potential to be translated into shortened functional protein isoforms. The role of post-transcriptional RNA cleavage in generating transcript diversity was first proposed (Fejes-Toth et al., 2009), around the time that cytoplasmic capping was discovered (Otsuka et al., 2009). Specifically, CAGE tags that mapped within exonic coding sequences were proposed to arise from RNA cleavage and subsequent secondary capping (Fejes-Toth et al., 2009). Post-transcriptional cleavage events were later shown to be widespread and important

contributors to the diversity of the transcriptome (Mercer et al., 2010). The numerous short 5' RACE products identified here could therefore result from differential transcription initiation and/or post-transcriptional RNA processing.

Another question raised following the results of 5' RACE is why there are numerous short products? One gene, generating multiple mRNAs with different 5' UTR may actually be fairly common. Accumulated CAGE data have demonstrated that a single gene can have highly heterogeneous 5' ends. The 5' UTR plays a key role in gene expression - influencing translational efficiency, mRNA processing, stability and export. As such, 5' heterogeneity is believed to be an important manifestation of complex transcriptional regulation in determining gene expression. Some may not be translated, or some may be more dominant than others.

6.3.2. AGXT: atypical targeting peptides

Throughout Chapter 6, I have highlighted the importance of organelle targeting for AGXT to carry out its metabolic functions and have suggested that targeting could be controlled by differential regulation of transcripts. So far, much of the focus has been at the organism level; how can targeting be regulated to meet the changing metabolic demands of an organism?

Organelle targeting is also acted on at the evolutionary level; the sequences of the targeting peptides have been subject to dietary selection pressures. The sequence and thus strength of the targeting peptides reflects the metabolic requirements of an organism. The major glyoxylate precursor in herbivores is synthesised in peroxisomes. As such, herbivores generally harbour a weak/cryptic MTS to favour peroxisomal targeting. The major glyoxylate precursor in carnivores is believed to be hydroxyproline which is converted to glyoxylate in the mitochondria. Therefore, carnivores generally harbour a strong MTS (Danpure, 2006).

While comparing targeting peptides between species, an interesting observation is that they often appear to be sub-optimal (e.g., AGXT known to localise to peroxisomes is often unidentified by predictive tools). The PTS1 of AGXT is atypical. A prototypal PTS1 consists of the C-terminal tripeptide SKL. This tripeptide mediates peroxisome targeting by binding to the TPR domain of the predominantly cytosolic PEX5 receptor. Cargo bound PEX5 then docks at the peroxisomal membrane for import (Dammai &

Subramani, 2001; Gould et al., 1996). In humans, the C-terminal tripeptide of AGXT is KKL. This tripeptide alone is not able to mediate peroxisomal import (unable to direct GFP to peroxisomes as part of a C-terminal fusion protein) and requires an additional ancillary sequence termed PTS1A located within the small C-terminal structural domain (Huber et al., 2005). The C-terminal tripeptides of AGXTs are poorly conserved compared to other PTS1 proteins and few species have the typical SKL tripeptide (Table 6.1). Therefore, it is likely that AGXT in other organisms would also require ancillary sequences for peroxisomal import (Danpure, 2006). The crystal structure of AGXT shows the PTS1A in close proximity to the C-terminal KKL. PTS1A may therefore improve stability of the KKL-TPR interaction (Zhang et al., 2003). It is conceivable that the atypical/suboptimal MTS and PTS1 observed in many organisms may have been selected for to allow flexibility of targeting. Targeting could be regulated via protein interactions that either functionally strengthens the MTS or weakens the PTS1; for example, by interfering with the PTS1A or by acting on the kinetic partitioning of AGXT (altering folding and/or dimerisation). This may allow shifts in the subcellular distribution of AGXT in response to changing metabolic demands via an alternative mechanism. This form of regulation could be particularly important in organisms that cannot regulate targeting transcriptionally (e.g., do not express short variants lacking MTS).

Table 6.1 Variation in the C-terminal tripeptide of mammalian AGXT enzymes

Adapted from Danpure, 2006

Species	C-terminal tripeptide
Human and other primates	KKL
Rat and other murine rodents	NKL
Rabbit	HRL
Guinea Pig	SQL
Cat	NKL
Dog	HKL
Cow	SKL
Prototypical PTS1	SKL

6.3.3. Nutritional regulation of *AGXT*

AGXT has been found to be nutritionally regulated in a number of high throughput studies in *Drosophila* - transcriptionally upregulated in response to starvation (Grönke et al., 2005; Sonn et al., 2018; Zinke et al., 2002) and downregulated in response to a HSD (Zinke et al., 2002). High throughput studies have also implicated mammalian *AGXT* in nutritional response, with *AGXT* found to be downregulated in response to a HFD in mice (Soltis et al., 2017a; Soltis et al., 2017b) and also in response to a combined HFD plus high fructose/glucose (Asgharpour et al., 2016). Although *AGXT* has been identified in a number of high throughput studies, only a few have specifically focused on the nutritional regulation of *AGXT* (Miyajima et al., 1989; Oda et al., 1982; Uchidas et al., 1994). These studies demonstrated that *AGXT* is upregulated in response to gluconeogenic stimuli.

The archetypal mammalian *AGXT* is encoded from a single *AGXT* gene that produces two transcripts from alternative TSSs (Oda et al., 1990). Two polypeptides are translated: the long form containing both MTS and PTS1, and the short form containing only a PTS1. In Chapter 6, I have described the presence of similar *AGXT* variants in *Drosophila*. Increased TAG storage in *CG1622* knockdown animals was associated with reduced expression of the full length *dAGXT* transcript, suggesting that differential regulation of transcripts could allow a response to changing metabolic demands by controlling organelle targeting in *Drosophila*. A similar mechanism of differential transcript choice has been described in mammals in response to low energy status; gluconeogenic stimuli including glucagon, high protein diet, insulin and glucocorticosteroids results in upregulation of specifically the long MTS-containing *AGXT* (Miyajima et al., 1989; Oda et al., 1982; Uchidas et al., 1994). Together, these results demonstrate that that *AGXT* targeting can be transcriptionally regulated via the inclusion/exclusion of the MTS by the use of alternative TSSs in response to nutritional cues.

The most 5' translation start site was lost during evolution in some species including humans. Interestingly the remaining N-terminal extension still acts as a weak/cryptic MTS in many of these species, which led me to wonder whether partial mitochondrial targeting may have been beneficial and selected for in species that primarily rely on peroxisomal targeting. The human Pro11Leu polymorphism for example - known to

strengthen the MTS (and associated with PH1 when homozygous) - is maintained at a high frequency in Sámi populations (an indigenous Finno-Ugric people). Sámi people have diets high in meat, therefore partial mitochondrial targeting may have been beneficial and selected for despite the deleterious recessive effects of this polymorphism (Caldwell et al., 2004).

The gluconeogenic role of AGXT takes place in the mitochondria. However, preventing mitochondrial targeting using the *AGXT*^[N-GFP;STOP] line (peroxisomal only at best) does not increase starvation sensitivity. This would suggest that serine catabolism through dAGXT is not a major contributor to gluconeogenesis. Redundancy between aminotransferases that contribute to gluconeogenesis is likely. The upregulation of *AGXT* observed in response to starvation may be part of a coordinated programme of amino acid catabolism to feed the gluconeogenic response. In the same way that *AGXT* may be upregulated in response to gluconeogenic stimuli, it is possible that the same programme is downregulated in response to a HFD and high energy storage levels (e.g., to shut down gluconeogenic pathways). This could explain the downregulation of *AGXT* described in response to obesogenic diets described in the literature (Asgharpour et al., 2016; Soltis et al., 2017a; Soltis et al., 2017b; Zinke et al., 2002). Downregulation of the long *AGXT* variant in response to knockdown of CG1622 (Chapter 4) could also be in response to the high levels of fat storage and downregulation of the gluconeogenic programme.

6.3.4. HFD-associated renal pathology

Nephrolithiasis currently affects ~10% of the world's population, and its incidence and prevalence is increasing. With high levels of morbidity and recurrence, it represents a significant disease burden. Obesity is a well-recognised risk factor for nephrolithiasis. Obesity is associated with a number of co-morbidities; therefore, it is difficult to delineate its overall contribution to the risk of renal stones. However, independent associations between visceral obesity and nephrolithiasis have been described (Poore et al., 2020). CaOx stones are the most prevalent form of nephrolithiasis in obese individuals (Poore et al., 2020) with the major risk factor for CaOx stone formation being elevated urinary oxalate excretion (hyperoxaluria). In general, there is a direct relationship between the level of hyperoxaluria and stone incidence (Kleinman, 2007). Positive correlations between obesity/body size and hyperoxaluria are well

documented (Lemann et al., 1996; Powell et al., 2000; Sakhaee, 2018) yet are of unknown aetiology. Although the precise mechanism of obesity-related hyperoxaluria remains unclear, diet, increased intestinal oxalate absorption, microbiota and increased hepatic oxalate synthesis are all suggested as contributing factors (Sakhaee, 2018).

I initially explored whether *Drosophila* could be used to model HFD-associated renal pathology. HFD feeding was associated with significant renal stone formation indicating that *Drosophila* could be a promising model. Using this system, I set out to explore the underlying mechanism of HFD-associated nephrolithiasis. More specifically, I explored the hypothesis that endogenous synthesis of oxalate contributes to HFD-associated renal stones. This hypothesis was based on previous studies suggesting that obesity-associated hyperoxaluria is only partly enteric in origin (i.e., dietary oxalate). Human studies have suggested that the impact of dietary oxalate on urinary oxalate is minimal (Taylor & Curhan, 2008), hinting towards an endogenous source. Ob/Ob mice (a commonly used genetic model of obesity) raised on oxalate-free diet still exhibited significant hyperoxaluria (Amin et al., 2018), also indicating an alternative endogenous source.

If endogenous oxalate synthesis contributes to HFD-associated renal stones, reducing the abundance of the major and direct oxalate precursor, glyoxylate, would be expected to reduce renal stone accumulation. To decrease the abundance of glyoxylate, I reasoned that increasing peroxisomal targeting of dAGXT could increase its metabolic clearance - increasing peroxisomal targeting of AGXT via vitamin B₆ treatment is known to improve glyoxylate clearance and reduce oxalate synthesis in PH1 patients. *AGXT^[N-GFP;STOP]* can only express a short variant lacking the putative MTS due to the presence of a transcription termination signal downstream of the most 5' TSS. Without the putative MTS, this variant is expected to exclusively target peroxisomes and was used to test my hypothesis. *AGXT^[N-GFP;STOP]* animals maintained on a HFD exhibited significantly improved pathology compared to WT controls, supporting the notion that endogenous oxalate synthesis contributes to the formation of renal stones on a HFD in *Drosophila*.

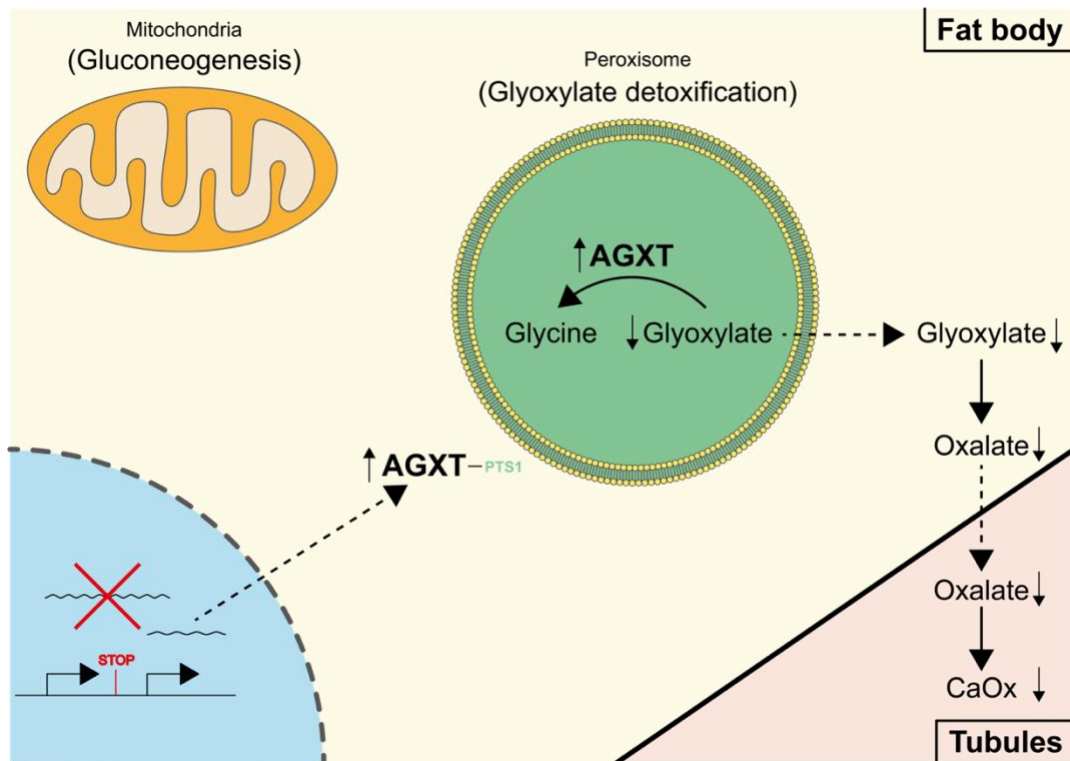


Figure 6.17 Working model: endogenous oxalate synthesis contributes to HFD-associated renal stones

Increasing peroxisomal dAGXT improves glyoxylate clearance and renal pathology on a HFD. Improved renal pathology as a result of increased clearance of the major and direct oxalate precursor, glyoxylate, suggests endogenous oxalate synthesis could contribute to HFD-associated renal stones. Downregulation of AGXT in response to a HFD could potentially result in elevated levels of glyoxylate and oxalate synthesis (see Chapter 7).

Chapter 7. General Discussion

7.1. Overview

A number of clinical studies have described an association between plasma ADMA levels and obesity (Eid et al., 2004; McLaughlin et al., 2006; Palomo et al., 2011; Stühlinger et al., 2002). Reducing the metabolic clearance of ADMA via *DDAH* inhibition/gene inactivation allows ADMA levels to be experimentally increased *in vivo*, which can be used as a strategy to investigate the role of ADMA in adiposity. *DDAH* deficiency in mice is associated with increased fat mass implicating ADMA in the regulation of adiposity (Dowsett, 2014 - PhD thesis). Following these results, the initial aim of this thesis was to utilise the genetically amenable model organism, *Drosophila*, to gain a mechanistic understanding into the role of ADMA in the regulation of adiposity. *CG1764* was identified as the *Drosophila* orthologue of mammalian *DDAH1*. *CG1764* KO animals (*DDAH^{XP}*) have elevated ADMA levels and display a range of metabolic phenotypes including a reduction in fat storage, increased mitochondrial content and elevated levels of respiration, inferred from elevated CO₂ production (Chapter 3). However, no phenotypes were observed from a second independently generated *CG1764* KO line, *DDAH^{Δe2-5}*, suggesting that the loss of *DDAH* is not responsible for the metabolic phenotypes observed in *DDAH^{XP}* animals as initially hypothesised (Chapter 4).

Crucially, *DDAH^{XP}* has a larger deletion than *DDAH^{Δe2-5}*. Within this larger deletion lies a highly conserved intronic region that likely represents *cis*-regulatory elements. Indeed, transcriptional upregulation of an uncharacterised adjacent gene, *CG1622*, in *DDAH^{XP}* suggests that repressor elements may have been removed in this allele. *CG1622* knockdown in the fat body results in increased fat mass (opposite to the *DDAH^{XP}* phenotype), indicating that upregulation of *CG1622* is likely to be responsible for the metabolic phenotypes observed in *DDAH^{XP}* (Chapter 5). Taken together, *DDAH* deficiency is not associated with changes in fat storage in *Drosophila* and *CG1622* is identified as a novel putative regulator of adiposity.

The aminotransferase *dAGXT*, which is also known to metabolise ADMA, was found to be transcriptionally upregulated in *DDAH^{XP}* but not *DDAH^{Δe2-5}* mutants, correlating

with the expression of *CG1622*. Knockdown of *CG1622* results in the transcriptional downregulation of specifically the long *dAGXT* variant, indicating that *CG1622* may differentially regulate the expression of *dAGXT* transcript variants, either directly or indirectly. AGXT is essential for the detoxification of the metabolic intermediate glyoxylate. Functional deficiency of AGXT is associated with PH1, a rare autosomal recessive disorder of glyoxylate metabolism (Cochat & Rumsby, 2013; Danpure, 2005). Despite its relevance to human disease, AGXT is almost completely unstudied in *Drosophila*. Having uncovered a correlation between the expression of *dAGXT* and *CG1622*, as well as TAG levels, I focused on characterising *dAGXT* and explored its role in nephrolithiasis in *Drosophila*. I found that knockdown of *dAGXT* results in the formation of renal stones, recapitulating the major pathological feature of PH1, making it a promising translational model to explore new treatments (Chapter 6).

The identification of previously unannotated short *dAGXT* transcripts lacking the putative MTS hints towards a mechanism whereby organelle targeting, and ultimately metabolic function, could be controlled by differential transcript choice resulting in the inclusion/exclusion of the MTS. Organelle targeting of AGXT is particularly relevant for human disease given that the most common mutations associated with PH1 cause mitochondrial mistargeting (Fargue, et al., 2013a). These results indicate that a *Drosophila* model could be used to study organelle targeting of *dAGXT* by manipulating the expression of transcript variants. This is exemplified in the *AGXT^[N-GFP;STOP]* mutant, which can only express a short variant lacking the putative MTS due to the presence of a transcription termination signal downstream of the most 5' TSS. *AGXT^[N-GFP;STOP]* is expected to retain its C-terminal PTS1 and exclusivity target peroxisomes. *AGXT^[N-GFP;STOP]* animals are partially protected from HFD-associated renal stones suggesting improved glyoxylate clearance, consistent with increased peroxisomal targeting of *dAGXT* since glyoxylate detoxification is predicted to occur primarily in peroxisomes.

The incidence and prevalence of nephrolithiasis is increasing. With high levels of morbidity and recurrence, it represents a significant disease burden. Obesity is a well-recognised risk factor of nephrolithiasis (Poore et al., 2020). I demonstrated that HFD feeding is associated with significant renal stone formation suggesting that *Drosophila* could be a promising translational model to study HFD-associated renal dysfunction. Using this model, I explored the underlying mechanism of HFD-associated

nephrolithiasis. CaOx stones are the most prevalent form of nephrolithiasis in obese patients (Poore et al., 2020), with the major risk factor for stone formation being hyperoxaluria. Positive correlation between obesity and hyperoxaluria is well documented (Lemann et al., 1996; Powell et al., 2000; Sakhaee, 2018), yet the aetiology is unknown. Human studies suggest that the impact of dietary oxalate on the levels of urinary oxalate is minimal (Taylor & Curhan, 2008), hinting towards an endogenous source. Ob/Ob mice raised on an oxalate-free diet still exhibit significant hyperoxaluria also supporting an endogenous source (Amin et al., 2018). I therefore explored the hypothesis that endogenous synthesis of oxalate contributes to HFD-associated renal stones, by testing whether reducing the abundance of the major and direct oxalate precursor, glyoxylate, leads to an expected decrease in renal stone accumulation. Peroxisomes are predicted to be the major site of glyoxylate detoxification. Therefore, increasing peroxisomal dAGXT is expected to improve glyoxylate clearance and reduce renal stone formation in the context of hyperoxaluria; increasing peroxisomal targeting by vitamin B₆ treatment is known to improve glyoxylate clearance and reduce oxalate synthesis and stone formation in PH1 patients (Cellini et al., 2014; Cochat et al., 2012; Fargue et al., 2013b; Hoppe & Langman, 2003). *AGXT*^[N-GFP;STOP] animals, which are expected to express a short *dAGXT* variant that exclusively targets peroxisomes, exhibit reduced renal stone formation on a HFD. These results suggest that hyperoxaluria, which is at least partly endogenous in origin, contributes to HFD-associated renal stones.

7.2. Future Perspectives

7.2.1. ADMA

In contrast to the mammalian model described by the Leiper lab, I found that *DDAH* deficiency is not associated with changes in adiposity in *Drosophila*. *DDAH* was targeted as a means to increase ADMA levels *in vivo* by reducing its metabolic clearance. It is possible that *Drosophila* exhibits redundancy in the enzymes that metabolise ADMA, and that its levels are not sufficiently elevated in *DDAH*-deficient flies. The aminotransferase *AGXT* has been described to metabolise ADMA in *Drosophila* (Leiper lab, unpublished). *dAGXT* may play a more significant role in the

metabolism of ADMA in *Drosophila* than in mammals. Additional insight might be gained from double mutants for *DDAH* and *dAGXT* to further increase ADMA levels, however these animals would be expected to accumulate renal stones due to the loss of *dAGXT*. Alternatively, dietary supplementation of ADMA could be used to elevate circulating levels, although this is likely to be expensive and therefore less feasible from a practical standpoint. Work here also highlights the importance of using independent mutations to ensure phenotypic consistency when inferring gene function.

7.2.2. CG1622

In this thesis, I identified *CG1622*, which was previously completely uncharacterised, as a novel putative regulator of adiposity. Although its function is unknown, *CG1622* is predicted to contain a pre-mRNA-splicing factor 38 (PRP38) domain based on sequence homology, and thus to be involved in splicing. If so, in what context could the protein encoded by *CG1622* be active, and what could its targets be? To address these questions, two broad approaches could be adopted. Firstly, genome-wide transcriptional profiling (e.g., on dissected fat body) in *FB-GAL4>CG1622^{PBac}* animals could be performed to help identify relevant pathways or biological processes. The limitation of this approach is that direct targets are not readily identified, and the data generated can be difficult to interpret. A second approach could be to identify RNA species bound by *CG1622*. Methods that couple UV crosslinking with high throughput sequencing (e.g., iCLIP) would allow direct targets to be identified and would even determine where the protein binds on the RNA molecule (i.e., identifying splice sites). Such methods would require a good antibody to pull down the crosslinked RNA-protein complexes by immunoprecipitation. No antibodies are currently available for *CG1622*, and developing efficient antibodies can be time consuming and costly. Alternatively, antibodies could be used against a tagged protein. A UAS *CG1622* flag-HA C-terminal fusion clone was obtained from the *Drosophila* Genomics Resource Center (DGRC) and injected into PhiC31 integrase expressing animals by Joachim Kurth at the Francis Crick Institute. Validation (sequencing and qPCR) of *CG1622* Flag-HA C-terminal fusion was carried out during this thesis. Anti-HA antibodies could then be used in the future to pull down crosslinked *CG1622* and identify its RNA targets. These

experiments would reveal whether CG1622 is definitively involved in splicing, and point towards the mechanism underlying its role in metabolic regulation.

7.2.3. The relationship between CG1622 and dAGXT

Transcriptional upregulation of *CG1622* in *DDAH^{XP}* animals was associated with transcriptional upregulation of *dAGXT*. Conversely, knockdown of *CG1622* results in the transcriptional downregulation of the long *dAGXT* variant specifically. These results indicate that *CG1622* may directly or indirectly regulated the expression of *dAGXT*. If there is a direct interaction (i.e., *CG1622* is predicted to be involved in splicing), one would expect to identify *dAGXT* by iCLIP (or other similar untargeted approaches). A direct interaction could also be assessed using a targeted approach where a crosslinked *CG1622*-RNA complex is pulled down by IP before performing RT-PCR using *dAGXT* specific primers. Alternatively, *CG1622* may indirectly regulate the expression of *dAGXT* (e.g., downstream of changes in energy homeostasis). Transcriptional upregulation of *CG1622* in *DDAH^{XP}* animals is associated with reduced TAG storage and downregulation of *CG1622* results in increased TAG levels. Given that *AGXT* has been found to be nutritionally regulated in a number of high throughput studies in both *Drosophila* and mammalian models, an indirect interaction between *CG1622* and *dAGXT* seems likely. Consistent with the transcriptional data from the literature, I found *dAGXT* to be upregulated at the protein level following starvation.

The subcellular localisation of *AGXT* reflects its dual metabolic role. In the mitochondria, *AGXT* is primarily involved in gluconeogenesis and in the peroxisomes, *AGXT* plays an essential role in the clearance of glyoxylate. The upregulation of *dAGXT* observed in response to a low energy status may be part of the coordinately regulated programme of amino acid catabolism to feed into gluconeogenesis. The same programme may be downregulated in response to a high energy status as gluconeogenic pathways are shut down. Interestingly, I found downregulation of specifically the long *dAGXT* variant in animals with elevated TAG levels following knockdown of *CG1622*. Downregulation of the long *dAGXT* variant harbouring the putative MTS would reduce mitochondria targeting while still allowing peroxisomal targeting and glyoxylate clearance. A converse mechanism of regulation has been described in mammalian models, where gluconeogenic stimuli results in upregulation of specifically the long MTS-containing *AGXT* (Miyajima et al., 1989; Oda et al., 1982;

Uchidas et al., 1994). Assessing the expression of *dAGXT* variants following HFD feeding would be necessary to draw further conclusions.

7.2.4. *dAGXT*

Obesity is a well-recognised risk factor of nephrolithiasis. I have shown that HFD feeding is associated with significant renal stone formation indicating that *Drosophila* could be a promising model to study the relationship between obesity and nephrolithiasis. I used this model to explore the underlying cause of HFD-associated nephrolithiasis and propose the following working model: HFD is associated with elevated glyoxylate levels, resulting in increased endogenous synthesis of oxalate which contributes to hyperoxaluria and CaOx nephrolithiasis. The *AGXT*^[N-GFP;STOP] allele is expected to express a short variant that harbours a C-terminal PTS1 but lacks the putative N-terminal MTS and therefore exclusively targets peroxisomes. Increasing peroxisomal AGXT is expected to improve glyoxylate clearance thus limiting oxalate synthesis and ultimately reducing the precipitation of CaOx stones. While this working model is consistent with the data at hand, additional experiments would be necessary to strengthen these conclusions. Firstly, although it is known that AGXT is essential for the clearance of glyoxylate, neither glyoxylate nor oxalate were specifically measured here. At this stage, glyoxylate clearance is inferred from the severity of renal stone formation. In the future, targeted metabolomics would allow changes in glyoxylate to be assessed. Secondly, while *AGXT*^[N-GFP;STOP] cannot express the long variant due to the presence of a transcription termination signal downstream of the most 5' TSS, I have not demonstrated that the short variant is expressed. This could be addressed by assessing the expression of *dAGXT* by qPCR using probes spanning exon junction 1-2 and 2-3. Lastly, organelle targeting of *dAGXT* requires definitive confirmation. The assumptions on organelle targeting of *dAGXT* in *Drosophila* are based on the literature for AGXT from other organisms, sequence analysis showing the presence of predicted targeting signal peptides, and preliminary imaging data. Although the N-terminal *dAGXT*-GFP fusion appears to localise to peroxisomes, co-localisation would be necessary to confirm this. Future work would aim to demonstrate mitochondria and peroxisomal targeting of *dAGXT* and relate organelle targeting to function. The necessary genetic lines and predicted outcomes are summarised below (Table 7.1). The lines generated/used during this thesis and

their known phenotypes are included (Table 7.1; *). The C-terminal GFP fusion with PTS1 was designed during this thesis and was generated by Cyrille Alexandre (Francis Crick Institute). This line can be converted into a PTS1 null line following Cre-Lox recombination.

The N-terminal GFP insertion is likely to disrupt the putative MTS of dAGXT. Therefore, a C-terminal GFP fusion protein expressing allele was designed to allow the function of mitochondrial targeting to be investigated. The C-terminal GFP fusion with PTS1 (internal GFP) was designed to leave both putative MTS and PTS1 unaltered by inserting GFP between the aminotransferase domain and the putative PTS1. Given the unusual PTS1 of dAGXT, there is a distinct possibility that ancillary peroxisomal targeting information is required; such additional targeting information has been described in humans (termed PTS1A) (Danpure, 2006). Interestingly, an SKL (residues 383-385) tripeptide exists nine residues upstream of the atypical SKI. Although the SKL tripeptide is a typical PTS1, its internal location is unusual. The SKL tripeptide could indeed be the *Drosophila* ancillary sequence, with both C-terminal SKI and SKL required for peroxisomal targeting. With this in mind, the internal AGXT fusion protein was designed to insert GFP downstream of the putative aminotransferase domain but upstream of the SKL and SKI tripeptides (>10 residues upstream of SKL). Such an insertion is predicted to have minimal impact on protein folding and dimerisation - the GFP insertion would not interfere with the large domain containing dimerisation interface - though this would need to be confirmed. It is worth noting that given the importance of protein folding/dimerisation on the kinetic partitioning of AGXT, even small changes could alter the subcellular distribution. Although correct targeting and protein folding cannot be guaranteed, such 'internal' fusion proteins containing both an MTS and PTS1 have been described in the literature and still target peroxisomes (using RFP) (Stehlik et al., 2020).

Table 7. 1 Genetic tools to explore AGXT organelle targeting and function in *Drosophila*

* Indicates the lines generated/used during this thesis and their known phenotypes.

Subcellular localisation

Genetic line	Predicted outcome
N-terminal GFP fusion (predicted to disrupt MTS) *	Targets peroxisomes
C-terminal GFP fusion (predicted to disrupt PTS)	Targets mitochondria
C-terminal GFP fusion (with PTS1)	Targets peroxisomes and/or mitochondria

Function

Genetic line	Predicted outcome
MTS null *	Renal stones ↓ *
PTS1 null	Renal stones ↑
ORF deletion KO	Renal stones ↑
RNAi *	Renal stones ↑ *

7.2.5. Glyoxylate and HFD

My working model proposes that HFD is associated with elevated glyoxylate levels resulting in increased endogenous synthesis of oxalate which contributes to hyperoxaluria and CaOx nephrolithiasis. Given that AGXT is essential for the metabolic clearance of glyoxylate, I turned to the literature to explore any known association between AGXT and animal models of obesity. Intriguingly, AGXT was found to be significantly down regulated in response to HFD in a number of high-throughput studies in mice, including (Soltis et al., 2017a; Soltis et al., 2017b) and also in response to a HFD plus high fructose/glucose (Asgharpour et al., 2016). AGXT downregulation to the extent of impaired glyoxylate detoxification could contribute to obesity-associated hyperoxaluria and nephrolithiasis. Indeed, it is well known that functional deficiency of AGXT causes CaOx nephrolithiasis (PH1) and I have shown

that downregulation of *AGXT* by RNAi results in renal stone accumulation in *Drosophila* (Chapter 6). This is a particularly interesting observation given that the underlying pathophysiologic mechanism of obesity-associated hyperoxaluria is currently largely unknown. Although a number of high-throughput studies have found *AGXT* to be downregulated in response to obesogenic diets, the relationship between *AGXT* and hyperoxaluria/nephrolithiasis in the context of obesity has not been explored. It would be important to assess the expression of *dAGXT* in our model of HFD-associated renal stones draw further conclusions. In addition to reduced clearance, obesity could also be associated with increased production of glyoxylate. Elevated glyoxylate is in fact a metabolite marker of T2D (Nikiforova et al., 2014). T2D is characterised by a range of metabolic defects that are closely linked to the formation of advanced glycation end-products (AGEs) via non-enzymatic glycation reactions of free amino groups on proteins, lipids and nucleic acids. A HFD has also been found to be associated with the production of AGEs in rats (Li et al., 2005). The numerous pathways involved in the detoxification of the AGE precursor, glyoxal, all directly or indirectly produce glyoxylate and is therefore hypothesised to be responsible for the elevated glyoxylate levels associated with T2D (Nikiforova et al., 2014). Elevated glyoxylate provides a causal link between the accumulation of AGEs associated with T2D and endogens synthesis of oxalate. Taken together, it is possible that both increased production and reduced detoxification of glyoxylate could lead to elevated levels of endogenous oxalate synthesis and contribute to obesity-associated hyperoxaluria and nephrolithiasis.

7.3. Closing remarks

Overall, my work on the molecular mechanisms underlying adiposity in *Drosophila* may contribute to an improved understanding of the aetiology of obesity and its associated diseases. The identification of a novel and completely uncharacterised regulator of adiposity opens avenues for future work, which could lead to the discovery of new pathways and ultimately an improved understanding of the regulation of adiposity. With the genetic tools I have developed, it has become possible to investigate the pathogenesis of obesity-associated nephrolithiasis at the cellular and molecular level. Work here highlights the usefulness of *Drosophila* as a model to study adiposity and obesity-associated disease.

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Appendix

Appendix 1.1 Oligonucleotides used for generating the constructs used in this thesis

Oligo name	Construct	Sequence 5'-3'
DDAH ^[N-GFP;STOP]		
pCFD4_T1_F_DDAH_N-GFP	pCFD4	TATATATAGGAAAGATATCCGGGTGAActtcGccgaggacgagcaccctagtGTTTTAGAGCTAGAAATAGCAAG
pCFD4_T2_R_DDAH_N-GFP		attttaacttgctatttctagctctaaaacTGTA ^{CTTGGGCGACATgg} tCgacgTTAAATTGAAAATAGGTC
HR1F_DDAH_N-GFP	Targeting vector	CCCGGGCTAATTATGGGGTGTCTGCCCTTCGCGCTCCAGGGCTCCATATGCTTCACCTGG
HR1R-4bp_DDAH_N-GFP		CCCGGTGAACAGCTCCTCGCCCTTGCTCACCATggtcggcggattggccactgg
HR2F_DDAH_N-GFP		AGTTCGGGGTCCAGCGGTTCTTCAGGCAGTTCGCCCAAGTACACACATGCGATTGTG
HR2R-4bp_DDAH_N-GFP		AATATCCAGTGACGCCGATTCTGGGTGTCgaagagcGTCAATCGAGTTCAAGGGC
DDAH ^{Δe2-5}		
pCFD4_T1_F_DDAH_KO_attP	pCFD4	TATATATAGGAAAGATATCCGGGTGAActtcGacttcaaaattggtatcctcGTTTTAGAGCTAGAAATAGCAAG
pCFD4_T2_R_DDAH_KO_attP		attttaacttgctatttctagctctaaaacCTCGGCTCCCAGAGAGATTTCgacgTTAAATTGAAAATAGGTC
HR1F_DDAH_KO_attP	Targeting vector	GGCCGCTACCGCGGGCTAGCACATATGcaGGAATAATctgcacgagacaagacg
HR1R_DDAH_KO_attP		CTCAAAGGTTACCCAgttggggcactacGtagatagtgttaaagacaggatccagg
HR2F_DDAH_KO_attP		TCGTATAATGTATGCTATACGAAGTTATcAGAGAAGCATTGCCATGAGAAACAT
HR2R_DDAH_KO_attP		GCACGCGTGCTTAATTAATCgacgtcGCAAAGATGTATAACCTTACCATCCCGC

Appendix 1.2 TaqMan Probes used in this thesis

Taqman Probe ID	Target
Dm01811991_m1	ACC
Dm02361072_s1	<i>alphaTub84B</i>
Dm01805233_m1	<i>bmm</i>
Dm01792734_g1	CG11241
Dm01838525_g1	CG1622
Dm01838526_g1	CG1622
Dm01838520_g1	CG1764
Dm01838521_g1	CG1764
Dm01838522_g1	CG1764
Dm01838523_g1	CG1764
Dm01838527_m1	CG1764
Dm01795381_g1	Clk
Dm02149911_m1	Cry
Dm01842654_g1	Cytochrome b5-related
Dm02361529_s1	Cytochrome c proximal
Dm01838904_g1	dob
Dm01801117_g1	FASN1
Dm01803727_g1	fu12
Mm03302249_g1	Gapdh
Dm01816893_g1	Hsl
Dm01818316_g1	Lpin
Dm01791906_m1	mdy
Dm02145573_g1	mino
Dm02136120_g1	mitochondrial transcription factor A
Dm01809992_g1	mtDNA-helicase
Dm01843681_g1	per
Dm01816546_s1	Phosphoenolpyruvate carboxykinase
Dm02151827_g1	Ribosomal protein L32
Mm01261022_m1	Rorc
Dm02151827_g1	RpL32
Mm01173149_m1	Slc5a4a
Mm00452283_m1	Slc5a4b
Dm02136710_g1	spargel
Dm01813076_g1	Spat (Agxt)
Dm01813077_g1	Spat (Agxt)
Dm01793857_g1	SREBP
Dm01816790_m1	Succinate dehydrogenase, subunit A (flavoprotein)
Dm01814246_g1	tim

Appendix 1.3 Oligonucleotides used for genotyping of *DDAH^{XP}*

Oligo name	Sequence 5'-3'
SOL894	GCGCAATTTTCTTTTCCTTCTAATG
SOL895	CACAAATCTCCACCCAAAAGTCCTAC
SOL952	ATTGAGGCGCGAATACGAGAAC