Bursicon – a novel regulator of intestinal homeostasis and systemic metabolism in adult *Drosophila*

Christin Bauer
MSc

CANCER
RESEARCH
UK

University of Glasgow

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Institute of Cancer Sciences
College of Medical, Veterinary and Life Sciences
University of Glasgow

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Abstract

Maintaining systemic energy homeostasis is crucial for the physiology of all living organisms. This process involves a tight control of cellular and organismal metabolic functions, which are required to coordinate energy intake and energy expenditure. Disruption of this balance can lead to major human pathologies, such as diabetes, obesity and lipodystrophy.

A central regulator of systemic metabolism is the intestine. The intestinal epithelium is responsible for nutrient absorption, as well as being a key-endocrine and immune tissue. Due to its endocrine function, the intestine orchestrates the communication between multiple organs, which is required to maintain organismal fitness in response to changing environmental and nutrient demands.

Functional studies on inter-organ communication are often challenging in mammalian systems, due to their complex physiology. A simpler, yet relevant organism like *Drosophila melanogaster* has proven to be an invaluable alternative model system to study complex physiological processes.

In this thesis we used *Drosophila melanogaster* as a paradigm to study how the intestine communicates with other tissues through its endocrine function to regulate systemic metabolic homeostasis.

We found that systemic secretion of the intestinal enteroendocrine derived hormone Bursicon is regulated by nutrients and maintains metabolic homeostasis via its neuronal receptor LGR2. Impairment of Bursicon/ neuronal LGR2 signalling resulted in extensive loss of stored energy resources, especially lipids.

Our data provides new insights into intestinal endocrine regulation of metabolic homeostasis. Our work identified a novel gut/brain axis controlling key metabolic tissues. Using *Drosophila* to identify gut-dependent hormonal metabolic networks will help to gain a deeper knowledge of how organs communicate with each other to maintain systemic metabolic homeostasis, which could impact the identification of therapeutic targets for metabolic disorders in humans.

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Publications arising from this work

Scopelliti, A., **Bauer, C.**, Cordero, J. B., & Vidal, M. (2016). Bursicon-alpha subunit modulates dLGR2 activity in the adult Drosophila melanogaster midgut independently to Bursicon-beta. *Cell Cycle, 15*(12), 1538-1544. doi:10.1080/15384101.2015.1121334

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Author's Declaration

I declare, that the work presented in this thesis is my own except where explicit reference is made to the contribution of others. None of the work has been previously submitted for another degree at any other institution.

Christin Bauer

Abbreviations

˚C	degrees centigrade
♀	female
♂	male
2-NBDG	2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
ADP	adenosine di-phosphate
AKH	adipokinetic hormone
AKHR	adipokinetic hormone receptor
AMP	adenosine monophosphate
ANOVA	Analysis of Variance
ATP	adenosine tri-phosphate
BSA	Bovine serum albumin
Burs	Bursicon
CA	corpora allata
cAMP	cyclic AMP
CC	corpora cardiac
CNS	central nervous system
CO_2	carbon dioxide
d	day(s)
DAPI	4',6-diamidino-2-phenylindole
DGRC	Drosophila Genetic Resource Center
Dilp	*Drosophila* insulin-like peptide
DNA	deoxyribonucleic acid
DSHB	Developmental Studies Hybridoma Bank
DTT	dithiothreitol
EBs	Enteroblast
ECs	Enterocyte
ee	Enteroendocrine cells
EGF	Epithelial growth factor
EGRF	Epithelial growth factor receptor
EMS	ethyl methanesulphonate
esg	escargot
FC	fold change

FFA	free fatty acids
FRET	Fluorescence Resonance Energy Transfer
GDP	guanosine di-phosphate
GFP	Green fluorescent protein
GMP	guanosine monophosphate
GSH	Glutathione
GSSG	Glutathione disulfide
GTP	guanosine tri-phosphate
GWAS	genome-wide association studies
h	hour(s)
HFD	high fat diet
H_2O	water
HSD	high sugar diet
IMP	inosine monophosphate
inr	Insulin receptor
IPC	Insulin producing cells
IR	RNA interference line
ISCs	intestinal stem cells
Jak	Janus Kinase
JH	juvenile hormone
JHA	juvenile hormone analogue
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LC-MS	liquid chromatography mass spectrometry
LGR	Leucine-rich repeat-containing G-protein coupled receptor
MAPK	Mitogen activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
min	minute(s)
µl	microliter
mM	milimolar
mtDNA	mitochondrial DNA
MyoIA	Myosin IA
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
nDNA	nuclear DNA

O_2	oxygen
OXPHOS	oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tritonX-100
PDF	Pigment Dispersing Factor
PI3K	Phosphatidylinositol 3-Kinase
Pros	Prospero
PVDF	Polyvinylidene fluoride
qPCR	quantitative Polymerase chain reaction
rk	rickets
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	reverse transcription
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Stat	Signal Transducer and Activator of Transcription
TAG	triacylglycerides
TBST	Tris-buffered saline Tween20
TF	transcription factor
TGF-β	Transforming growth factor beta
ts	temperature sensitive
tub	tubulin
UAS	upstream activating sequence
VDRC	Vienna Drosophila RNAi Center
VM	visceral muscle

1 Introduction

1.1 *Drosophila melanogaster* as a model organism

1.1.1 History of *Drosophila*

Since Morgan's discovery of the *white* mutation and the corresponding *white* gene on the X-chromosome in 1910, *Drosophila melanogaster*, also known as the fruit fly, has become an invaluable tool for genetic research (Green, 2010; Morgan, 1910; Schneider, 2000). In the second half of the 20th century the fruit fly was used in different biological disciplines. Everything seemed to be possible: from behaviour, development and molecular mechanisms; many aspects of biology are conserved between *Drosophila* and higher organisms (Arias, 2008; Bellen et al., 2010). Due to this conservation, *Drosophila* has become a great model organism to identify key biological processes conserved in humans.

Fruit flies are relatively simple to maintain and a low-cost model organism. Importantly, their amenable genetics make flies a popular model to perform large scale *in vivo* genetic screens to uncover new components of signalling pathways or disease loci, among others (Adams et al., 2000; Bier, 2005; Reiter et al., 2001; Vidal and Cagan, 2006). However, as it is not possible to freeze *Drosophila* at any stage of development, animals must be constantly maintained as live-stocks. To achieve this, we keep our stocks and experimental animals in temperature and humidity-controlled incubators under a daily 12h light-12h dark cycle. Flies are kept in vials or bottles containing food made of an agar-based mix of yeast and sugars, which is regularly replaced (between a few days and two weeks), depending on the temperature of the incubation. Most fly stocks originate from the laboratories that have generated them and/or from public *Drosophila* stock centres, which keep important fly lines for researchers to order.

Drosophila is one of the best-studied and widely used non-mammalian model organisms to uncover new development and disease related processes.

1.1.2 *Drosophila* life cycle

One key advantage of *Drosophila* is their fast life cycle. Fruit flies start their life as an egg/embryo, which further develops into a larva, followed by a pupal stage until the animal emerges as an adult fly (Figure 1-1). This life cycle takes approximately 10 days at 25 °C, and it varies with temperature. Lower temperatures slow down developmental timings.

At room temperature (22 °C), adult *Drosophila* can live up to 100 days. Within that time period females can lay many hundreds of eggs to ensure species survival. Fertilised eggs/embryos need about 16 h to hatch into a larva, which will constantly eat and grow for approximately 6 days. During that time, larvae undergo 3 molting stages followed by the formation of the pupae. Larvae consist of imaginal discs (Weigmann et al., 2003), which are 'bags' of undifferentiated epithelial cells forming precursors of the external adult structures, which emerge during pupal metamorphosis. These structures include the head, thorax, wings, legs, halteres, eyes and antennae. Interestingly, other tissues, like the nervous system, fat body and gut are preserved during metamorphosis, but will undergo extensive remodelling.

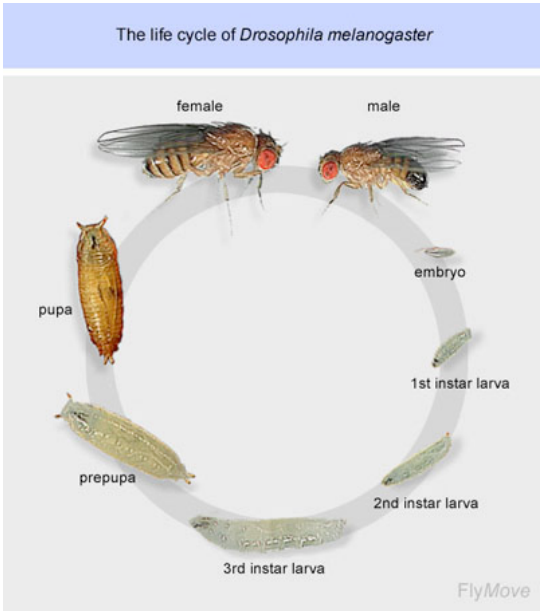

Figure 1-1: The life cycle of *Drosophila melanogaster*.

The image shows the life cycle of *Drosophila melanogaster*. Development from egg to adult takes approximately 10 days at 25 °C. Image taken from (Weigmann et al., 2003).

1.1.3 *Drosophila* - a powerful genetic tool

The *Drosophila melanogaster* genome was sequenced in 2000 and contains approximately 14.000 genes (Adams et al., 2000), which are dispersed among 4 chromosomes: the X (1st), the 2nd, the 3rd and the 4th chromosome, the latter being largely composed of heterochromatin. The *Drosophila* genome displays low genetic redundancy compared to higher organisms, which makes it easier to study the role of one particular gene and gene family.

Even before the genome was fully sequenced, *Drosophila* was already widely used to study genes affecting development. With the use of mutagenic agents, like ethyl methanesulphonate (EMS), X-ray irradiation and transposable P-elements, the discovery of new genes important for *Drosophila* development started to bloom.

Another advantage of using *Drosophila* is the ability to use easily visible phenotypic markers and balancer chromosomes. Balancer chromosomes carry many inversions and rearrangements, which lead to their inability to recombine with a partner chromosome. They also carry one or more visible phenotypic markers, which facilitates the mapping and following of a gene or transgene of interest. Balancers also carry recessive lethal markers and can therefore only be present in one copy. Combinations of balancer chromosomes with chromosomes carrying a mutation in a gene of interest allows maintenance of lines heterozygous for a recessive lethal mutation or mutations which lead to unhealthy or weak animals when homozygous.

1.1.4 The Gal4-UAS system

In the early 1990's, the introduction of the Gal4-UAS system into *Drosophila* was a 'stepping stone' into understanding the tissue and cell specific roles of genes (Brand and Perrimon, 1993).

The Gal4-UAS system consists of the Gal4 gene, which is a yeast transcriptional activator that can bind to upstream activating sequence (UAS). This system enables researchers to express any gene or transgene of interest containing a UAS

sequence and to express such transgenes in a spatially restricted manner by the promotor specific Gal4 lines (Brand and Perrimon, 1993) (Figure 1-2).

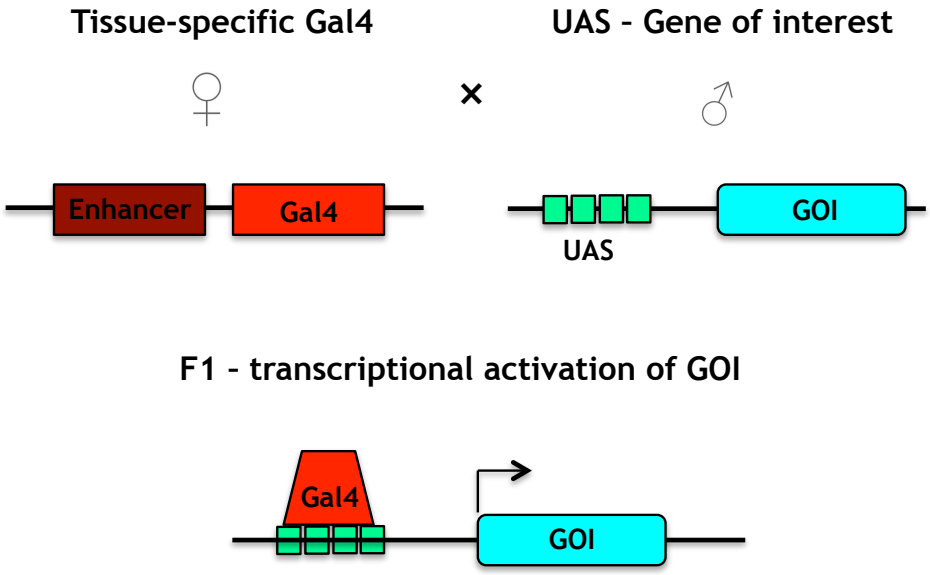

Figure 1-2: The Gal4/ UAS system for targeted gene expression.

Schematic description of the Gal4/ UAS system, which allows targeted gene expression. The Gal4 is a transcriptional activator (red pentagon), which can bind to the Upstream Activating Sequence (UAS) (green squared) to activate transcriptional expression of the gene of interest (GOI). Tissue specific expression is achieved by placing a tissue specific enhancer element upstream of the Gal4 (dark red rectangle).

1.1.4.1 Temporal regulation of the Gal4-UAS system: The addition of Gal80

The Gal4-UAS system is ideal for studying the role of genes in development. But many genes, if absent during development, lead to lethality. Most importantly, to be able to study gene function within a restricted developmental window requires an obligate component that allows temporal regulation of transgene expression. To study the function of a gene in a temporal and spatially controlled manner, researchers adapted a protein from yeast, Gal80, to use in *Drosophila*. Gal80 can bind to the transcriptional activation domain of Gal4 and represses its activity (Ma and Ptashne, 1987). Additionally, the introduction of temperature sensitive Gal80 proteins ($Gal80^{ts}$) allowed temporal control over the Gal4-UAS expression due to changes in temperature (Matsumoto et al., 1978). At the permissive temperature $Gal80^{ts}$ becomes inactive and allows Gal4-UAS expression.

1.2 *Drosophila* - pioneering work to uncover conserved biological processes during development

The use of *Drosophila* as a developmental biology tool started to bloom in the 1980th with the discovery of genes involved in normal patterning of embryos and larvae, which could be hierarchical clustered (Nusslein-Volhard and Wieschaus, 1980; St Johnston and Nusslein-Volhard, 1992). For this research Christiane Nusslein-Vollhard, Eric Wieschaus and Ed Lewis were awarded with a Nobel Prize in 1995, the 3rd in history for research in *Drosophila*. Christiane Nusslein-Vollhard and Eric Wieschaus took advantage of the defined patterns and segments in the *Drosophila* larva and asked which genes are important for this precise segmentation. They used damaging agents, such as X-rays to manipulate the DNA, monitored the effects of these random mutations in the *Drosophila* embryo and uncovered genes controlling developmental patterning (Nusslein-Volhard and Wieschaus, 1980). They discovered new genes, which when mutated led to duplication of segments, like *gooseberry*, *hedgehog* and *patch* (Nusslein-Volhard and Wieschaus, 1980). They also identified new mutants causing loss of alternating segments (*even-skipped*, *odd-skipped*, *paired*, *barrel*, *runt*) and deletion of neighbouring segments (*knirps*, *hunchback*) (Nusslein-Volhard and Wieschaus, 1980). This research was ground breaking and the start of understanding how a complex organism develops from a single cell. Ed Lewis tried to understand the evolutionary aspect of genes. He was intrigued by the fact that *Drosophila* looked different to many of their ancestors. For example, he imagined that there must be "haltere-promoting" and "leg-suppressing" genes (Lewis, 1978), because *Drosophila* has 2 wings and 6 legs, instead of 4 wings and multiple legs as seen in ancestors of the fruit fly. So he was interested in finding mutations, which lead to the development of 4 wings instead of the usual 2 in *Drosophila* and discovered the importance of the HOX genes, which control segmentation of the embryo (Lewis, 1978).

An additional line of research pioneered by *Drosophila* was devoted to the discovery and understanding of the 24 h circadian rhythm that each organism has. Konopka and Benzer found the first 'clock' gene, controlling the length of eclosion times, which they called *period* (Konopka and Benzer, 1971). In the 1990s many more circadian rhythm controlling genes were identified such as *timeless* (Sehgal et al., 1994), *clock* (Allada et al., 1998) and *cycle* (Rutila et al., 1998). Intriguingly,

all major genes regulating the circadian clock have been identified in *Drosophila* first, before homologs were found in vertebrates. Understanding the mechanisms involved in circadian rhythm is key, due to its appreciated importance in many physiological processes, such as sleep, activity and metabolism (Potter et al., 2016).

Interestingly, all abovementioned genes involved in patterning, segmentation and the circadian clock, were found to be conserved between fruit flies and higher organisms and important for normal development and disease (Lander et al., 2001; Nusslein-Volhard, 1994; Venter et al., 2001).

1.2.1 Anatomy of adult *Drosophila* and link to mammals

The fundamental physiological requirements between fruit flies, humans and all living animals are the same. They all need to breath, eat, move and reproduce in order to stay alive and ensure species survival.

As previously mentioned, larval imaginal discs are transformed into adult structures during metamorphosis. For example, the larval wing discs metamorphose into adult wings and thorax, the eye imaginal discs will give rise to the adult eyes and antennae, and leg discs go on to form the equivalent adult tissues. Adult *Drosophila* tissues are functionally equivalent to their human counterparts due to our shared evolutionary history.

The *Drosophila* nervous system represents a simplified version of its mammalian counterpart but both share many essential molecular functional characteristics. The fly's central nervous system (CNS) is divided into brain and ventral/spinal nerve cord, functioning through neurons. Fun fact, humans and *Drosophila* can develop alcohol addictions in a similar fashion (Heberlein, 2000). This clearly shows, that *Drosophila* can be and is used to uncover genes involved in regulation of behaviour (Saltz, 2013), circadian rhythm (Wager-Smith and Kay, 2000), metabolism (Schlegel and Stainier, 2007) and neuronal disease (Fortini and Bonini, 2000), to name a few.

Humans, as flies, need to produce energy to survive. This is mainly achieved by metabolic processes within the mitochondria of a cell, which uses oxygen to

produce cellular energy in the form of ATP and CO_2 as a by-product. In contrast to mammals *Drosophila* doesn't have a closed, but an open circulatory system. This means breathing oxygen and removing CO_2 from cells is less complex and is achieved by the trachea, which are homologous to lungs in mammals, highly branched tubules, reaching into each organ and supplying the cells directly with oxygen.

The circulatory system, consisting of blood and lymph in mammals is represented by an open circulatory system in *Drosophila*, known as the hemolymph, which bathes all organs and is necessary to pass nutrients, hormones and immune cells through the body. *Drosophila* only consists of innate immune cells, which are leukocyte-like hemocytes, and lacks an adaptive immune system.

Last but not least, the digestive tract is highly similar between humans and fruit flies. Humans, as well as *Drosophila* have salivary glands to help digest the food. Furthermore, the intestine is divided into the same regions: foregut, stomach, mid- and hindgut, all necessary for the uptake of ingested nutrient. In humans, kidneys are responsible for the excretion of urine, for which flies have the Malpighian tubules. Digestive enzymes are released from the liver, the homolog to the fly's fat body. The pancreas is also responsible to secrete different hormones, like Insulin and Glucagon, which is achieved by the Insulin producing cells (IPCs) in the *Drosophila* brain and the corpora cardiaca cells situated in close proximity to the foregut.

1.3 *Drosophila* - a model for disease study

Reiter and colleagues concluded that 'approximately three-quarter of the known human disease genes are clearly related to genes in *Drosophila* (Reiter et al., 2001), which makes it a valuable model to study human diseases.

Human Genome-Wide Association Studies (GWAS) revealed many genomic loci associated with human disease (Hardy and Singleton, 2009). These GWAS are highly valuable in discovering disease mechanisms, which in the future could be used to design targeted therapies. GWAS have already helped to elucidate the risk alleles for many human diseases, like multiple sclerosis (De Jager et al., 2009), Alzheimer's disease (Naj et al., 2011) and many more. However, due to the vast

amount of data presented in GWAS, it is necessary to generate animal models to identify genes, which are key drivers of diseases, rather than passengers.

Drosophila is an excellent model organism to study diseases due to the close genomic conservation with mammals, low genetic redundancy, quick life cycle, cost effectiveness and genetic amenability. Using flies as a model organism for human diseases has been proven successful in many fields, including neurodegenerative diseases (Jaiswal et al., 2012; Shulman et al., 2003), cardiac dysfunction (Neely et al., 2010), cancer (Vidal and Cagan, 2006) and metabolism (Pendse et al., 2013), among others.

A great example of the effectiveness and efficiency of using *Drosophila* as a model to study disease comes from the discovery of genes responsible for heart defects in Down syndrome (Trisomy 21) (Grossman et al., 2011). Here, *Drosophila* was used as a screening tool for genetic interactions and it was found that overexpression of DSCAM and COL6A2 was responsible for heart defects in Down syndrome, which than led to further investigation in a mouse model of the disease (Grossman et al., 2011). Uncovering such complex genetic interactions would have been extremely challenging in murine models.

The following overview aims to highlight the demonstrated power of *Drosophila* as a model organism, which helped to understand the complex signalling pathways involved in multiple disease processes.

1.3.1 Metabolic disease models in *Drosophila*

The term metabolic disease includes every disease affecting normal metabolism, which is the conversion of food into cellular energy. Metabolic diseases are clustered into inherited and endocrine disease and include diabetes mellitus, hypothyroidism and malnutrition.

Metabolism can be disrupted in many ways by interfering with the uptake, transport or processing of proteins, carbohydrates or lipids. Such processes are regulated by thousands of enzymes, which take part in extremely complex networks of chemical reactions where each enzyme is responsible for the conversion of a substrate into a product, which will then become a substrate for

the following enzyme. Therefore, if an enzyme is missing or has low activity, it will lead to the accumulation of its substrate and depletion of its product. Cells contain a great number of pathways regulating metabolism, which are highly interconnected. Therefore, the deregulation of a single enzyme can alter many pathways simultaneously, which could cause severe phenotypic effects.

To achieve normal metabolic homeostasis, the mammalian liver and adipose tissue shift their metabolism dramatically in response to nutritional state. After a meal, Insulin is produced by pancreatic ß-cells, to promote the absorption of energy molecules. In mammals, carbohydrates are stored as glycogen mainly in the skeletal muscle and liver, whereas lipids are stored by the adipose tissue in the form of triacylglycerides (TAG). Under starvation condition, Insulin levels are low and Glucagon levels rise, which dramatically changes the metabolism within the liver and adipose tissue to fatty acid oxidation to produce energy (Ikeda et al., 2014; Owen et al., 1979). Furthermore, the muscle and liver induce gluconeogenesis and breakdown of glycogen, the main form of stored glucose, which is used as a source of energy for organs such as the CNS and heart. If the breakdown or synthesis of glycogen is disrupted due to dysfunctional enzymes involved, this will lead to glycogen storage disease.

Metabolic homeostasis in *Drosophila* is achieved in a similar fashion. The *Drosophila* muscle and fat body serve as major organs for glycogen storage, while lipids are accumulated in the fat body. Energy can be released in times of demand. After feeding, Insulin producing cells in the brain release Insulin-like peptides (Dilps) and when the animal is starving and circulating glucose levels drop, the corpora cardiaca (CC) secrets Adipokinetic Hormone (AKH), the fly Glucagon, to release energy from muscles and fat body. Due to these similarities flies have been used to understand metabolic disorders such as glycogen storage disease and diet-induced Insulin resistance (Musselman et al., 2011; Ruaud et al., 2011). Mutants for *Drosophila hr38*, the ortholog of the mammalian nuclear receptor subfamily 4 group A, display reduced muscular glycogen levels due to misregulation of glycogen synthesis, while glucose and TAG levels are unaffected (Ruaud et al., 2011).

Some of the major metabolic diseases the western world is facing, are hyperlipidemia, obesity and Type II diabetes for which many *Drosophila* models

were developed in order to understand their underlying mechanism and genes involved.

As already indicated, Insulin signalling has a major effect on metabolism, including carbohydrate and lipid metabolism, reproduction and growth. Diabetes mellitus is a metabolic disorder causing prolonged high circulating sugar levels. Increased circulating glucose levels can be achieved by either loss of Insulin production (Type I diabetes), for example through the death of pancreatic ß-cells or a diet-induced Insulin resistance (Type II diabetes) due to constant secretion of Insulin.

Human GWAS (Hardy and Singleton, 2009) have been useful to design *Drosophila* studies to uncover genes regulating metabolism. In one of the first studies, GWAS for diabetes were used as a basis for an RNAi screen in *Drosophila* to uncover genes involved in sugar metabolism (Pendse et al., 2013). Also, Park and colleges used GWAS to analyse Type II diabetes associated genes through a loss-of-function genetic screen in *Drosophila* (Park et al., 2014). Interestingly, *Drosophila* can also be used as a screening tool for drugs (Cagan, 2016; Tickoo and Russell, 2002), including anti-obesity drugs (Men et al., 2016).

Type I diabetes is characterised by the loss of Insulin-producing pancreatic ß-cells, which causes high circulating glucose levels. Many studies have shown that *Drosophila* is a great organism to study this disease. Ablation of fly IPCs causes loss of Insulin production and increased circulating carbohydrate levels in larvae (Broughton et al., 2005; Rulifson et al., 2002) and adults (Haselton et al., 2010). Haselton and colleagues subjected wild type and IPC-ablated adult animals to fasting before feeding them with a glucose solution to perform an oral glucose tolerance test. After an initial peak of high circulating glucose upon glucose feeding, glucose levels quickly returned back to baseline in wild type animals, while remaining high in IPC-ablated adult flies, a characteristic feature of diabetes (Haselton et al., 2010). Consistently, deletion mutants for *insulin-like peptides 1-5* (*dilp1-5*) also led to a diabetic-like phenotype (Zhang et al., 2009). Interestingly, it has also been shown that *Drosophila* can perform Insulin dependent glucose uptake by vesicular trafficking of glucose transporters to the membrane of fat cells as seen in mammals (Crivat et al., 2013; Dawson et al., 2001). Expression of a double-tagged version of human glucose transporter 4 (hGlut4) in fat cells of

flies showed translocation of hGlut4 to the membrane in response to mammalian Insulin (Crivat et al., 2013).

Modelling Type II diabetes using fruit flies has also proven successful. High sugar diet (HSD) and high fat diet (HFD) are commonly used in the *Drosophila* field to model western diet induced obesity. *Drosophila* fed with a HSD displayed hyperglycemia, Insulin resistance and high TAG levels (Musselman et al., 2011; Musselman et al., 2013). Furthermore, feeding flies with a HSD has been shown to induce heart defects (Na et al., 2013) and HFD caused increased cardiac TAG levels and problems in heart contraction (Birse et al., 2010), which phenocopies the outcome of diet-induced cardiac dysfunction in humans.

Signalling and metabolic pathways involving lipid metabolism are also conserved between mammals and *Drosophila*. Mutants for *brummer* (*bmm*), the mammalian homolog of the adipocyte triglyceride lipase (ATGL), are obese with a defective lipid mobilisation phenotype (Gronke et al., 2005). Furthermore, a *Drosophila* screen identified Sir2, the silent information regulator 2, encoding a protein deacetylase, as a modulator of lipid metabolism (Reis et al., 2010). It was shown that fat body derived Sir2 led to transcriptional alteration of lipases, like *brummer*, to regulate lipid mobilisation and therefore controlling starvation survival (Banerjee et al., 2012). On the contrary, the blockage of lipases in the intestine leads to anti-obesity phenotypes, due to the defective uptake of lipids from the diet (Sieber and Thummel, 2012).

Lipodystrophy is the counterpart to obesity and is characterised by the loss of lipids. Mutations in the human gene *Seipin* are believed to cause a severe form of lipodystrophy. However, the exact functions of *Seipin* remain unclear. Results using *Drosophila seipin* mutants showed loss of lipids in the fat body and accumulation of lipids in non-fat tissue, arguing that Seipin works by preventing ectopic lipid droplet formation (Tian et al., 2011b). Another lipodystrophy gene is *lipin*, which when mutated leads to reduced fat body and total lipid content in *Drosophila* (Ugrankar et al., 2011). It has been shown that upon starvation *lipin* is transcriptionally upregulated to promote survival (Ugrankar et al., 2011).

Altogether, the above data highlights the value and power of *Drosophila* as a model organism to study metabolic diseases.

1.3.1.1 Mitochondrial disorders and their study in *Drosophila*

The main intracellular organelles for energy production are the mitochondria. Mitochondria contain circular DNA molecules (mtDNA), which encode for a number of components of the oxidative phosphorylation system (OXPHOS), while nuclear DNA (nDNA) encodes for the remaining components of OXPHOS. Proper interaction between nucleus and mitochondria are key for the maintenance of metabolic homeostasis.

Defects in intergenomic communication between nDNA and mtDNA can lead to the depletion of mtDNA, as observed in Toni-Fanconi syndrome. In flies, overexpression of the mitochondrial DNA polymerase *pol γ-a* led to depletion of mtDNA content (Lefai et al., 2000). Interestingly, depletion of mtDNA in the muscle by tissue specific overexpression of *pol γ-a* resulted in pupal lethality, while overexpression of *pol γ-a* in the CNS did not (Lefai et al., 2000). Additionally, flies mutant for *technical knockout* (*tko*), encoding the mitochondrial ribosomal protein S12, displayed decreased OXPHOS and ATP synthesis (Jacobs et al., 2004).

Leigh syndrome is a mitochondrial disease leading to severe neurological conditions in which patients lose their mental and locomotor abilities, and usually die in early life due to respiratory failure. In most Leigh syndrome cases the mutation of the *surf1* gene is predominant (Bohm et al., 2006). *surf1* knockdown in *Drosophila* leads to larval developmental defects, decreased locomotion and increased death before pupariation (Zordan et al., 2006). Targeted knockdown of *surf1* within the CNS resulted in adult animals displaying deficiency of cytochrome c oxidase, an important enzyme in the respiratory electron transport chain, in the brain (Zordan et al., 2006).

Friedreich's ataxia is a severe mitochondrial disease caused by Frataxin insufficiency. Frataxin is an iron binding protein important to prevent mitochondrial iron overload, which can cause protein damage (Campuzano et al., 1996; Pandolfo, 2002). An RNAi based method was used to knockdown *frataxin* in *Drosophila* globally or within specific tissues (Anderson et al., 2005; Navarro et al., 2010). Global *frataxin* knockdown led to developmental arrest in larvae, leading to many characteristic phenotypes of Friedreich's ataxia, like reduced activity of respiratory complexes and iron containing mitochondrial proteins

(Anderson et al., 2005). When knocking down *frataxin* in the peripheral nervous system, larvae developed normally but resulting adults were short lived (Anderson et al., 2005). Interestingly it has also been reported that glial-specific *frataxin* knockdown led to the accumulation of lipid droplets in glia cells and increased lipid peroxidation (Navarro et al., 2010).

Studying *Drosophila* to uncover underlying signalling pathways in mitochondrial diseases is in its infancy and likely to grow in the near future.

1.4 Metabolism of nutrients in *Drosophila*

Tissues within a complex organism have a specialised function, which are required to maintain metabolic homeostasis. For example, the mammalian intestine and the *Drosophila* midgut are responsible for nutrient absorption and processing into smaller metabolites, which are released into the blood stream (hemolymph in *Drosophila*). The liver and adipose tissue (fat body and oenocytes in *Drosophila*) further process, uptake, store and lastly release stored nutrients for use by peripheral tissues.

1.4.1 Nutrient intake and absorption

Nutrient intake in *Drosophila* is very similar to mammals. Food is ingested through the mouth and digestive enzymes released by the salivary glands and the intestinal enterocytes (ECs) help to break down macromolecules into easier accessible nutrients. The *Drosophila* midgut is functionally regionalised, which can be determined by specific markers and histological structure (Lemaitre and Miguel-Aliaga, 2013). Furthermore, the *Drosophila* intestine is directly innervated by the CNS, which produces neuroendocrine peptides like Insulins (Buchon et al., 2013; Cognigni et al., 2011).

After nutrients are ingested, the salivary glands and ECs produce and secrete enzymes to digest the food and nutrients are uptaken by the intestinal epithelium. The remains further travel along the gastrointestinal tract to be finally excreted. The expression and release of Glucosidases into the gut lumen is necessary to break down sugars into monosaccharides, which are then transferred through specialised sugar transporters into the ECs and released into the hemolymph in

Drosophila. Proteins are broken down into amino acids by proteases and absorbed by the intestine. Lipases and other enzymes are important to breakdown lipids into free fatty acids (FFA) and glycerol. Dietary lipids can be stored for a short term within ECs in the form of triacylglycerol (TAG) containing lipid droplets, which can be mobilised in times of starvation (Sieber and Thummel, 2009). Lipids are modified in the gut and transported as lipoproteins by so called lipophorins (Lpp) (Palm et al., 2012). Lpps transport lipids mostly in form of diacylglycerols (DAG) through the hemolymph (Carvalho et al., 2012). Interestingly, Lpps and other important lipid cargo proteins, like the lipid transfer particle (Ltp), which are necessary to transport gut-derived lipids through the hemolymph, are made and modified by the fat body (Palm et al., 2012). This shows, that communication between different organs is key to maintain energetic organismal homeostasis.

1.4.2 Nutrient storage and usage

Once nutrients have been absorbed by the intestine and released into the mammalian blood or *Drosophila* hemolymph, they need to be stored or used by the organism.

Glucose is taken up and stored by the fat body and muscles as glycogen and lipids accumulate in form of TAG containing lipid droplets in the fat body. Nutrients are used by the muscles and the heart for contraction, by the Malpighian tubules for water balance and removal of waste, by the ovaries for reproduction, by the CNS for neuronal function and by other tissues for growth and homeostasis.

The uptake and release of stored energy is regulated by Insulin and Glucagon signalling in humans. In the last 15 years, it has become clear that *Drosophila* exhibits cells functioning like mammalian pancreatic cells and Insulin and Glucagon signalling was discovered in the fruit fly (Brogiolo et al., 2001; Ikeya et al., 2002; Kim and Rulifson, 2004; Lee and Park, 2004; Rulifson et al., 2002).

In *Drosophila*, circulating sugar concentration is sensed by specialised neurons. A small cluster of neurons, the median neurosecretory cells, produce Insulin-like peptides (Dilp2, 3 and 5) (Rulifson et al., 2002). Therefore, those cells are also known as Insulin producing cells (IPCs). Insulins are secreted in response to high circulating sugar levels to promote cellular sugar uptake. Insulin signalling is

counterbalanced by the fly's Glucagon-like adipokinetic hormone (AKH). AKH gets released from its production site, the corpora cardiaca (CC) in response to low circulating sugar levels to initiate glycogen and lipid breakdown within the fat body in a calcium dependent manner (Kim and Rulifson, 2004).

1.4.3 Regulation of metabolism by the intestine

The major roles of the intestine are nutrient digestion, vitamin and mineral absorption, detoxification, immune response and hormone regulation. Digestion and absorption of nutrients is achieved with the help of many enzymes and transporters. Therefore, reduction in lipases, glucose transporters or others molecules involved in nutrient processing, will affect the health and metabolism of the whole individual.

It has been shown that DHR96, the othologue of the mammalian nuclear receptors Pregnane X and constitutive Androstane receptor, positively regulates the expression of the lipase *magro*, the homolog to mammalian Lipase A (Sieber and Thummel, 2012). Mutation in *dhr96* or knockdown of *magro* in the midgut led to reduced stored lipid content, whereas overexpression led to the opposite effect (Sieber and Thummel, 2009, 2012; van der Veen et al., 2009), which is similar to the results obtained in *lipA* mutant mice (Du et al., 2001).

Additionally, Tachykinins (TK) were found to play a major role in midgut lipid homeostasis (Song et al., 2014). Tachykinins are expressed within the midgut and the CNS (Asahina et al., 2014; Birse et al., 2011; Reiher et al., 2011; Winther et al., 2006). Song et al further described, that Tachykinin is expressed in a subset of enteroendocrine (ee) cells to regulate intestinal lipid production (Song et al., 2014). Knocking down *tk* specifically in TK-producing ee cells or knockdown of its receptor *tkr99d* within ECs led to a dramatic increase of intestinal lipid production and whole fly TAG levels, due to increased transcription of digestive lipases and enzymes for lipogenesis (Song et al., 2014). This could be reversed by activation of PKA signalling within the ECs (Song et al., 2014). Interestingly, they found that TK within the ee cells is dramatically increased after 24h starvation. This is restored when flies were re-fed with yeast, but not sucrose or coconut oil (Song et al., 2014).

It has been shown that the *Drosophila* midgut has the capacity to sense nutrients through local Insulin signalling, which directly impacts ISC homeostasis. Just after adult animal eclosion, the fly gut heavily proliferates and increases in size if enough nutrients are available. Interestingly, when flies were starved for the first 4 days after eclosion midguts failed to increase its size due to decreased Dilp3 production by the visceral muscle (VM), which led to impaired expansion of the stem cell compartment (O'Brien et al., 2011).

Gut dysplasia can also lead to altered nutrient absorption. Aging causes hyperproliferation of the midgut and it has been shown that Insulin and JNK signalling influence overall survival of flies, due to Foxo activation (Biteau et al., 2010). Furthermore, Foxo activation within ECs has been shown to be required for inhibition of *magro* (Karpac et al., 2013), the homolog of the mammalian LipA. This signalling becomes activated in the aging midguts due to increased JNK signalling, causing a reduction of dietary lipid uptake and therefore disruption of lipid homeostasis (Karpac et al., 2013).

All together the above data demonstrate the key role of the intestine in the regulation of local and systemic metabolic homeostasis.

1.5 Endocrine and neuroendocrine regulation of metabolism in *Drosophila*

It is necessary for all living organisms to be able to sense and respond to different environmental cues and changing organismal demands. These processes are highly dependent on complex inter-organ communication programs. Understanding how tissues communicate with each other is necessary to understand human physiology and pathology. Due to their simpler physiology and genetic amenability, *Drosophila* has pioneered inter-organ communication studies.

1.5.1 Gut derived factors regulating metabolism

The gut is the first organ sensing nutrients and many gut-derived secreted factors are known to act on distant tissues to regulate metabolism.

It has been shown that Hedgehog (Hh) is increased within the larval gut upon starvation, it is secreted into the hemolymph and binds its receptor Patch on the

fat body to mobilise lipid stores under starvation condition (Rodenfels et al., 2014). Furthermore, circulating Hh also regulated Ecdysone levels through Patch binding on the prothoracic gland, thus regulating pupariation (Rodenfels et al., 2014).

Recent work on the adult *Drosophila* midgut has shown that ISC proliferation impacts brain derived Insulin signals and therefore has major effects on metabolism. Activation of the Hippo pathway, by overexpressing an activated form of *yorkie* in stem progenitor cells (ISCs and EBs) induced ISC proliferation, which was shown to induce ImpL2 production within the gut (Kwon et al., 2015). Imaginal morphogenesis protein Late 2 (ImpL2), the homolog to the mammalian IGFBP7, belongs to the immunoglobulin-superfamily and is a secreted Insulin/IGF antagonist, therefore leading to reduced nutrient uptake from the circulation, which consequently also impacts nutrient storage and induces tissue wasting (Kwon et al., 2015). In agreement with reduced Insulin signalling, animals bearing *yorki*-driven hyperproliferative midguts were hyperglycemic and showed reduced lipid and glycogen levels, which was independent from feeding behaviour (Kwon et al., 2015). Furthermore, intestinal hyperproliferation led to ovary and muscle wasting, which could be rescued by the introduction of a mutant allele for *impl2* (Kwon et al., 2015).

Those relatively recent studies provided a great foundation and opened up the field of endocrine regulation of metabolism in *Drosophila* research, but yet many more investigations are needed to fully understand and uncover the endocrine system in the fruit fly.

1.5.2 Non-gut derived factors regulating metabolism

The *Drosophila* fat body and oenocytes, the homolog to the mammalian adipose tissue and liver, are the primary sites for energy storage and release, which needs to be carefully regulated by hormones. In *Drosophila*, many fat body derived peptides, for example Unpaired 2 (Upd2), Dawdle, ImpL2 and Dilp6 have been identified.

In mammals, Leptin, the satiety hormone has been identified as a hormone responding to Insulin levels. It was shown that adipose derived Leptin binds to its

receptor in neuroendocrine organs to regulate metabolism (Ahima et al., 1996; Tartaglia et al., 1995). Interestingly, there is no *Drosophila* protein, which has recognisable sequence similarity with mammalian Leptin. But recently, in *Drosophila* larvae it was shown, that Unpaired 2 (Upd2) acts in a similar fashion as human Leptin (Rajan and Perrimon, 2012). Fat body derived Upd2 can bind to its receptor Domeless (Dome, JAK/Stat receptor) on $GABA^{+ive}$ neurons to mediate Dilp secretion from IPCs (Rajan and Perrimon, 2012).

Drosophila Dawdle is a TGF-ß/ Activin-like ligand, which is produced by the fat body in response to dietary sugars (Chng et al., 2014). Dawdle was shown to act on midgut enterocytes through Baboon/Punt receptors to supress digestive enzymes, thus working as a negative feedback loop (Chng et al., 2014). This sugar sensing mechanism has been found to be specific to nutritious sugars and dependent on Smad2 activation, but independent to Insulin- or AKH- (Glucagon) signalling (Chng et al., 2014). Interestingly, it has also been shown that muscle derived *dawdle* is a Foxo target and therefore being controlled by Insulin (Bai et al., 2013). Finally, *dawdle*, and its receptor *baboon* were expressed throughout various larval tissues (Ghosh and O'Connor, 2014). *dawdle* mutant larvae showed increased levels of Dilp2 within IPCs, thus suggesting that wild type Dawdle protein promotes Insulin secretion (Ghosh and O'Connor, 2014). Furthermore, *dawdle* mutant larvae displayed higher TAG, glycogen and glucose levels compared to control or heterozygous *dawdle* mutants (Ghosh and O'Connor, 2014). Those studies demonstrate that TGFß/ Activin-like signalling is a regulator of metabolism in *Drosophila*.

ImpL2, the homolog to the mammalian IGFBP7 has been found to act as an inhibitor of Insulin signalling by binding extracellular Dilp2 (Honegger et al., 2008). ImpL2 in the fat body was increased upon 24h starvation, suggesting that ImpL2 is important for regulating the starvation response of the animal (Honegger et al., 2008). Consistently, mutant larvae for *impl2* showed decreased survival when fed with 1% sugar or PBS only, compared to fully fed animals (Honegger et al., 2008). Another inhibitor of circulating Insulin is secreted decoy of insulin receptor (Sdr), therefore leading to the inhibition of growth (Okamoto et al., 2013). Sdr is expressed by the CNS and is also necessary for adequate response to starvation (Okamoto et al., 2013). Interestingly, Sdr and Impl2 act independently from each

other and they bind circulating Insulins (Dilp1-7 tested) with different affinities (Okamoto et al., 2013). Furthermore, Impl2 was also found as a secreted factor from the muscles as a protective mechanism upon mitochondrial stress (Owusu-Ansah et al., 2013).

Dilp6, which displays structural similarity to the Insulin-like growth factor (IGF) was highly increased in non-feeding stages of the larval fat body (Okamoto et al., 2009). *dilp6* expression in the fat body is essential to achieve normal overall animal size, lipid metabolism and survival upon starvation (Chatterjee et al., 2014; Okamoto et al., 2009; Slaidina et al., 2009).

It has recently been discovered that terminal tracheal branches, akin to mammalian vasculature, play an important role in nutrient sensing and systemic metabolism in *Drosophila* (Linneweber et al., 2014) Nutrients were sensed by enteric neurons producing Insulin-like peptide 7 (Dilp7) and Pigment Dispersing Factor (PDF). These neuropeptides bind to Insulin and PDF receptors within gut-associated trachea, which increased or decreased their branching in conditions of abundant or poor nutrients, respectively (Linneweber et al., 2014). Reducing terminal gut-tracheal branching throughout animal development by inhibition of Insulin or PDF signalling led to the reduction of organismal TAG levels in larvae and adult flies (Linneweber et al., 2014).

The endocrine system in *Drosophila* also consists of endocrine glands, called corpora allata (CA) and corpora cardiaca (CC), which produce key factors to maintain metabolic homeostasis. Those factors are Adipokinetic Hormone (AKH), Limostatin and Juvenile Hormone (JH).

AKH is a Glucogon-like peptide and therefore works as an opposing factor to Insulins. AKH is produced and released by the CC (Galikova et al., 2015; Kim and Rulifson, 2004) and binds to its receptor AKHR in various tissues to increase the release of stored nutrients (Galikova et al., 2015; Gronke et al., 2007; Kim and Rulifson, 2004; Lee and Park, 2004). AKH ablation in *Drosophila* larvae led to reduction of circulating sugar levels (Kim and Rulifson, 2004). *akh* and *akhr* mutants also showed higher lipid content compared to control flies, which was responsible for increased starvation resistance (Galikova et al., 2015). Furthermore adult flies carrying a mutation for *akh* or *akhr* showed reduced

circulating sugar levels, which wasn't due to an upregulation in stored glucose (Galikova et al., 2015). Interestingly, AKH ablated flies didn't show the starvation induced hyperactivity as seen in control flies (Lee and Park, 2004). This shows that adequate AKH signalling is essential in *Drosophila*.

Limostatin (Lst), another CC produced peptide, is secreted in response to nutrient deprivation to reduce Insulin secretion by binding to its receptor LstR on IPCs and therefore acts as a Decretin (Alfa et al., 2015). Limostatin signalling was found to be ortholog to mammalian Neuromedin U/ Neuromedin U receptor signalling (Alfa et al., 2015). *lst* expression was upregulated upon starvation and reduced again specifically after re-feeding with carbohydrates, but not proteins (Alfa et al., 2015). *lst* mutants showed decreased glucose levels and increased *dilp2* transcription, as well as increased circulating Dilp2 protein, increased stored lipid content and reduced lifespan (Alfa et al., 2015). *lst* mutant phenotypes were rescued upon blockage of Dilp2 secretion or when *lst* was overexpressed in CC cells in a *lst* mutant background (Alfa et al., 2015). The differential regulation of Lst by nutrients represents an excellent paradigm to understand nutrient sensing mechanism.

The corpora allata produces Juvenile Hormone, which regulates larval growth and adult reproduction through its receptor Germ cell-expressed (Gce)/ Methoprene-tolerant (Met) (Jindra et al., 2015; Mirth et al., 2014; Reiff et al., 2015). Ablation of JH producing CA cells led to smaller larvae due to Foxo dependent reduction in growth rate (Mirth et al., 2014). Recently, it was found that JH is important for proliferation, growth and metabolic status of the midgut of mated females in preparation for reproduction (Reiff et al., 2015). Virgin flies displayed smaller and less proliferative midguts as well as lower lipid content compared to mated females (Reiff et al., 2015). Interestingly, this lack of growth and proliferation could be rescued by feeding virgin flies with JH supplemented food (Reiff et al., 2015). Knockdown of the Juvenile hormone receptor *met* or *gce* specifically in stem/ progenitor cells or enterocytes resulted in flies with reduced fecundity, showing that JH acts on the midgut to increase its size, which leads to increased lipid metabolism for functional fecundity (Reiff et al., 2015).

All above mentioned hormones and signalling pathways uncovered and characterised show the great advantage of using *Drosophila* as a model organism

to study complex inter-organ communication leading to the regulation of local and systemic metabolic homeostasis through highly conserved molecular mechanism.

1.6 Regulation of intestinal homeostasis in *Drosophila*

1.6.1 Structure of the gut – comparison between mammals and *Drosophila*

The gastrointestinal tract comprises the mouth, oesophagus, stomach, small intestine and colon, which are necessary to ingest food and absorb nutrients to provide the organism with energy. The mouth, oesophagus and stomach are responsible for intake, passaging and first digestion of the food. Further digestion and nutrient absorption is achieved by the mammalian small intestine, which displays finger-like protrusions, known as villi to increase its absorptive surface. Villi are comprised of enterocytes, secretory enteroendocrine and goblet cells. The intestinal epithelium is constantly exposed to harmful substances and bacteria. In order to protect intestinal cells from infection by pathogens, goblet cells secrete a mucus layer (Allen and Flemstrom, 2005). If epithelia cells are damaged, they will undergo apoptosis and shed into the lumen. In order to remain a constant number of cells and epithelial homeostasis, the lost cells need to be replenished. This is achieved by intestinal stem cells, situated at the base of the crypt, which are able to self-renew the intestinal epithelia within a week. Adjacent to the small intestine is the colon, which displays a smooth epithelium and is responsible for reabsorption of water and excretion of the faeces.

The *Drosophila* gut is organized into 3 subregions: the foregut, the midgut and the hindgut (Demerec, 1950) (Figure 1-3). The *Drosophila* foregut consists of the pharynx, esophagus and crop, which is the adult structure to store food. The main function of the *Drosophila* midgut is food digestion and nutrient absorption. The posterior end of the midgut is followed by the hindgut, homolog to the mammalian colon, which is responsible for water reabsorption from food prior to excretion (Cognigni et al., 2011). Attached to the midgut-hindgut junction are the Malpighian tubules, which act similarly to the mammalian kidney.

The fly intestine is able to self-renew every 2-4 weeks through the activity of the intestinal stem cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006,

2007). As its mammalian counterpart the fly intestinal epithelium is subject to self-renewal due to its exposure to internal and external challenges (Hakim et al., 2010). In order to protect intestinal cells from pathogens, *Drosophila* has a relatively thin protective layer called peritrophic matrix, akin to the vertebrate mucosa (Kuraishi et al., 2011).

Damage can also be induced in the laboratory by feeding *Drosophila* with damaging agents, like Bleomycin and dextran sodium sulfate or by expressing apoptotic genes within intestinal cells. Damaging the epithelium leads to the regulation of multiple signalling pathways to ensure quick regeneration.

1.6.2 Intestinal stem cells and their niche

Maintaining epithelial homeostasis in the intestine is essential for proper functioning of the tissue and overall organismal wellbeing. Pluripotent ISCs are located on the basement membrane and in close contact with the underlying visceral muscle cells, which produce multiple stem cell niche components, including Wingless (Wnt signalling), Vein (EGFR signalling) and Dilp3 (Insulin signalling) (Lin et al., 2008; O'Brien et al., 2011; Scopelliti et al., 2014; Xu et al., 2011). ISCs are able to self-renew and to give rise to undifferentiated progenitor cells, which are called enteroblasts (EBs). These EBs then differentiate directly into either the absorptive enterocytes (EC) or the secretory enteroendocrine (ee) cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) (Figure 1-3).

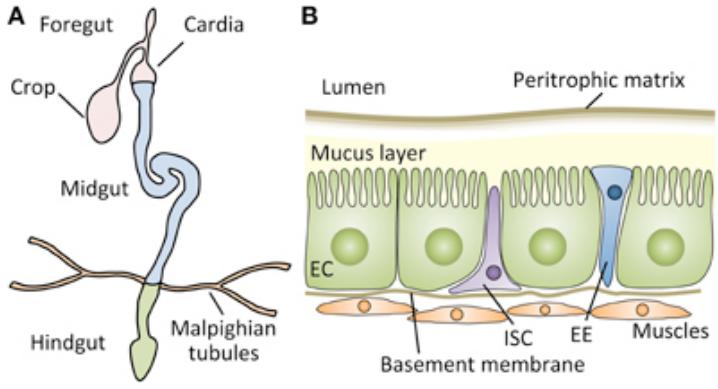

Figure 1-3: The intestine of *Drosophila melanogaster*.

(A) The *Drosophila* intestine is divided in 3 sub-regions: the forgut (rose), the midgut (blue) and the hindgut (green). (B) The intestinal epithelial monolayer with its different cell types. ISC = intestinal stem cell (purple), EC = enterocyte (green), EE = enteroendocrine cell (blue), Muscles (orange). Image taken from (Kuraishi et al., 2013).

All cell types of the midgut can be distinguished through the expression of specific markers and their morphological differences. *Drosophila* ISCs express the Notch ligand Delta (Dl) and the transcription factor (TF) Escargot (Esg, also expressed by EBs). ISCs have a small nucleus and are diploid (Ohlstein and Spradling, 2007; Perdigoto et al., 2011). Enteroblasts are also diploid, have a small nucleus and express the DNA binding protein Suppressor of hairless (Su(H)), a component of the Notch signalling pathway. ECs are marked by expression of the TF Pdm1, are polyploidy and endoreplicative cells with large nuclei. ee cells have a diploid nucleus and can be distinguished by the expression of the TF Prospero (Pros) (Ohlstein and Spradling, 2007).

It has been described that 2 types of interconvertable ISCs are present in the crypts of the mammalian epithelium: the fast-cycling LGR5^{+ive}, which are located at the bottom of the crypt and the slow-cycling Bmi^{+ive} stem cells located at position '+4' (Takeda et al., 2011; Tian et al., 2011a). LGR5^{+ive} stem cells are intercalated with Paneth cells, secreting important ISC niche factors for growth and proliferation (Sato et al., 2011). In *Drosophila* Paneth cells are not present. However, EBs, which are in direct physical contact with ISCs, appear to play a similar role of that of Paneth cells in mammals by providing stem cell niche components including EGF and Wnt ligands (Cordero et al., 2012b; Jiang et al., 2011).

1.6.3 The mechanisms regulating stem cell proliferation: Parallels between flies and mammals

Many signalling pathways, such as the Notch (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), Wnt (Cordero et al., 2009; Lee et al., 2009; Lin et al., 2008), JNK (Biteau et al., 2008; Jiang et al., 2009), Jak/Stat (Beebe et al., 2010; Jiang et al., 2009), EGFR (Jiang and Edgar, 2009) and Hippo signalling pathways (Staley and Irvine, 2010) are involved in homoeostasis and regeneration of the *Drosophila* midgut. These pathways are often highly interconnected and appear to be non-redundantly required to drive ISC proliferation during normal tissue homeostasis and/or in the proliferative response of ISC to regenerate the tissue upon damage.

Damage to the ECs causes upregulation of JNK signalling, release of cytokines, like the IL-6 ortholog Unpaired 3 (Upd3), induction of EGF-like ligands within the epithelium and VM, and secretion of Wg from EBs (Apidianakis et al., 2009; Biteau et al., 2008; Biteau and Jasper, 2011; Buchon et al., 2009a; Buchon et al., 2009b; Jiang and Edgar, 2009). All these signals are required for the promotion of ISC proliferation by activating their cognate signalling pathways, such as JAK/Stat, EGFR/MAPK and Wnt signalling, within ISCs (Cordero et al., 2012a; Cordero et al., 2012b; Jiang and Edgar, 2009; Jiang et al., 2011). Similar to *Drosophila*, the mammalian intestine also reacts to damage with a rapid upregulation of cytokines, like IL-6 and Stat signalling (Grivennikov et al., 2009).

Notch signalling is another key pathway regulating ISC homeostasis in *Drosophila* (Ohlstein and Spradling, 2007). Notch is expressed in ISCs and EBs, but the Notch ligand Delta is only present in EBs (Ohlstein and Spradling, 2007). High Notch signalling activation in ISCs/EBs leads to the differentiation of ISCs into ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), whereas low signalling promotes ISC and ee cell fate at the expense of ECs (Ohlstein and Spradling, 2007). Similarly, activation of Notch signalling in the mammalian intestine impairs secretory cell differentiation, whereas inhibition promotes their differentiation (Fre et al., 2005; Stanger et al., 2005). Delta is expressed in Paneth cells (Sato et al., 2011), and Notch is active within the stem cells, which was determined by lineage tracing (Vooijs et al., 2007). This once again, clearly shows the conservation of signalling pathways in different species.

Inhibition of Wnt signalling in mammals leads to reduced proliferation and depletion of the transient amplifying cells, which ultimately results in villi and crypt loss (Fevr et al., 2007; Ireland et al., 2004; Korinek et al., 1998). On the contrary, activation of Wnt signalling or loss of the negative effector of the Wnt pathway APC (adenomatous polyposis coli) results in overproliferation and intestinal adenoma formation (Andreu et al., 2005; Harada et al., 1999; Shibata et al., 1997). Wnt signalling hyperactivation is a key driver of human colorectal cancer (CRC). Loss of function mutations of APC are present in 80-90 % of hereditary and sporadic forms of CRC. Similarly, inhibition of Wnt signalling in the *Drosophila* intestine impairs regeneration following damage of the intestinal epithelium (Cordero et al., 2012b), while loss of APC, overexpression of activated

ß-catenin (*arm*^{S10}) or *wingless* leads to gut dysplasia and an increase in ISC proliferation, even though more modest than that observed in mammals (Cordero et al., 2009; Lee et al., 2009; Lin et al., 2008).

Furthermore, Hippo signalling has been shown to be important for epithelial homeostasis and for regeneration upon damage in both mammals (Cai et al., 2010; Zhou et al., 2011) and *Drosophila* (Karpowicz et al., 2010; Shaw et al., 2010).

This overview of signalling pathways affecting stem cell homeostasis highlights once more the similarity in key biological processes between *Drosophila* and mammalian tissues.

1.7 Neuroendocrine control of adult intestinal homeostasis in *Drosophila*: The unexpected role of Bursicon signalling

All arthropods have an exoskeleton as a barrier against microorganisms and to protect them from injury and desiccation, as well as to generate the attachment site for muscles. After emergence of the *Drosophila* adult, the exoskeleton is soft and weak, which leaves the flies vulnerable to the environment. Therefore, this newly formed cuticle must undergo hardening following adult eclosion. In 1965 a hormone called Bursicon (Burs) was discovered in blowflies to be the initiator for cuticle hardening and tanning after adult emergence (Fraenkel, 1965). Bursicon was later shown to be conserved among insects (Fraenkel et al., 1966). Elegant experiments showed that neck-ligating flies just after eclosion led to animals unable to harden and darken their cuticle. This effect was reversed when injecting animals with hemolymph from newly born adults, containing high titers of circulating Bursicon (Fraenkel et al., 1966; Fraenkel, 1965). From these experiments it was concluded that the hormone Bursicon is released from the brain to mediate cuticle tanning and hardening in newly eclosed adults. Later work showed that Bursicon corresponds to a 30 kDa protein in many insects (Kostron, 1995). The purification of Burs (Honegger et al., 2002; Kostron et al., 1999) was key to obtain the first sequencing of the hormone (Honegger et al., 2004; Honegger et al., 2002). The former, together with the discovery of *CG13419* as the gene encoding for Bursicon (Burs) (later named *burs-a*) in *Drosophila* (Dewey et al., 2004), represented a major breakthrough in the field of ecdysis.

Additionally, Burs was shown to also modulate wing expansion (Dewey et al., 2004). Furthermore, in 2005 it was shown that the active Burs tanning hormone was made of a heterodimeric complex of two cysteine knot proteins, Burs-α and Burs-ß (the latter encoded by *CG15284*, also known as *partner of bursicon* (*pburs*) and hereafter called *burs-ß*). This heterodimeric complex acts as a ligand to its receptor LGR2 (leucin-rich repeat-containing G-protein coupled receptor 2, encoded by the *rickets* (*rk*) locus) (Baker and Truman, 2002; Luo et al., 2005; Mendive et al., 2005). It has been shown that only the heterodimer of Burs can activate LGR2 leading to an increase in cAMP (Luo et al., 2005; Mendive et al., 2005). Furthermore, only a Burs-α and –ß combined solution was able to induce tanning, when injected into neck-ligated flies, compared to solutions containing either Burs-α or Burs-ß (Mendive et al., 2005). These results clearly demonstrate, that the bioactive Burs hormone controlling ecdysis is a heterodimeric complex of the cysteine knot proteins Burs-α and –ß.

As per the evidence described above, it was assumed that Burs/ LGR2 signalling had no role beyond development. Initial work leading to the discovery of Bursicon reported undetectable levels of the hormone beyond 10 hours after adult eclosion (Fraenkel, 1965). Consistently, it was shown that Burs-α and –ß producing neurons undergo apoptosis soon after animal eclosion (Honegger et al., 2011).

Surprisingly, we recently showed that Burs-α has an essential role in adult *Drosophila* midgut homeostasis, which is independent from the role of the hormone during development and it is also independent from the subunit Burs-ß (Scopelliti et al., 2016; Scopelliti et al., 2014). We demonstrated, that Burs-α was exclusively expressed in a subset of enteroendocrine (ee) cells in the adult fly midgut, whereas the receptor LGR2 was expressed within the visceral muscle (VM) surrounding the gut (Scopelliti et al., 2014). We showed that Burs-α from ee cells binds LGR2 in the VM to block the production of the EGF-like growth factor Vein through activation of cAMP, which is necessary to maintain stem cell quiescence (Scopelliti et al., 2014). Knockdown of *burs-a* alone was able to induce stem cell proliferation in the midgut (Scopelliti et al., 2014), while *burs-ß* was neither expressed in adult animals nor required for intestinal homeostasis (Scopelliti et al., 2016).

The question as to whether Bursicon othologs exist in mammals remains open. In vertebrates, there are many cysteine knot protein families, such as the glycoprotein hormones Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), Chorionic Gonadotropin (CG) and Thyroid-stimulating hormone (TSH); growth factors including Nerve Growth Factor (NGF), Platelet-derived Growth Factor (PDGF) and Transforming Growth Factor-beta (TGF-ß); Mucins and Bone Morphogenic Factors (BMPs) (Vitt et al., 2001). Mucin like-BMP- antagonists were found to be the closest potential orthologs of Burs proteins (Vitt et al., 2001). And the *Drosophila* receptor LGR2 displays structural homology with the human LGRs 4, 5 and 6 (Eriksen et al., 2000; Nishi et al., 2000). However, genetic and functional experiments still remain to be done to determine the degree of functional homology between the insect and vertebrate proteins.

1.8 Aim of this Thesis

The project presented in this thesis was conducted in collaboration with Dr. Alessandro Scopelliti, a postdoctoral researcher in our laboratory. At the time I joined the laboratory in October 2012, Dr. Allessandro Scopelliti, Dr. Julia B. Cordero and Dr. Marcos Vidal were working on the role of Bursicon/ LGR2 signalling in regulating intestinal stem cell quiescence (Scopelliti et al., 2014).

Persuaded by the findings of a novel and unexpected non-developmental adult-specific function of Burs/ LGR2 signalling in *Drosophila*, we decided to explore further function of this endocrine system in mature adult animals, which represents the core of my PhD thesis.

I was able to contribute equally to this work and we demonstrated that enteroendocrine derived Bursicon is regulated by nutrients and acts via its neuronal receptor LGR2 to maintain metabolic homeostasis.

Experiments presented in this thesis were designed, performed and analysed together with Dr. Alessandro Scopelliti. Furthermore, Dr. Yachuan Yu, the senior scientific officer in the laboratory helped with lipid measurements.

2 Material and Methods

2.1 Drosophila melanogaster

2.1.1 Fly husbandry

Flies were mated and kept on standard food in incubators with various temperatures ranging from 18 – 29 °C with a controlled humidity and a 12h-12h light-dark cycle. Animals were anaesthetised using CO_2 under a Leica dissection microscope and pushed using a feather. Stocks were maintained at 18 °C. Experiments with *burs* and *rk* mutants were carried out at 25 °C. Crosses with flies carrying an adult specific targeted knockdown were kept at 18 °C (no transgene expression) and F1 was shifted to 29 °C to start the activation of the transgene.

Standard food: 10g Agar, 15g Sucrose, 30g Glucose, 15g Maize meal, 10g wheat germ, 30g treacle and 10g Soya flour per litre of distilled water.

The fly food was kindly prepared according to this recipe by Central Services of the Beatson Institute.

2.1.2 Fly stocks

Fly stocks were kindly provided by our colleagues and bought from the stock collection centres Bloomington, *Drosophila* Genome Resource (DGRC) and Vienna *Drosophila* RNAi Centre (VDRC). A full list of stocks and their sources used for the work in this thesis can be found in Table 2-1.

Genotype	Description	Source
w^{1118}	wild type strain of *Drosophila*	Vidal lab stocks
Mutants		
$burs^{z5569}$	hypomorphic mutant allele	(Dewey et al., 2004)
$burs^{1091}$	hypomorphic mutant allele	(Dewey et al., 2004)
w; cn, bw, rk^{1}	hypomorphic mutant allele	Bloomington 3589
$pupal^{6}$	loss of function allele of *burs-Β*	DGRC 101309
Df(2) 110	excision allele of *burs-Β*	(Lahr et al., 2012)
Df(2) Excel6035	excision allele of *burs-Β*	Bloomington 7518
RNA interference		
UAS-rk^{IR-1}	RNAi transgene for *rk*	Vidal lab stocks (4753)
UAS-rk^{IR-2}	RNAi transgene for *rk*	VDRC 2993 GD
UAS-rk^{IR-3}	RNAi transgene for *rk*	VDRC 105360 KK
UAS-$burs^{IR}$	RNAi transgene for *burs*	VDRC 102204 KK
UAS-$burs^{IR}$	RNAi transgene for *burs*	VDRC 13520 GD
UAS-$glut1^{IR}$	RNAi transgene for *glut1*	VDRC 13326 GD
UAS-akh^{IR}	RNAi transgene for *akh*	VDRC 11352 GD
UAS-$ccklr17\text{-}d1^{IR}$	RNAi transgene for *ccklr17-d1*	VDRC 102039 KK
UAS-$ccklr17\text{-}d3^{IR}$	RNAi transgene for *ccklr17-d3*	Bloomington 60405
Gal4 driver lines		
how-Gal4	visceral muscle expression	(Jiang et al., 2009)
voila-Gal4	enteroendocrine expression	Irene Miguel-Aliaga
MyoIA-Gal4	enterocyte expression	Bruce Edgar
dilp2-Gal4	dilp2/IPC expression	Bloomington 48030

nsyb-Gal4	pan-neuronal expression	Irene Miguel-Aliaga
elav-Gal4	pan-neuronal expression	Bloomington 8760
rkPAN-Gal4	expression in *rk*$^{+ive}$ cells	Benjamin White
FB-Gal4	fat body expression	Vidal lab stocks
Lsp2-Gal4	fat body expression	Vidal lab stocks
dsrf-Gal4	expression in terminal tracheal branches	Irene Miguel-Aliaga
btl-Gal4	tracheal expression	Irene Miguel-Aliaga
ccklr17-d3-Gal4	expression in *ccklr17-d3*$^{+ive}$ cells	Truman Lab (Texada)
ccklr17-d1-Gal4	expression in *ccklr17-d1*$^{+ive}$ cells	Truman lab (Texada)
UAS-transgenes		
UAS-Epac1-camps	calcium sensor	Bloomington 25407
UAS-gal80^{ts}	temperature regulated GAL80 repressor	Bloomington 7019
UAS-dicer2	transgene expressing Dicer2, enhances RNAi expression	Vidal lab stocks
UAS-*rk*	overexpression of *rk*	(Scopelliti et al., 2014)
UAS-*burs77*	overexpression of *burs*	(Scopelliti et al., 2014) Benjamin White
UAS-GFP	transgene expressing GFP	Bloomington 6874
UAS-CD8-GFP	transgene expressing membrane GFP	Vidal lab stocks
UAS-nRS	transgene expressing Red Stinger in the nucleus	Benjamin White
UAS-*inr*^{DN}	transgene expressing a dominant negative form of Insulin receptor	Bloomington 8251
UAS-*dp110*^{DN}	transgene expressing a dominant negative form of Dp110, a subunit of PI3K	Irene Miguel-Aliaga
Fosmids		
burs-gfp fosmid	GFP tagged *burs*	generated by Vidal lab
dilp2-gfp fosmid	GFP tagged *dilp2*	Vidal lab stocks

Table 2-1: Fly stocks used in this study.

2.1.3 Lifespan analysis

F1 with the required genotypes from crosses at 18 °C were collected within 48h of eclosion using CO_2 anaesthesia. F1 was transferred to 29 °C and dead flies were counted every 1-2 days. Statistical tests were performed using Graph Pad Prism to compare survival curves. Log-rank (Mantel-Cox) test was used to analyse statistical significance.

2.1.4 Starvation sensitivity assay

Flies of required genotypes were collected and aged for 2-3 days at 25 °C for *burs* and *rk* mutants and their controls, or aged for 10 days at 29 °C for flies with an adult specific targeted knockdown and transferred into 1% agar containing vials. Dead flies were counted multiple times a day. Statistical tests were performed using Graph Pad Prism to compare survival curves. Log-rank (Mantel-Cox) test was used to analyse statistical significance.

2.1.5 Cold stress assay

Flies were aged for 3 days at 25 or 29 °C. Females were collected, placed in a fresh vial and subjected to an ice-water bath for 10 min. Recovery time, measured by the fly's ability to stand, was recorded. Statistical tests were performed using Graph Pad Prism. Log-rank (Mantel-Cox) test was used to analyse statistical significance.

2.2 Immunofluorescence of *Drosophila* tissues

2.2.1 Fixation and antibody staining

Adult tissues were dissected in PBS using number 5 forceps (Dumont) and a Leica dissection microscope and fixed in 9-well glass plates in 4% para-formaldehyde (Polysciences, Inc.) for at least 30 min. After fixation, tissues were transferred first into fresh PBS for 5 min and after into PBS + 0.2% TritonX-100 (PBST) for 20 min. Samples were incubated overnight at 4 °C with primary antibodies in PBST + 2% Bovine Serum Albumin (BSA) (Sigma). The next day, samples were washed in PBST for 1h on a horizontal shaker and incubated with secondary antibodies in PBST for 2h at room temperature. Samples were washed in PBST for 1h and

mounted onto polylysine glass slides (Thermo Fisher) with 13mm x 0.12mm spacers (Electron Microscopy Schience) and Vectrashield mounting media containing DAPI (Vector Laboratories, Inc).

Midguts stained for Bursicon were incubated in a series of ethanol washes ranging from 10% to 90% (steps of 20%) on ice after fixation in 4% para-formaldehyde. Dissected midguts in 90% ethanol were kept over night at -20 °C and the next day the ethanol series was inverted starting from 90% and going down to 10%. Quick wash in PBS and from here standard staining protocol as described above was used.

LipidTOX (life technologies) stainings were performed using PBS containing 0.005% saponin instead of PBST after fixation. Cuticles were stained with LipidTOX (diluted 1/500 in PBS + saponin) for 2h at room temperature. Three 15 min washes in PBS + saponin were performed and tissues were mounted without spacers. Antibodies and fluorescent stains used in this study can be found in Table 2-2.

Antibody	Description	Dilution	Source
Primary antibodies			
anti-GFP	chicken, polyclonal against GFP	1:4000	abcam 13970
anti-Pros	mouse, monoclonal against Prospero	1:20	Developmental Studies Hybridoma Bank (DSHB) C594.9B
anti-pH3S10	rabbit, polyclonal against Ser10 phosphorylated Histone 3	1:100	Cell Signalling 9701
anti-pH3S28	rabbit, polyclonal against Ser28 phosphorylated Histone 3	1:100	Cell Signalling 9713
anti-Burs	rabbit, polyclonal against Bursicon	1:250	Ben White (for immuno-fluorescence)
anti-Burs	rabbit, polyclonal against Bursicon	1:500	(Scopelliti et al., 2016) (for Western Blotting)
anti-Dilp2	rabbit, polyclonal against Dilp2	1:500	Stocker Lab
anti-AKH	rabbit, polyclonal against AKH	1:250	J. Park
anti-αTub	mouse, monoclonal against α Tubulin	1:1000	DSHB E7-c

Anti-Bruchpilot (Brp)	stains neuro-muscular junction	1:20	DSHB nc82
Secondary antibodies			
anti-ch-IgG-488	goat, polyclonal against chicken IgG, conjugated to Alexa Fluor 488	1:200	Invitrogen A11039
anti-ms-IgG-488	goat, polyclonal against mouse IgG, conjugated to Alexa Fluor 488	1:200	Invitrogen A11029
anti-ms-IgG-594	goat, polyclonal against mouse IgG, conjugated to Alexa Fluor 594	1:100	Invitrogen A11032
anti-rb-IgG-488	goat, polyclonal against rabbit IgG, conjugated to Alexa Fluor 488	1:200	Invitrogen A11008
anti-rb-IgG-594	goat, polyclonal against rabbit IgG, conjugated to Alexa Fluor 594	1:100	Invitrogen A11037
Phalloidin-488	High affinity F-actin probe, conjugated to Alexa Fluor 488	1:500	Invitrogen A12379
IRDye 680RD-anti rabbit	donkey, conjugated with IRDye 680RD	1: 10 000	LiCor 926-68073
IRDye 800RD-anti mouse	donkey, conjugated with IRDye 800RD	1: 10 000	LiCor 926-32212
fluorescent staining			
lipidTOX	neutral lipid stain (red)	1:500	life technologies H34476

Table 2-2: Antibodies used in this study.

2.2.2 Confocal Microscopy

All confocal images were taken using the Zeiss 710 LSM confocal microscope in the Beatson Advanced Imaging Resource (BAIR). Raw data was stored as LSM files and confocal maximumprojection images are presented in this study.

2.2.3 Quantification of $pH3^{+ive}$ cells in the posterior midgut

Antibodies against phosphorylated Histone 3 were used to assess proliferation in the posterior midguts of different genotypes ($n \geq 10$).

Statistical tests were performed using Graph Pad Prism to compare proliferation rates. To analyse statistical significance of 2 genotypes, unpaired t-tests were

performed. If 3 or more genotypes were compared, one-way-ANOVA analysis and Turkey's multiple comparisons test was used.

2.2.4 FLIM-FRET

The fluorescent lifetime of the Epac1-camps biosensor was recorded as previously described (Scopelliti et al., 2014). Briefly, flies expressing the Epac1-camps biosensor in the VM were dissected in S2 media and their midguts exposed onto glass-bottom 3,5 cm dishes (MatTek Corporation MA, USA). Images were taken on a Nikon Eclipse TE 2000-U microscope to measure CPF lifetime changes based on CFP/YFP FRET and a 445 nm intensity modulated LED was used for illumination. The frequency domain was analysed using the Lambert Instruments fluorescence attachment to measure FLIM-FRET. A standard with a known lifetime of 4.0 ns (10 mM Fluorescein solution in 0.1 M Tris-Cl) was used as a reference. Upon administration of recombinant Burs-α or S2 media, every minute for 30 min images of 5 regions of interest were taken. Two-way ANOVA test with Bonferroni correction was applied to analyse statistical significance.

2.3 Protein analysis

2.3.1 Protein extraction

Flies were collected using CO_2 anaesthesia and immediately frozen on dry ice. 5 flies were homogenised in cold RIPA buffer (150 mM NaCL, 1% TritonX100, 0.5% Sodiumdeoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) on ice using a pestel. Homogenates were incubated for 30 min on ice, vortexed from time to time and centrifuged for 10 min at 13 000 g and 4 °C. Supernatant was transferred into a new Eppendorf tube, if protein solution still contained debris, homogenate was centrifuged a second time.

Hemolymph was extracted by decapitating flies using CO_2 anaesthesia and transferring them upside down into a 10 µl filter pipette tip, which is inserted in a 20 µl pipette tip (tip was cut in a 45° angle) and placed in a 2 ml Eppendorf tube on ice. Decapitated flies were centrifuged at 10 000g for 15 min at 4 °C. Hemolymph was transferred in a fresh Eppendorf tube and immediately frozen in dry ice.

Protein concentration was quantified using Bradford (Abcam) and a spectrophotometer.

2.3.2 SDS-PAGE and Protein transfer

DTT and loading buffer (NuPAGE, Invitrogen) was added to protein extracts and heat treated for 10 min at 95 °C. Samples were centrifuged at 13 000 g and 4°C for 10 min and loaded onto a 10% Bis-Tris pre-cast gel (life technologies). The run was performed at 100 V for 45 min in 1x NuPAGE MES running buffer using Invitrogen XCell *SureLock*™ electrophoresis system. 4 µl of PageRuler™ prestained marker (Thermo Scientific) was used to estimate protein size. Gels were transferred onto a PVDF membrane (Amersham) and blocked with 5 % BSA in TBST (TBS containing Tween 20) for 1 h at room temperature. Membranes were incubated with primary antibody in TBST containing 5 % BSA over night at 4 °C. The next day, membranes were washed 3 times for 5 min in TBST and incubation with secondary antibody was performed for 2 h at room temperature in TBST containing 5% BSA. Membranes were washed as before and bands were visualised using the ODYSSEY Clx from LiCor to image fluorescent intensity, which was used to analyse statistical significance using Graph Pad Prism and unpaired t-test.

2.3.2.1 Non-reducing Western Blotting

50 ng of recombinant Burs-α protein (Cusabio) was either dissolved in loading buffer containing ß-mercaptoethanol, DTT and SDS for reducing conditions, or SDS only for non-reducing conditions. Samples were treated for 10 min at 70°C, shortly centrifuged and loaded onto a 10% Bis-Tris pre-cast gel (life technologies). From here the standard protocol described above was used.

2.3.3 Liquid Chromatography Mass Spectrometry (LC-MS)

Tissues were dissected as stated above and metabolites from them or from whole flies were extracted using -80 °C pre-cooled extraction buffer (Methanol, Acetonitrile and H_2O in a ratio 5:3:2) kindly provided by Dr. Saverio Tardito. Lysates were centrifuged at 13 000 g and 4 °C and supernatant was transferred into a fresh Eppendorf tube and analysed by means of LC-MS by Dr. Saverio Tardito. Protein amount in fly debris was measured as described above. Obtained data from Dr. Saverio Tardito was normalised by protein concentration.

2.4 Nucleic acid extraction and quantification

2.4.1 DNA extraction from whole flies

Total DNA was extracted from biological triplicates of 5 female flies using E.Z.N.A Insect DNA Kit (OMEGA bio-tek) according to manufacturer's instructions. DNA was quantified using a NanoDrop Spectrophotometer.

2.4.2 RNA extraction from midguts

Total RNA was extracted from biological triplicates of 5 female flies using the Qiagen RNAeasy kit (Qiagen) according to manufacturer's instruction. RNA was quantified using a NanoDrop Spectrophotometer.

2.4.3 RNA extraction from brains and heads

Biological triplicates of around 50 brains were dissected in cold PBS or 80 heads immediately snap frozen in dry ice. Total RNA was extracted using a combined method of Trizol and the Qiagen RNAeasy kit. The tissues were homogenised in 100 µl Trizol using a pestel and additional 700 µl of Trizol were added. Samples were centrifuged at 12 000 g and 4 °C for 10 min. Supernatant was transferred into a fresh Eppendorf tube and incubated for 5 min at room temperature. 200µl of chloroform were added, vortexed and incubated for 2min at room temperature. Homogenates were centrifuged at 12 000 g and 4 °C for 15 min. The transparent aqueous phase was transferred into a fresh tube, equal volumes of 70 % ethanol added and transferred onto an RNeasy spin column. From here the manufactures protocol for the Qiagen RNeasy kit was followed. RNA was quantified using a NanoDrop Spectrophotometer.

2.4.4 RNA extraction from cuticles

Biological triplicates of around 80 cuticles were dissected in cold PBS and immediately snap frozen in dry ice. Total RNA was extracted using Trizol. 1ml Trizol was added to each sample and vortexed. Centrifugation at 12 000 g and 4°C for 10 min followed, and supernatants were transferred into a new tube and incubated for 5 min at room temperature. 200 µl of Chloroform was added, rigorously vortexed and centrifuged at 12 000 g and 4 °C for 15 min. The

transparent aqueous phase was transferred into a fresh tube, 500 µl isopropanol added, rigorously vortexed and incubated for 10 min at room temperature. Samples were centrifuged for 15 min at 14 000 g and 4 °C and supernatant discarded. Pellet was washed with 1 ml 75% ethanol, vortexed and centrifuged for 5 min at 14 000 g and 4 °C. Ethanol was discarded and the remaining ethanol is removed after quick centrifugation with a smaller pipette. After the pellet was air-dried for 2 min in the hood, and treated with DNase TURBO (Thermo Fisher) and RNAsine (Promega) for 30 min at 37 °C. 2.8 µl of inactivation buffer was added, vortexed every minute within a 5 min incubation time at room temperature and centrifuged at 13 000 g and 4 °C for 5 min. Supernatant was transferred into a fresh Eppendorf tube. RNA was quantified using a NanoDrop Spectrophotometer.

2.4.5 RNA sequencing and gene enrichment analysis

1 µg of sample RNA in 50 µl RNase free water was handed to Billy Clark for cDNA library preparation and RNA sequencing. Furthermore, a few microliter of 100 – 300 ng/µl were used to analyse RNA Integrety Number (RIN). After successful library preparation and sequencing, the data was handed over to Ann Hedley to do the analysis. She prepared excel sheets of the data and we analysed it further. Using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.8 (Huang da et al., 2009a, b) we performed gene set enrichment analysis.

2.4.6 Reverse transcription - cDNA synthesis

cDNA was synthesised in triplicates for each biological replicate using the High-Capacity cDNA reverse transcription kit (Applied Biosystems – life technologies). cDNA synthesis reactions were pooled for each biological replicate.

2.4.7 Quantitative PCR

Expression of target genes was measured and normalised to *rpl32*, *sdha* or *actin5c* and primers used for RT-qPCRs were designed using the pearlprimer software and are shown in Table 2-3. A standard curve was produced by a series of 10-fold dilutions of pooled cDNA samples. Quanta SYBR green Master Mix (Low ROX) was used following manufacture'rs instructions. Data were extracted and analysed

using Applied Biosystems 7500 software and melt curves were used to make sure only one PCR product was produced by each primer pair.

Target	Primer name	Sequence 5’ ▫ 3’
rpl32	rpl32 F1	AGGCCCAAGATCGTGAAGAA
	rpl32 R1	TGTTGCACCAGGAACTTCTTGAA
actin5c	actin5c F1	GAGCGCGGTTACTCTTTCAC
	actin5c R1	CCATCTCCTGCTCAAAGTCG
Sdha	sdha F1	AATGCCCAGATGACTATTGTGAG
	sdha R1	GCTTGCTGAAATCGTATTCATCC
rpl39	rpl39 F1	AAAGATTGGACGAAATGGCTG
	rpl39 R1	GCTTAGCGTTGTAACGAATAGTG
burs-a	burs F1	CATCCATGTGCTCCAGTATCC
	burs R1	GGCTTCACTTTGGGACAGAA
burs-ß	burs-ß F1	AGGATTGTGCAACAGTCAGG
	burs-ß R1	AGCAATGGGTTAGAGTGATGAC
Rk	rk F1	GTCAATCTTCCCAACGAGGTG
	rk R1	GGACAAAGTTAGCTCCTCCAG
dilp2	dilp2 F1	CCTGCAGTTTGTCCAGGAGT
	dilp2 R1	AGCCAGGGAATTGAGTACACC
dilp3	dilp3 F1	GTATGGCTTCAACGCAATGAC
	dilp3 R1	GAGCATCTGAACCCAACTATCAC
dilp5	dilp5 F1	CGTGATCCCAGTTCTCCTGT
	dilp5 R1	ACCCTCAGCATGTCCATCAA
Thor	thor F1	CCAGGAAGGTTGTCATCTCG

	thor R1	TGAAAGCCCGCTCGTAGATA
Inr	inr F1	GGTGCTGGCATCATAGGTCT
	inr R1	CCTGCCTCTGAGTGATAGAAGG
sut2	sut2 F1	GGTTGTTGTAATGCACGTGAC
	sut2 R1	CCCAAAGAAATAGCCCACTG
Rfabg	rfabg F1	AAGTAGATGTCATTGGAAGTGGGA
	rfabg R1	CGATTCAACAATATGCCAGAAACC
lsd1	lsd1 F1	CCGCCCGAAATGATGTACTG
	lds1 R1	GCATAAGTGGTAAGTGGACTCTC
yp3	yp3 F1	CGGCGATTTGATCATCATTGAC
	yp3 R1	TTGTTGGTCAGATCGATCAGG
CG6283	CG6283 F1	TTTGTTCTTGCCGCCTTACTG
	CG6283 R1	CATCCTGCATGTCCATCCAC
CG6543	CG6543 F1	TTGCTAAGATCTTCGCTAGCC
	CG6543 R1	CCACTTCGGTCTTGATGTACTC
CG7720	CG7720 F1	GCCAACTGTTCATGTCCCTC
	CG7720 R1	GTCAGCATAACTCGACGCAC
CG6805	CG6805 F1	CGACCAGTTGAATCTGCTCC
	CG6805 R1	AAGTTGTAGTCATTAGTGCCTTCC
Tpi	tpi F1	CCACTTCTGCATCAGGACAC
	tpi R1	CTTGGGTTTGTCATTTATGGTGGA
plc21c	plc21c F1	GCTTCTTCCTCTACTGGGTC
	plc21c R1	CTTGTTGTCCTTTGGTCGCT
slc5a11	slc5a11 F1	GTTCTCGGCTCTTCAAGTACG

	slc5a11 R1	AAAGGCAGAAACAGAATCTCATCC
Stim	stim F1	TGAACAACAATGGTCTGCCC
	stim R1	GGTAATACGTGCTCTAGAACCC
Ipp	ipp F1	TCGATCCAATTGACGCTACC
	ipp R1	GTAGACGCCAATTAGCACGG
rpl32 (genomic DNA)	rpl32 gen. F1	AGGCCCAAGATCGTGAAGAA
	rpl32 gen. R1	TGTGCACCAGGAACTTCTTGAA
mtDNA	mitoDNA F1	ATTTCGTCCAACCATTCATTCC
	mitoDNA R1	ATATAAAGTCTAACCTGCCCACTG

Table 2-3: Primers used in this study.

2.5 Lipid quantification

5 female flies were collected in biological triplicates for each genotype. Free fatty acids (FFA) per sample were quantified using the Free Fatty Acid Kit (Abcam) according to manufacturer's instructions. We also assessed total FFA, after lipase (Abcam) treatment of the samples, which we refer to as total lipids or triacylglycerides (TAG) in this study.

2.6 Glucose quantification

Total body glucose levels of 5 female flies or hemolymph glucose levels (see 2.3.1) were quantified in biological triplicates using the Glucose Colorimetric Assay Kit (Cayman Chemical) according to manufacturer's instructions.

2.7 Glycogen quantification

5 female flies were collected in triplicates, lysed in 50 µl PBS + 0.2% TritonX100 and centrifuged to removed debris at maximum speed and 4 °C for 10 min. 20 µl of the supernatant was used, 20 µl of 0.5 M Na_2CO_3 added and boiled for 4 h at 95°C. 24 µl of 1 M acetic acid and 96 µl of 0.2 M sodium acetate were added. 80 µl each for control and experimental measurements were used and experimental solution was incubated with 1 unit/ml amyloglucosidase (Sigma) and samples were

rotated or shaken at 57 °C. Glycogen amount was measured using the Glucose Colorimetric Assay Kit (Cayman Chemical) according to manufacturer's instructions.

2.8 O_2-consumption assay

We measured O_2-consumption in extracted mitochondria from flies of desired genotypes. To extract mitochondria, we used the differential centrifugation technique and the Mitochondria Isolation Kit for Tissues (Abcam) according to manufacturer's instructions. Mitochondria from 10 females were re-suspended in 110 µl of the kit-supplied buffer, containing succinate. Each sample was measured in duplicates, as only 50 µl were used to measure O_2-consumption using a Clark-type oxygen-sensitive electrode (Hansatech) with the help of Dr. Björn Kruspig. After the measurement, mitochondrial solution was transferred into a new tube and protein levels within were measured to normalise O_2-consumption.

2.9 Locomotor assay

A single female fly was transferred into a food-containing 6 cm tissue culture dish (Falcon) and movement of 2 flies in 2 separate dishes was recorded with a Samsung S4 phone for 500 sec. Distance between the 6 cm dish and camera was kept constant. Obtained videos were converted into an Image sequence (2 frames per second) using QuickTime Pro and locomotor activity was measured using the manual tracking plugin in Fiji.

2.10 Egg laying assay

One female was housed together with one wild type male in vials containing normal food, or normal food containing 10% ethanol or ethanol plus methopren (JHA, 0.02 mg/ml ethanol), flies were transferred every day into a new vial and eggs were counted.

2.11 Feeding assays

2.11.1 Glucose absorption assay

2-NBDG is a non-metabolisable, fluorescent-labelled deoxyglucose analogue. 2-NBDG was diluted in a 5 % sucrose solution, also containing Allura red to monitor feeding, and applied to Whatman paper circles to feed flies overnight. The next day, flies were transferred onto vials containing normal fly food and aged for another 1.5-2 days. Flies still displaying a red belly were discarded. Biological triplicates or quadruplicates of 5 female flies fed on 2-NBDG diet and one replicate of control diet fed females were collected. Flies were lysed in 60 µl PBST. Lysates were centrifuged and supernatant collected into a new Eppendorf tube. Fluorescent intensity was measured using the TECAN Safire^2 plate reader. Fluorescent intensity of control diet fed animals (auto-fluorescence) was subtracted from measurements obtained from 2-NBDG fed animals.

2.11.2 Food intake assay

To measure food intake per fly, 25 flies per replicate were fed with 5% sucrose solution with Allura red for 2 h at desired temperature. Control flies were fed with 5% sucrose only. Flies were frozen at -80 °C till processing. Flies were lysed in PBST, centrifuged to remove debris and supernatant loaded into a 96-well plate. Absorbance of Allura red was measured using the TECAN Sunrise plate reader and absorbance from control samples subtracted from the experimental ones.

2.11.3 Collecting excrements of flies

We have developed a method, which enables us to collect the excrement from flies of desired genotypes to perform downstream analysis. For this we de-capped 1.5 ml Eppendorf tubes and filled the lids with standard fly food containing a blue dye (Brilliant Blue FCF) to allow us to normalise lipid and glucose content by excrement volume. The lids containing the blue food had to be pierced with a needle big enough to allow oxygen to enter the tube, otherwise the flies died. Flies were reared the day before the excrement was collected in dye-containing food. Then, 5 female flies were put into an Eppendorf tube, which was closed with a blue food-containing lid, and kept at 29 °C overnight. The next day, flies and food-containing lid were removed. 100 µl of PBST was added and tube closed with

a fresh lid, vortexed and absorbance of Brilliant Blue FCF was measured as a read-out for amount of excrement using the TECAN Sunrise plate reader. Furthermore, lipids and glucose were measured as described above.

2.12 Statistics

To statistically analyse proliferation, mRNA and protein levels, and metabolic measurements we used Graph Pad Prism 5 for results shown in Chapter 3, or 7 software and applied an unpaired t-test to compare two groups, or One-way ANOVA followed by Turkey's multiple comparisons test for 3 or more groups. Survival curves were analysed using curve comparison and Log-Rank (Mantel-Cox) test. Error bars mean ± standard error of the mean [SEM].

3 Bursicon-ß is not required in the adult *Drosophila* midgut

3.1 Short Summary

The active Bursicon molting hormone requires a heterodimeric complex of Bursicon-α and Bursicon-ß subunits to mediate post-eclosion events. Previous work from our laboratory reported for the first time a role for Bursicon-α in the adult fly, independent to its role during development (Scopelliti et al., 2014). Bursicon-α alone was able to induce ISC quiescence (Scopelliti et al., 2014), indicating a Bursicon-ß independent function.

We therefore further investigated whether Bursicon-ß is expressed and has a role in regulating adult ISC quiescence. Our results clearly demonstrate, that Bursicon-α alone is able to maintain adult midgut homeostasis and that Bursicon-ß is dispensable during adulthood (Scopelliti et al., 2016).

3.2 Introduction

3.2.1 The role of Bursicon during development

All arthropods undergo multiple molting cycles to shed their exoskeleton in a process known as ecdysis, which allows growing of the animal. Just after ecdysis, a tightly regulated neurohormonal cascade induces the systemic release of the hormone Bursicon to mediate hardening and melanisation of the adult cuticle (Fraenkel, 1965; Kostron, 1995; Luo et al., 2005; Mendive et al., 2005) and, in the case of winged insects, to expand their wings (Arakane et al., 2008; Bai and Palli, 2010; Dewey et al., 2004). Consequently, impaired Bursicon signalling, experimentally obtained in *Drosophila melanogaster* mutants for *bursicon* or its receptor *lgr2* — encoded by the *rickets* (*rk*) locus (Truman, 2005) — results in poor cuticle hardening and impaired wing expansion. The active Bursicon tanning hormone consists of a heterodimer of two related cysteine knot proteins, Bursicon-α (Burs-α) and Bursicon-ß (Burs-ß) (Luo et al., 2005).

3.2.2 The role of Bursicon in the adult *Drosophila* midgut

Work from our laboratory previously reported, that Bursicon-α is a regulator of ISC homeostasis, representing the first described role of Bursicon signalling beyond development (Scopelliti et al., 2014). Furthermore, we showed, that Bursicon-α is mainly expressed in the posterior midgut in a subset of enteroendocrine (ee) cells, which is in line with 2 transcriptomic databases of whole and cell type specific midgut expression (Buchon et al., 2013; Chintapalli et al., 2007; Dutta et al., 2015). In short, our previous work demonstrated that ee-specific Burs-α signals via its receptor LGR2 in the visceral muscle to regulate cAMP, which limits production of the EGF ligand Vein, leading to ISC quiescence (Scopelliti et al., 2014).

3.3 Aim of the project

It is well characterised, that the ecdysal tanning hormone is a heterodimeric complex of Bursicon-α and –ß. But, unlike Burs-α, transcription of its heterodimeric binding partner Burs-ß was not detectable in neither whole midgut nor cell-specific databases available online (Buchon et al., 2013; Chintapalli et al., 2007; Dutta et al., 2015). Therefore, we hypothesised that Bursicon-α alone is biologically active and able to activate a LGR2 mediated response and that Bursicon-ß is dispensable for the maintenance of intestinal stem cell homeostasis.

3.4 Burs-β is not required for adult midgut homeostasis and Burs-α alone is able to induce ISC quiescence

Bursicon-ß is highly expressed during metamorphosis. We therefore collected dark pupae as a positive control for quantitative RT-PCR and confirmed high *burs-ß* expression (Figure 3-1). In contrast, expression levels of *burs-ß* were very low to undetectable in adult heads (Figure 3-1). This is in line with published data showing, that Burs^{+ive} neurons undergo apoptosis shortly after adult eclosion (Honegger et al., 2011; Peabody et al., 2008). In our recent study we found that Bursicon-α within the midgut is regulated in an age-dependent manner (Scopelliti et al., 2014). However, contrary to our findings on *burs-α* (Scopelliti et al., 2014), we did not detect significant *burs-ß* mRNA in either 3 or 14 day-old whole adult *Drosophila* midguts (Figure 3-1). Our results were consistent with published

midgut transcriptomic databases and suggested a Burs-ß independent activity of Burs-α within the adult midgut.

This differential expression of the two subunits is in sharp contrast with the classical notion that only the heterodimeric Bursicon is able to activate its receptor LGR2 and elicit a biological activity.

Next, we wanted to examine if Burs-ß plays any role in controlling adult midgut homeostasis. We stained midguts with an antibody against phosphorylated Histone 3 (pH3) to quantify ISC proliferation. Consistent with our previous report, we found that two independent Bursicon-α loss of function mutants (*burs*^{z5569}, *burs*^{1091}) showed ISC hyperproliferation and epithelial multilayering (Scopelliti et al., 2014) (Figure 3-2). On the contrary, Burs-ß loss of function, achieved by the trans-heterozygotic combination of two deletion alleles *Df(2)110* (Lahr et al., 2012) and *Excel6035* (Bloomington 7518) spanning the *burs-ß* locus, or by combination of the point mutations *pupal6* (loss of function allele of *burs-ß*, DGRC 101309) with the deletion *DF(2)110*, displayed no defects in midgut homeostasis and were indistinguishable from wild type tissues (Figure 3-2). This was remarkable, as *burs-ß* mutant flies displayed the same developmental phenotypes as *burs-α* or *rk* mutants. Those results further supported the notion that Burs-ß is dispensable for Burs-α/ LGR2-dependent adult midgut homeostasis, which is uncoupled from the well-characterised developmental function of the signalling pathway.

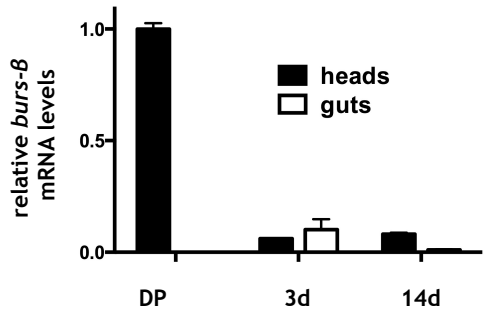

Figure 3-1: No *burs-ß* expression in the adult *Drosophila* head and gut.

RT-qPCR analysis for *burs-ß* relative to *rpl32* from dark pupae, 3 and 14 day-old adult heads and guts of *w*^{1118} control flies. High *burs-ß* expression is detectable in DP (dark pupae), while *burs-ß* levels are low to undetectable in adult heads and guts at all stages tested.

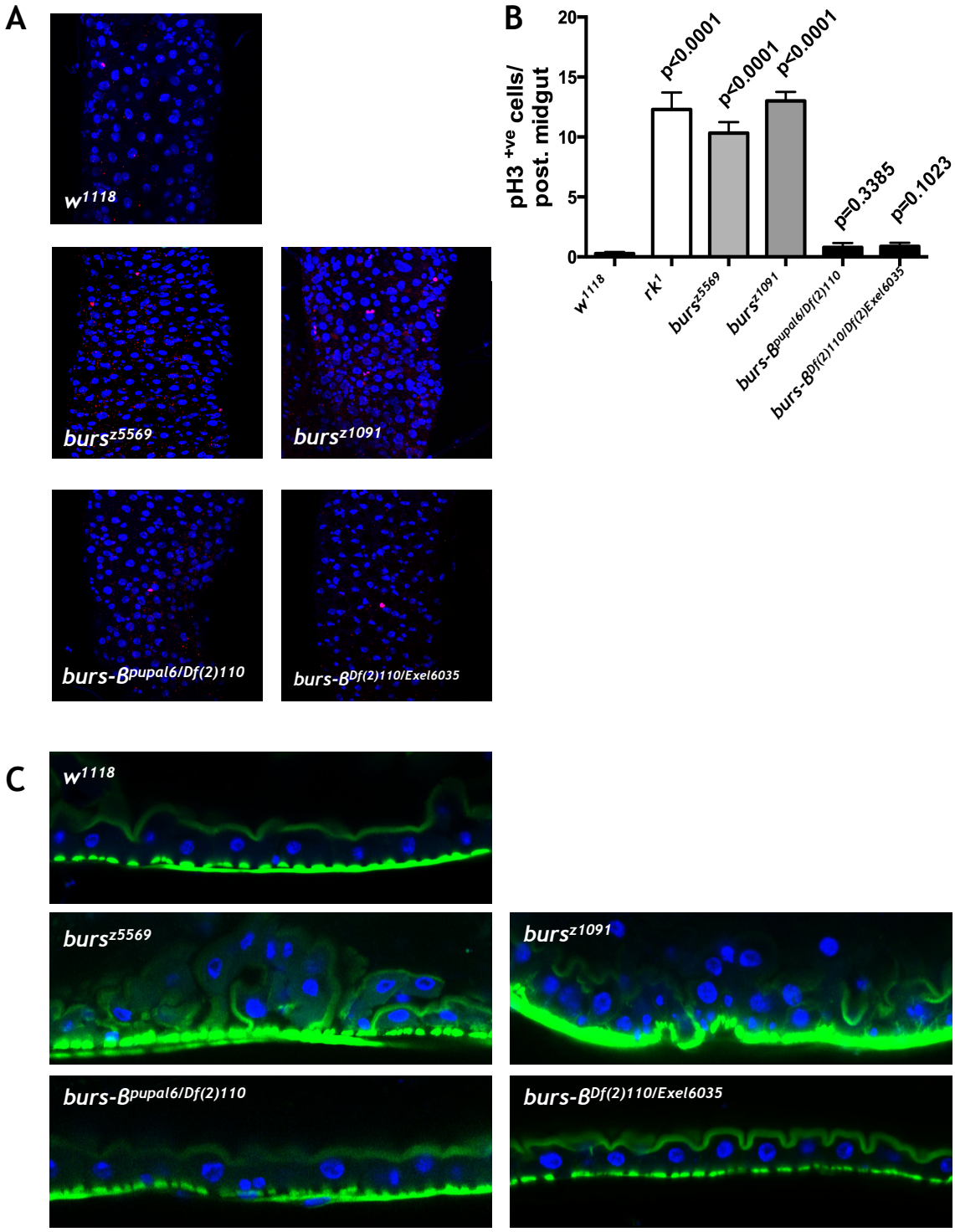

Figure 3-2: Burs-β is dispensable for adult midgut homeostasis.

(A) Representative confocal maximum projection images of adult midguts of the indicated genotypes stained for pH3 (red), DAPI (blue) and quantified in (B). Please note, that *burs-α* mutant midguts displayed a hyperproliferative phenotype ($burs^{z5569}$, $burs^{z1091}$), while *burs-β* mutants have wild type like (w^{1118}) midguts ($burs\text{-}\beta^{pupal6/Df(2)110}$, $burs\text{-}\beta^{Df(2)110/Exel6035}$) (B) Quantification of $pH3^{+ve}$ cells per posterior midgut as a read-out for ISC proliferation of indicated genotypes. P-values to the control are indicated above the genotypes (n > 10) and were calculated using the standard error of the mean [SEM]. w^{1118} control and *burs-β* mutant midguts displayed a low mitotic index, while *rk* and *burs-α* mutant midguts are hyperproliferative. (C) Transversal confocal sections of the midgut of indicated genotypes. *burs-α* mutant midguts showed multilayering of the epithelium, while w^{1118} and *burs-β* mutant guts displayed a wild type like phenotype. Phalloidin (green), DAPI (blue).

So far, our data suggest that Burs-ß doesn't play a role in Burs signalling in the adult midgut, but we can't exclude that Burs-α might heterodimerise with an unknown partner to be biologically active and activate its receptor.

We have previously shown, that ee-specific overexpression of *burs-a* is sufficient to suppress of ISC proliferation in the adult *Drosophila* midgut (Scopelliti et al., 2014). To understand if Burs-α needs an endogenous dimerization partner, we mis-expressed *burs-a* in enterocytes (using the *MyoIA-gal4*^{ts} driver) and visceral muscle (using the *how-gal4*^{ts} driver), 2 cytotypes where we don't see any Burs-α immunoreactivity (Scopelliti et al., 2014), to test if Burs-α needs an endogenous dimerisation partner to accomplish its role in the midgut. We examined proliferation after DSS damage, which displayed high pH3 counts compared to sucrose controls (Figure 3-3 A, B). Overexpressing *burs-a* in each of the exogenous domains resulted in clear reduction of proliferation upon damage when compared to DSS treated control guts (Figure 3-3 A, B).

Ageing animals display intestinal hyperplasia characterised by increased ISC proliferation and mis-differentiation (Ayyaz and Jasper, 2013; He and Jasper, 2014). We therefore used this paradigm to assess the outcome of gain of function Bursicon signalling by mis-expressing *burs-a* in the VM. We checked 30 day-old midguts and found *burs-a* overexpression significantly reduced ISC proliferation when compared to control aging midguts (Figure 3-3 C). Altogether, our results suggest that Burs-α is solely responsible for the maintenance of ISC quiescence in the midgut.

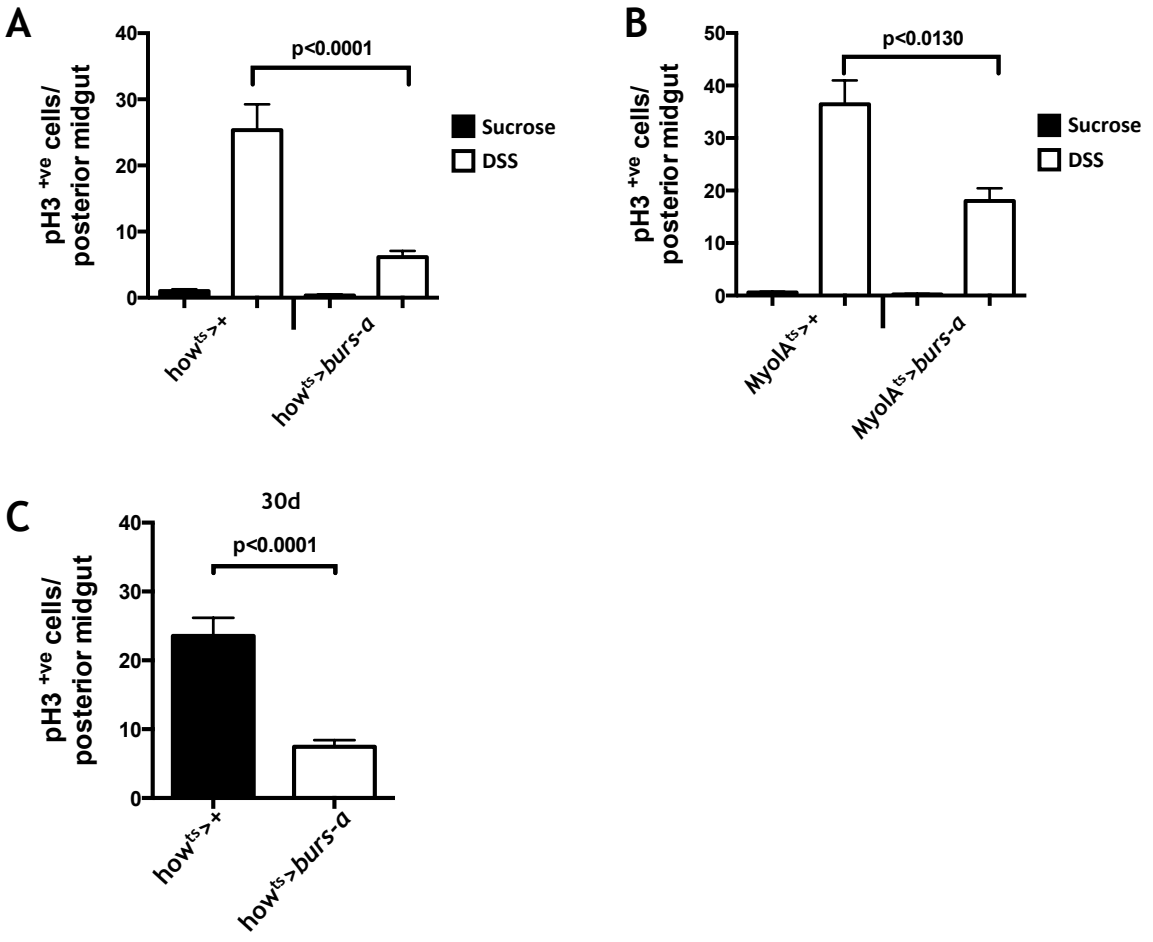

Figure 3-3: Mis-expression of *burs-a* reduced damage- and age-dependent hyperproliferation.

(A and B) Quantification of ISC proliferation, evaluated by pH3 counts in the posterior midgut of flies overexpressing *burs-a* within (A) the visceral muscle (how^{ts}>) and (B) the enterocytes (Myo1A^{ts}>) treated with sucrose (black bars) or DSS (white bars) compared to their controls. (C) pH3^{+ive} cells were counted in the posterior midgut to quantify ISC proliferation in 30d old control flies (black bar) and flies overexpressing *burs-a* (white bar) within the VM. Data is presented as average values of at least 10 guts using SEM, p-values are indicated for each graph.

3.5 Bursicon-α is sufficient to activate cAMP production in an LGR2 dependent manner in the adult *Drosophila* midgut

We next tested whether Burs-α alone could activate its receptor LGR2 and trigger cAMP production within the VM, where the receptor LGR2 is expressed (Scopelliti et al., 2014). It is known, that just after eclosion Burs-α and –ß heterodimer concentration is highest in the open circulation of the fly, known as hemolymph. In our previous study we have used hemolymph from newly born control animals to show that it is able to sustain cAMP levels in the VM in an LGR2-dependent manner (Scopelliti et al., 2014). To meticulously exclude Burs-ß involvement in this process, we made use of a recombinant His-tagged Burs-α protein, which was produced in yeast and purified using the His-tag. We confirmed that the solution contains Burs-α by immunoblotting assay using a Burs-α specific antibody (Figure 3-4). We tested if purified Burs-α could homodimerise by performing Western Blotting under reducing and non-reducing conditions. Under reducing conditions, we only detected one band at the expected size for the tagged Burs-α homodimer (~25 kDa) (Figure 3-4). Importantly, under non-reducing conditions, where the protein is able to sustain its cysteine bridges, we were able to detect a fraction of the recombinant protein at ~50 kDa, consistent with the size of a homodimeric complex and, which is in line with previous reports (Honegger et al., 2011).

We then performed FLIM-FRET experiments, but instead of using hemolymph of newborn control flies as in our previous work (Scopelliti et al., 2014), we used His-tagged Burs-α recombinant protein solution. For this, we expressed the YFP/CFP FRET biosensor UAS-Epac1-camps (Ponsioen et al., 2004) specifically in the visceral muscle, where LGR2 is expressed to monitor cAMP levels. Epac1 is activated by cAMP and the YFP/CFP FRET sensor enables us to monitor cAMP levels specifically in the VM due to changes in fluorescence. We found that cAMP levels in the VM are significantly increased when treated with Burs-α recombinant protein whereas no, or significantly reduced signal was obtained upon buffer-only incubation or when *lgr2* was specifically knocked down in the VM (Figure 3-5).

This recently published work (Scopelliti et al., 2016) clearly demonstrated that Burs-ß has no effect on adult midgut homeostasis and that Burs-α, most likely in its homodimeric confirmation, is able to induce VM cAMP production in a LGR2

dependent manner. This represents the first evidence of a role of Burs-α, which does not involve its classical dimerisation partner.

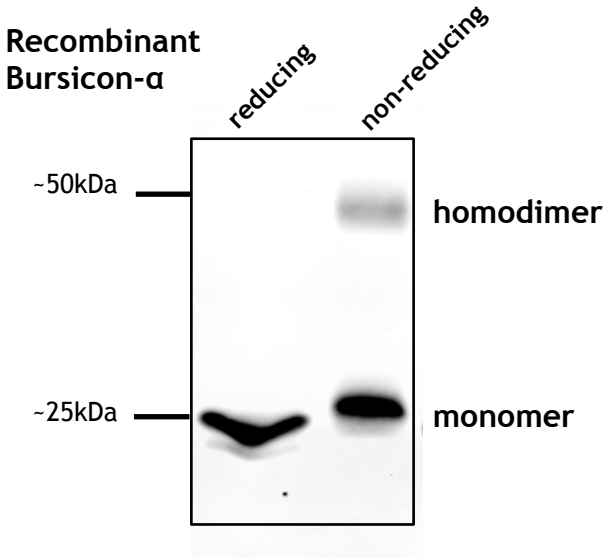

Figure 3-4: Recombinant Burs-α can build homodimers *in vitro*.

Western Blotting analysis under reducing (left lane) and non-reducing (right lane) conditions of recombinant Burs-α protein detected with an anti-Burs-α antibody. Note, that non-reducing conditions showed a band at around 50 kDa, detecting Burs-α homodimers.

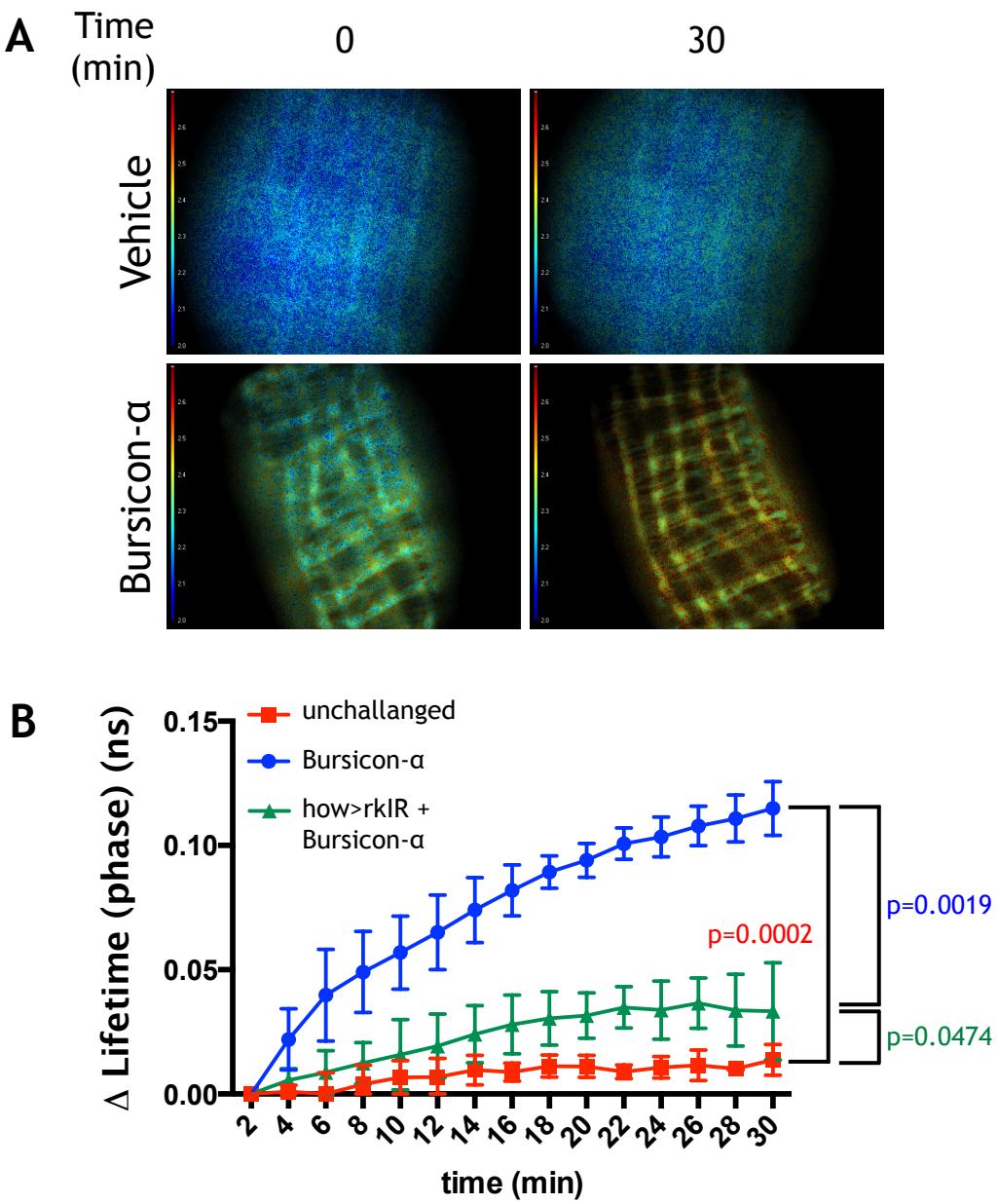

Figure 3-5: Recombinant Burs-α can activate cAMP production in an LGR2 dependent manner in the adult *Drosophila* midgut.

(A) Activation of the Epac1-biosensor in the VM was measured by time lapse FLIM-FRET. Burs-α administration resulted in cAMP production, which is reported by a colour shift from blue to red. 0 and 30 min after administration are shown. (B) Quantification of (A). Experiments were done in biological triplicates and p-values from 2-way ANOVA with Bonferroni correction are displayed. Note, that upon knockdown of *rk* within the VM, Burs-α is no longer able to increase cAMP production.

4 Metabolic importance of Bursicon/ LGR2 signalling

4.1 Short Summary

Our laboratory found that Bursicon is expressed in enteroendocrine (ee) cells in the adult midgut and functions as a suppressor of ISC proliferation (Scopelliti et al., 2014). We were intrigued by these findings and wanted to further explore this endocrine signalling in adult flies. The endocrine system regulates many physiological functions like growth, metabolism, development and reproduction, amongst other things.

Our data demonstrate a novel role for Burs/ LGR2 signalling, independent of LGR2 in the visceral muscle, in regulating whole organismal metabolism. We found that systemic secretion of Bursicon is nutrient dependent and loss of Burs/ LGR2 signalling resulted in excessive loss of stored energy depots, especially lipids, which is unrelated to animal feeding or physical activity.

4.2 Introduction

Hormones are critical regulators of all physiological functions in all Metazoans. Despite long lasting and intensive research done in the field of endocrinology within the last century, the description of new hormones regulating unexpected physiological processes is still ongoing (Lee et al., 2015; Romere et al., 2016). The intestine is a key endocrine tissue, which produces multiple hormones in response to nutritional status or signalling pathways and orchestrates systemic metabolic regulation across tissues.

Since its discovery in insects in the 1960th Bursicon has been thought to be exclusively involved in developmental processes such as wing expansion and cuticle tanning and hardening, which are critical in insect physiology (Fraenkel et al., 1966; Mills, 1967). Recently, we demonstrated a role for *Drosophila* Bursicon/ LGR2 signalling during adulthood, which was not linked to its effects on animal development: adult intestinal ee cells express Bursicon-α (from now on referred to as Bursicon or Burs) mRNA and protein to regulate paracrinally the intestinal stem cell niche via its receptor LGR2 (encoded by the *rickets* (*rk*) locus; LGR2 = protein; *rk* = gene) in *Drosophila* expressed by the visceral muscle (VM) (Scopelliti

et al., 2014). Further investigations by our group also highlighted a novel role for Bursicon/ LGR2 in mediating the local responses of the intestine to organismal nutritional status, as well as systemic metabolic homeostasis in adult flies.

4.3 Bursicon/ LGR2 signalling mediates local intestinal responses to nutrients

Scopelliti et al. showed that *bursicon* and *rickets* mutants displayed a hyperproliferative phenotype within the intestinal stem cells (ISCs) leading to multilayering of the digestive epithelium under normal feeding conditions (Scopelliti et al., 2014). Interestingly, levels of *burs* mRNA inversely correlated with the proliferative status of ISCs in the midgut of unchallenged animals over time (Scopelliti et al., 2014). From this work it was concluded that Burs/ LGR2 signalling was acting as a permissive signal required for the maintenance of ISC quiescence.

We next asked whether there were conditions driving active regulation of Burs/ LGR2 signalling to fit various physiological and metabolic demands leading to ISC proliferation versus quiescence in the intestine.

It has been demonstrated that the adult *Drosophila* midgut undergoes significant re-sizing, including growth, increased ISC proliferation (O'Brien et al., 2011) and low *burs* expression (Scopelliti et al., 2014) in the first five days following animal eclosion. This growing phase of the intestine is greatly dependent on nutrient availability. Midguts from animals subjected to nutrient deprivation are smaller in size and enter ISC quiescence, a process reversible by the re-supplementation of nutrients (O'Brien et al., 2011).

We hypothesised that nutrients would be a key signal dictating Burs/ LGR2 activity and that in turn Burs/ LGR2 signalling would mediate local gut intrinsic and systemic responses to nutrients.

First we asked, whether disruption of Burs/ LGR2 signalling has an effect on midgut ISC quiescence upon starvation in animals undergoing intestinal growth in their first days of adult life. To examine this hypothesis, we carried out immunostainings on *burs* and *rk* mutant midguts using an anti-pH3 antibody, which

is the gold standard for assessing ISC proliferation in the adult fly midgut. Interestingly, we found that, contrary to wild type animals adult midguts from *burs* and *rk* mutants failed to induce ISC quiescence upon starvation and rather sustained ISC proliferation during this growing phase in spite of the lack of nutrients (Figure 4-1 A). This data suggested to us that Burs/ LGR2 signalling was essential to sense nutritional status in the midgut and regulate tissue homeostasis accordingly.

Furthermore, we also noticed that *burs* and *rk* mutant animals were hypersensitive to starvation (Figure 4-1 B), suggesting a potential systemic role of this signalling pathway in addition to its local role in midgut homeostasis.

We next used RNA interference and temperature-controlled tissue specific drivers to achieve adult specific knockdown of *burs* in ee cells (Dicer2; *voila*^{ts}> *burs*^{IR}, hereafter referred to as ee^{ts}> *burs*^{IR}) and the receptor *rk* in the visceral muscle (Dicer2; *how*^{ts}> *rk*^{IR}, hereafter referred to as VM^{ts}> *rk*^{IR}) and assessed starvation sensitivity of adult animals following a sustained period of 10 days of transgene activation. Unexpectedly, while ee^{ts}> *burs*^{IR} animals recapitulate the starvation sensitivity of *burs* and *rk* mutant animals (Figure 4-1 C), VM^{ts}> *rk*^{IR} animals displayed normal sensitivity to starvation, when compared to their control counterparts (Figure 4-1 D). Altogether, this data suggested that, while gut intrinsic Burs/ LGR2 signalling is mediating local responses that impact ISC proliferation/ quiescence decisions in the intestine, there is a midgut independent and likely endocrine signalling mediated by ee-derived Burs to respond to systemic changes in organismal nutritional status.

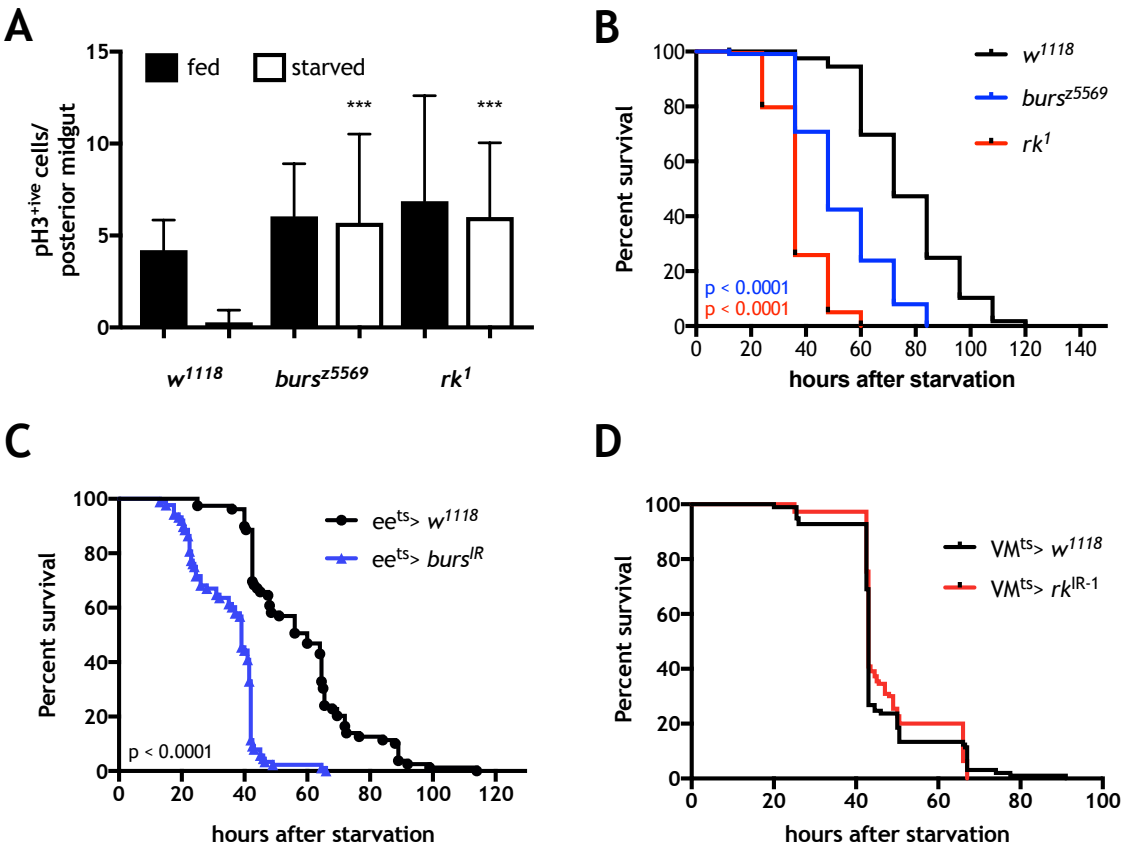

Figure 4-1: Burs/ LGR2 signalling is necessary for an adequate starvation response independent to LGR2 in the VM.

(A) Quantification of ISC proliferation, assessed by pH3 counts in fed and 24h starved, 5d old flies of indicated genotypes. 1-way ANOVA with Turkey's multiple comparisons test was performed to obtain significance score ($n > 10$). P-value is reported compared to starved w^{1118} midguts. Note, *burs* and *rk* mutant midguts fail to undergo stem cell quiescence upon starvation. (B) Flies were aged for 2 days before starvation started ($n > 100$). Dead flies were counted. *burs* and *rk* mutant flies are hypersensitive to starvation compared to controls. (C and D) Starvation sensitivity tests in flies of indicated genotypes. Animals were aged for 10 days to activate the transgene prior to starvation. Dead flies were counted ($n \geq 80$). Note that starvation sensitivity is independent to LGR2 in the VM.

4.4 Enteroendocrine cells sense nutritional status and regulate Bursicon in response to nutrients

Our data suggested, that Burs is an endocrine regulator of metabolism. To better understand the physiological function of Burs in this process, we looked at upstream signalling regulating hormone production. Since we found that disrupting Bursicon signalling resulted in hypersensitivity to starvation (Figure 4-1 B, C), we hypothesised that Bursicon is regulated upon starvation to mediate appropriate responses to nutrient deprivation in an endocrine manner. Therefore, we analysed protein levels of Bursicon by immunostainings in midguts from control animals (*esg*-Gal4) under normal feeding and upon 24h starvation. Under normal feeding conditions Burs immunoreactivity is rather low and restricted to a small subset of ee cells within the adult posterior midgut (Scopelliti et al., 2014). Strikingly, Bursicon is significantly upregulated within ee cells of starved animals (Figure 4-2 A). This data suggested, that Burs is regulated in response to nutrient intake.

Next we wanted to rigorously test how Burs is regulated. More Burs immunoreactivity upon starvation could be due to [1] upregulation of *burs* mRNA, [2] stabilisation or less degradation of Burs protein and/or [3] reduction in Burs secretion. To test these hypotheses, we first analysed *burs* mRNA levels upon different length of starvation and found that transcript levels are very quickly decreased upon starvation (Figure 4-2 B), excluding the possibility that transcriptional regulation of the *burs* gene was responsible for Burs protein increase observed upon starvation. This data also makes it unlikely that the protein is stabilised or less degraded upon starvation while corresponding gene transcription is inhibited. Then we moved on to test the hypothesis of Burs being regulated at the secretion level through retention of the protein. To rigorously assess this hypothesis, we need to be able to quantify Burs protein within the hemolymph of fed and starved animals.

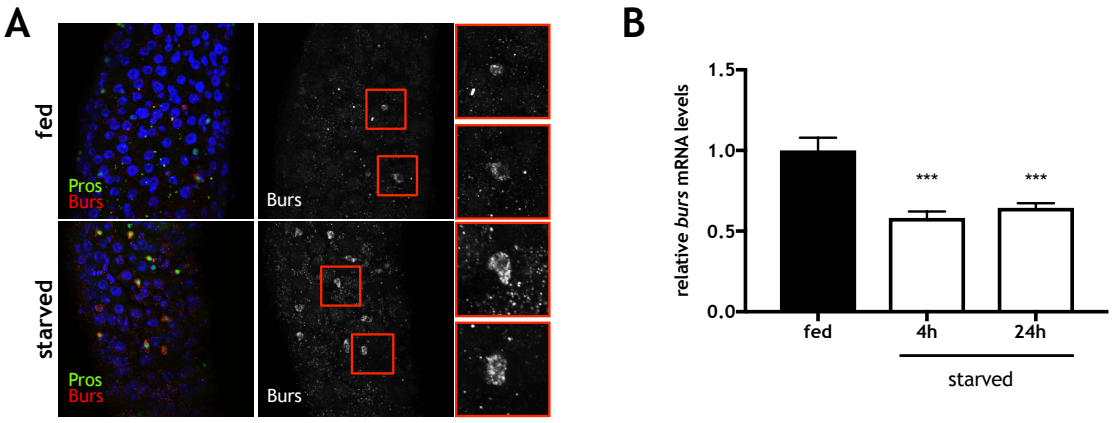

Figure 4-2: Bursicon is retained in ee cells in response to starvation.

(A) Burs immunoreactivity in fed and 24h starved *esg*-Gal4 wild type animals. Burs immunoreactivity in ee cells is increased upon starvation. Prospero (green), Burs (red/white), DAPI (blue). (B) RT-qPCR analysis for *burs* in fully fed, 4h and 24h starved w^{1118} midguts (n = 3). Values are relative to *rpl32* mRNA levels. P-values compared to fed midguts are given.

We have already validated our laboratory-generated Burs specific antibody by its capacity of recognising the recombinant Bursicon-α protein by Western Blotting (Scopelliti et al., 2016). We next validated the antibody in a more complex protein solution. It is known that Bursicon is expressed in neurons throughout development and that it is secreted just after adult eclosion to mediate ecdysis (Mills, 1967; Peabody et al., 2008). For that reason, we knocked down *burs* within neurons throughout development and analysed Burs protein levels in newborn flies. Knocking down *burs* in developing neurons led to a significant reduction in Burs protein compared to newborn control flies (Figure 4-3 A, B). This confirms, that Burs is expressed in neurons throughout development and highly abundant in flies just after eclosion and that the antibody is specifically recognising Burs protein.

Next, we validated if the antibody is able to recognize Burs protein from hemolypmh. For this, we collected hemolymph from flies bearing ee specific knockdown of *burs* and their controls. When knocking down *burs* specifically in ee cells during adulthood, we found a significant reduction of circulating Bursicon protein (Figure 4-3 C, D). These results fully validated the specificity of our antibody, and most importantly, showed that adult circulating Burs is secreted from ee cells.

To test if Burs is retained upon starvation, we analysed hemolymph from fed and 24h starved w^{1118} animals and found a significant reduction of Burs within the hemolymph of animals upon starvation (Figure 4-4). Overall, this data clearly demonstrated that Burs is secreted as an endocrine signal into the hemolymph under normal feeding conditions and retained, when animals are starved.

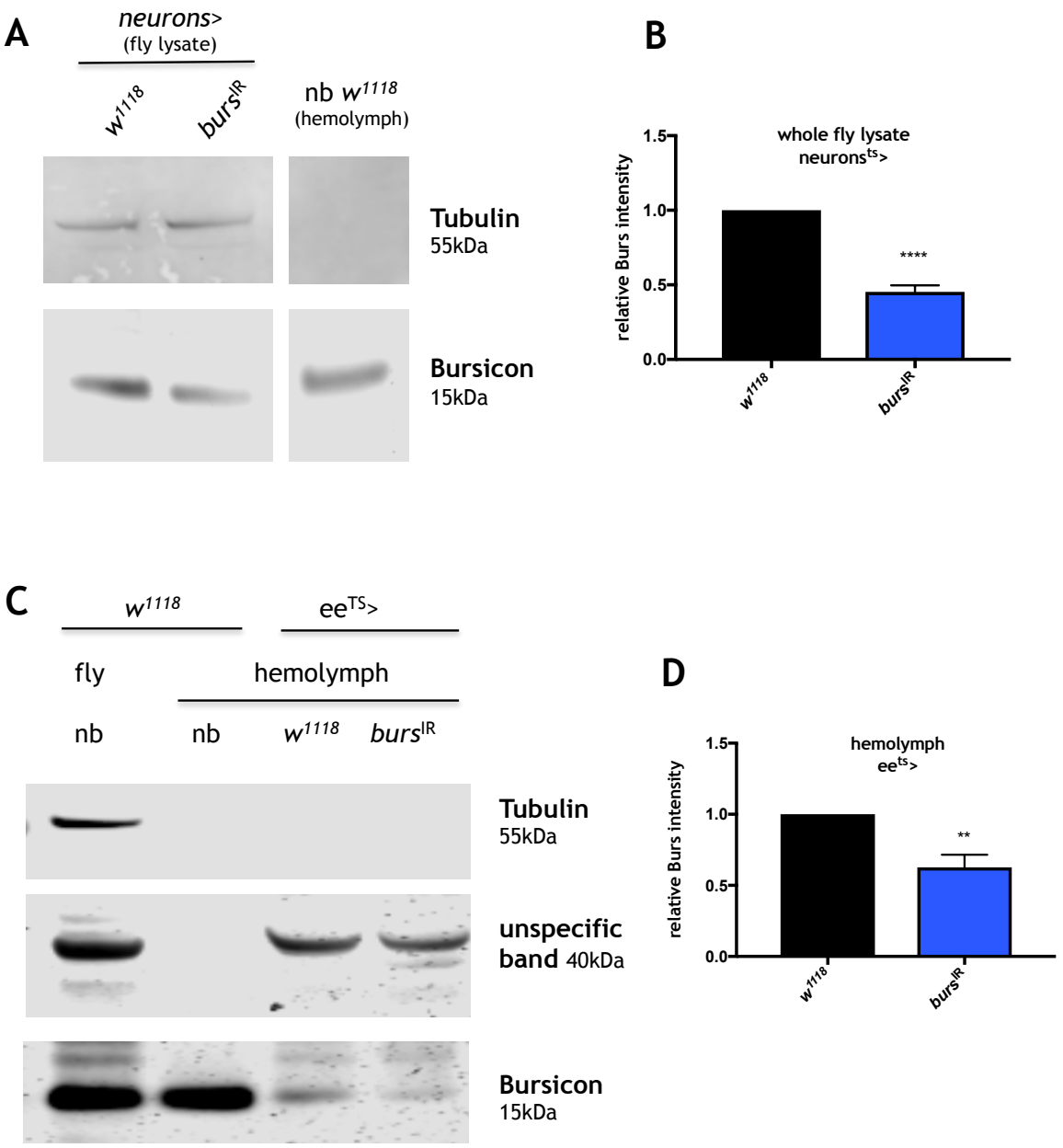

Figure 4-3: Confirmation of Bursicon expression in developing neurons and adult ee cells.

(A) Western Blotting analysis of Burs levels in whole fly lysates of newborn flies in which *burs* was knocked down throughout development within the neurons compared to its control. Newborn w^{1118} hemolymph was used as a control to identify Burs. (B) Quantification of (A) relative to Tubulin intensity. Experiments were performed in biological triplicates and significance was analysed using unpaired t-test. (C) Western Blotting analysis of Burs levels in hemolymph from flies with ee specific *burs* knockdown and their controls. Newborn w^{1118} whole fly lysates and hemlymph were used to control for Burs specificity and Tubulin contamination of extracted hemolymph. nb = newborn. (D) Quantification of (C) relative to unspecific band. Experiments were performed in biological triplicates and unpaired t-test was used to analyse significance.

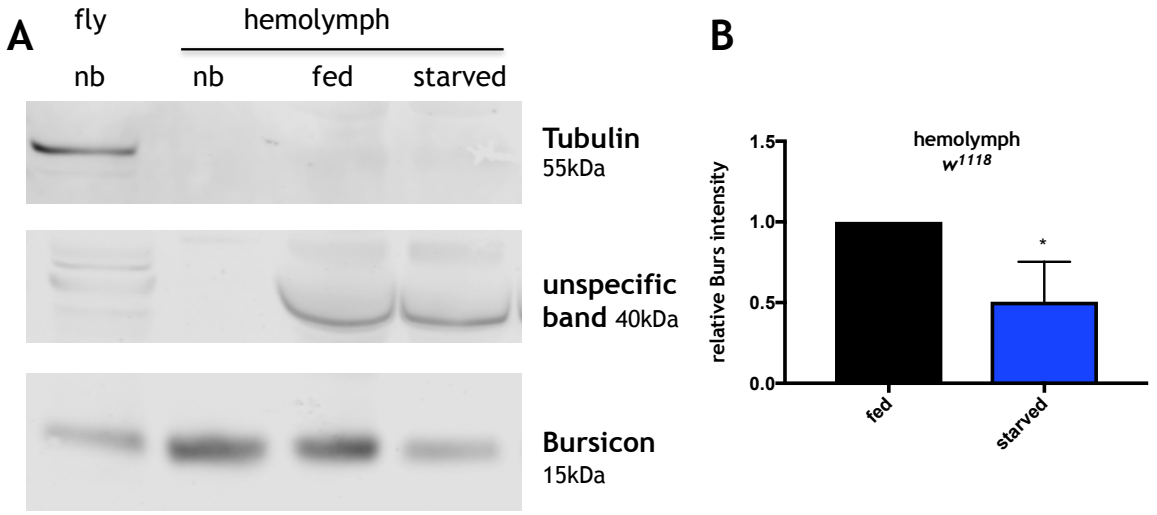

Figure 4-4: Burs is secreted into the hemolymph in response to feeding and retained in ee cells upon starvation.

(A) Circulating Burs levels were analysed by Western Blotting. Newborn whole fly extracts and hemolymph were used to control for Burs specificity and Tubulin contamination in the hemolymph. Upon starvation, less circulating Burs is detected. nb = newborn. (B) Quantification of (A) relative to the unspecific band. Experiments were performed in biological triplicates

Next, we wanted to examine if the observed increase in Burs immunoreactivity upon starvation is due to complete starvation or to the lack of specific components within the food. We noted that Bursicon immunoreactivity under normal feeding conditions is variable among flies and also within different genetic backgrounds (data not shown). Therefore, we next overexpressed burs in ee cells using voila-Gal4; gal80ts> UAS-burs77 (hereafter referred to as eets> burs77) to get more reliable Burs immunostainings to assess protein levels in midguts of animals subjected to different feeding conditions. Staining for Bursicon in adult midguts from normally fed animals overexpressing burs in ee cells showed levels of protein staining that were comparable to the basal ones (Figure 4-5 A), compare with Figure 4-2 A). We attributed this to the likely high secretory rate of the hormone in these conditions. However, and matching our staining of endogenous protein (compare to Figure 4-2 A), Bursicon immunoreactivity in *burs* overexpressing midguts was significantly increased upon 24h starvation (Figure 4-5 A). These results suggested that overexpression of *burs* within ee cells mimics the nutrient-dependent regulatory pattern observed with the endogenous Bursicon. Strikingly, starved animals subjected to re-feeding for just 2h with a 20 % sucrose solution displayed Bursicon levels that were similar to the ones observed in flies kept on standard food. Interestingly, this wasn't the case if we re-fed animals with a 20 % BSA containing solution, where Burs staining pattern was comparable to the one

observed upon 24h starvation (Figure 4-5 A). This led us to hypothesise that ee cells can sense carbohydrate availability and thus modulate organismal energetic homeostasis.

Therefore, we next asked if ee cells were able to sense carbohydrates directly. To test this hypothesis, we knocked down the *glucose transporter 1* (*glut1*) specifically in adult ee cells and stained for endogenous Bursicon. Bursicon levels in ee cells increased dramatically in *glut1* knockdown midguts compared to controls (Figure 4-5 B). Interestingly, we also found, that those flies are hypersensitive to starvation as seen upon ee specific *burs* knockdown flies (Figure 4-5 B). These results indicated that ee cells are able to directly sense carbohydrate availability via the glucose transporter Glut1 to modulate Burs levels.

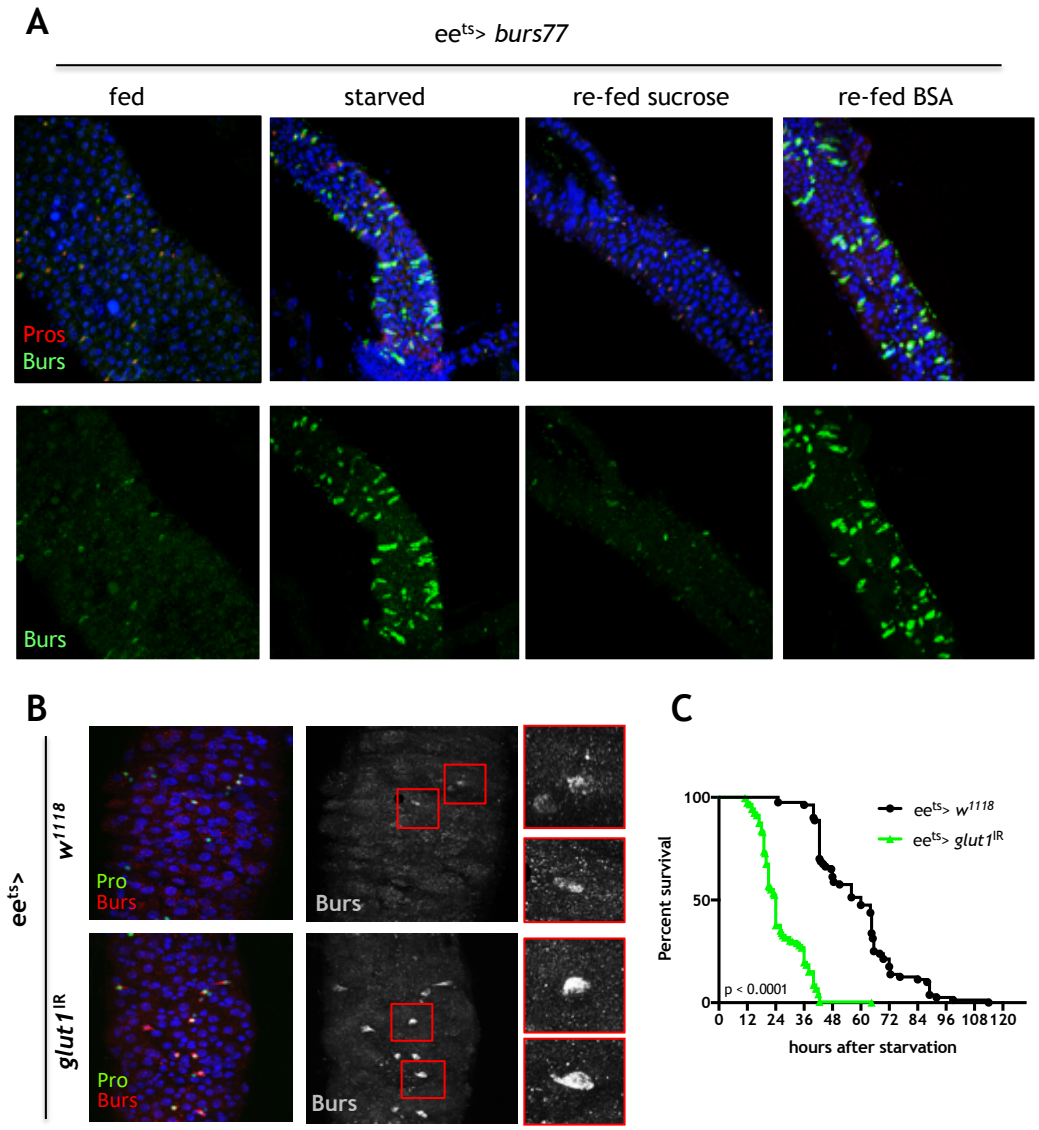

Figure 4-5: Bursicon in ee cells is regulated by carbohydrates.

(A) Representative confocal maximum projection images of adult posterior midguts upon different feeding conditions. After 24h starvation Burs immunoreactivity is high in ee cells (Prospero positive). Note that after 2h of re-feeding with 20 % sucrose Burs levels within ee cells are indistinguishable from the fed state, whereas 20 % BSA still showed high Burs levels within ee cells. Pros (red), Burs (green), DAPI (blue). (B) Representative confocal maximum projection images of adult posterior midguts upon ee specific *glut1* knockdown. *glut1* knockdown resulted in high Burs immunostaining within ee cells, phenocopying the starved state in figure (A). Pros (green), Burs (red/white), DAPI (blue). (C) Starvation survival test of flies with ee specific *glut1* knockdown compared to controls ($n \geq 80$). *glut1* knockdown led to similar starvation sensitivity as seen with ee specific *burs* knockdown.

Next, we asked if this Glut1 dependent regulation of Burs was dependent on cell autonomous Insulin signalling in ee cells. We disrupted Insulin signalling within ee cells using a dominant negative form of Dp110 (*dp110^{DN}*) or Insulin receptor (*inr^{DN}*) and monitored starvation sensitivity. Preliminary results showed that expression of *dp110^{DN}* resulted in slight but significant hypersensitivity to starvation (Figure 4-6). However, expression of *inr^{DN}* didn't (Figure 4-6). Even though *dp110^{DN}* flies displayed slight starvation sensitivity, this is not comparable with the observed hypersensitivity to starvation of flies with an ee specific knockdown of *burs* or *glut1*, suggesting that Insulin signalling within ee cells is unlikely to mediate the effects of Glut1 on Burs regulation.

Altogether, these data showed for the first time, that Burs within the adult midgut is regulated by retention and secretion in response to carbohydrate availability, which is sensed by Glut1 to maintain metabolic homeostasis.

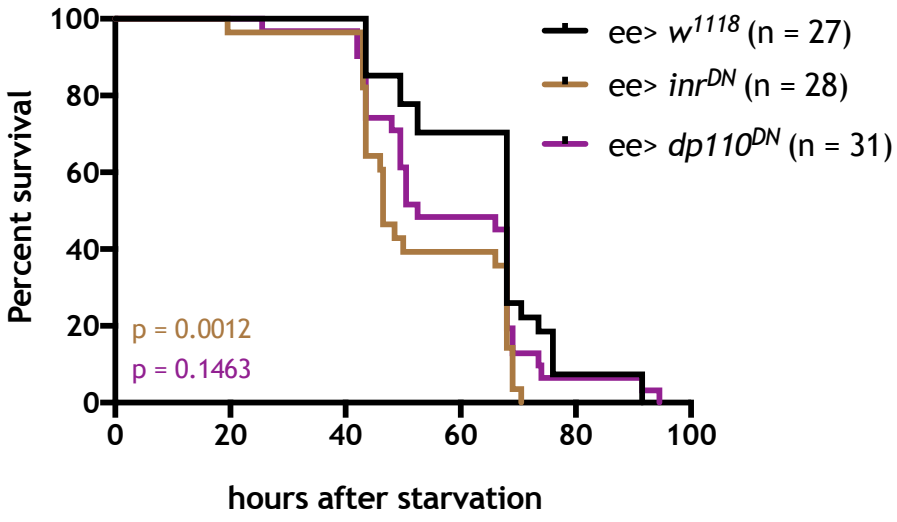

Figure 4-6: Insulin signalling within ee cells has no or little effect on starvation sensitivity.

Flies of indicated genotypes were subjected to starvation tests after 10d of transgene activation. Dead flies were counted. *inr^{DN}* = dominant negative form of Insulin receptor, *dp110^{DN}* = dominant negative form of Dp110, a subunit of PI3K.

4.4.1 Burs secretion is dependent on protease activity

The laboratory has generated flies containing a *burs-gfp* fosmid construct to help us analyse Burs regulation within the midgut and beyond. First, we tested the activity of the fosmid by examining its potential to rescue developmental and midgut phenotypes of *burs* mutant animals. As expected, *burs* mutants showed the typical developmental defects and hyperproliferation of the midgut. Strikingly, both phenotypes were rescued when *burs-gfp* was combined with *burs* mutants (Figure 4-7 A and personal communication by Dr. A. Scopelliti and Dr. J. B. Cordero). Given our previous data demonstrating independency between developmental and gut associated phenotypes derived from impaired Burs/ LGR2 signalling (Scopelliti et al., 2016; Scopelliti et al., 2014), the observed phenotypic rescue of *burs* mutants is likely due to fosmid activity in the CNS of the developing animal and the adult midgut. However, when using a GFP antibody to analyse Burs-GFP protein, we observed detectable fosmid expression in the CNS (Figure 4-7 B), but not in the adult midgut. We hypothesised that, given the GFP-tag in the fosmid construct is located at the C-terminus of Burs, there might be a protease, cleaving the GFP-tagged Burs protein, which leads to the rapid degradation of the GFP protein, making it undetectable. To test this hypothesis, we imported the Burs-GFP fosmid protein sequence into the ProP server (Duckert et al., 2004), which is able to identify candidate cleavage sites targeted by known proteases. This analysis revealed a cleavage site for proprotein convertase 2 (PC2) at the C-terminus of the Burs sequence. Amontillado (Amon), the homolog of the mammalian proprotein convertase 2 (PC2) (Siekhaus and Fuller, 1999) has been already shown to be required for protein processing of secreted proteins like Slit (Ordan and Volk, 2016) and AKH (Rhea et al., 2010). Intriguingly, *amon* is highly expressed in ee cells in *Drosophila* (Dutta et al., 2015) and we hypothesised that Amon could be involved in Burs processing and secretion. We next analysed if Amon was involved in Burs secretion. To test this, we collected hemolymph from flies overexpressing *burs* in fat body cells (FB-Gal4; gal80^{ts}> *burs77*, referred to as FB^{ts}> *burs77*) and from flies overexpressing *burs* and *amon* (FB^{ts}> *burs77* + *amon*) simultaneously and measured Burs protein within the hemolymph by Western Blotting. Preliminary data showed, that overexpression of *burs* and *amon* in combination led to more circulating Burs protein when compared to overexpressing *burs* alone within fat body cells (Figure 4-7 C). Even if not yet

conclusive, this data suggested, that Amon is a potential candidate protease regulating Burs secretion by cleaving its C-terminus and, more generally, that Bursicon needs to be post-translationally processed in order to be secreted and perhaps being biologically active.

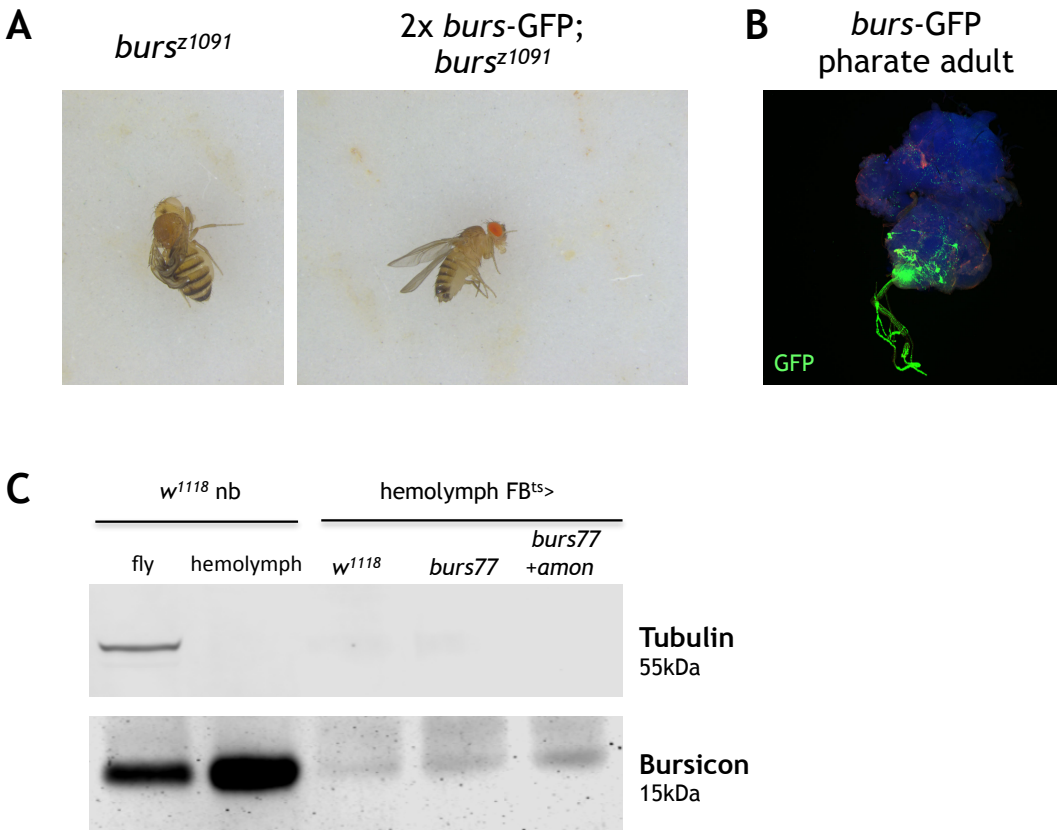

Figure 4-7: Burs-GFP fosmid is functional and Bursicon secretion is potentially regulated by Amontillado.

(A) *burs* mutant flies showed developmental wing inflation defects, which is rescued when 2 copies of the *burs-gfp* fosmid are expressed within those mutants. (B) Representative confocal maximum projection image of Burs-GFP expression in the CNS of pharate animals, showing that the fosmid is expressed. (C) Western Blotting analysis of indicated samples. Hemolymph from flies with *burs* overexpression in the adult fat body (FB^{ts}> *burs77*) showed increased circulating Burs levels, compared to w^{1118} control hemolymph, which is even further increased when *burs* and *amon* are overexpressed simultaneously. Newborn (nb) w^{1118} fly lysate and hemolymph were used to control for hemolymph contamination with Tubulin and for specificity of the Burs antibody. This preliminary experiment was performed once.

4.5 Bursicon/ LGR2 signalling is required to maintain systemic metabolic homeostasis

To maintain systemic metabolic homeostasis, the tissues within an organism need to be able to sense the nutritional status at a cellular and organismal level, and further communicate with other tissues to respond appropriately.

Drosophila tissues work in very similar ways compared to their mammalian counterparts. The major signalling pathways regulating metabolism are functionally conserved. Muscles store energy in form of glycogen, which is important for quick release of energy in times of high-energy demand, such as flying. *Drosophila* ovaries are a storage organ for lipids and proteins, which are used to produce offspring and therefore ensure species survival. After a meal, the intestine absorbs nutrients, where they can be stored short-term in form of TAG containing lipid droplets. The intestine releases ingested nutrients into the hemplymph, where the fat body stores them for later use. The fat body of the fly is the major storage organ for lipids and carbohydrates, mainly in form of TAG containing lipid droplets and glycogen, respectively. It responds to nutritional cues to release energy into the hemolymph in times of starvation for peripheral organs to use. The CNS is a high energy-demanding organ responsible to maintain neurological functions, which are important for life. In times of prolonged starvation energy is mobilised from all tissues but the CNS, because nutrient deprivation within the brain leads very quickly to the death of the organism. Therefore, while the CNS is spared, muscles, fat body and ovaries release their stored reserves to maintain the function of the CNS. Due to loss of energy storage, flies stop laying eggs, become lean and eventually stop moving and die. Interestingly, as an initial response to starvation, flies become more active trying to find new food sources.

Possible reasons for the increased starvation sensitivity observed in *burs* and *rk* mutant animals may be an incapacity of these animals to either absorb nutrients from the diet and/or to generate, store or use energy sources obtained from ingested nutrients.

To test these various possibilities we analysed energy reserve content in 3 day-old *burs* and *rk* mutant animals and 14d old adults with adult ee specific *burs* knockdown, except otherwise stated.

We started by analysing carbohydrate storage, which is mainly stored as glycogen, an energy-rich branched polysaccharide. We measured glycogen levels in *burs* and *rk* mutants and in control flies. Neither mutant showed any differences compared to control w^{1118} flies when they were fully fed (Figure 4-8 A). This suggested, that Burs/ LGR2 signalling does not influence glycogen storage.

Next, we asked if Trehalose levels, the main circulating sugar in insects (Bedford, 1977), were changed. Trehalose is synthesised by combining 2 glucose molecules, which mainly derive from glycogen breakdown. In collaboration with Dr. Saverio Tardito we analysed Trehalose levels from whole fly extracts using Liquid Chromatography Mass Spectrometry (LC-MS). We didn't detect any changes in Trehalose, when comparing whole *burs* and *rk* mutant to control fly lysates (Figure 4-8 B). This suggests that Trehalose metabolism under fed conditions is not influenced by Burs/ LGR2 signalling.

Another important source of energy are lipids. Lipids are mainly stored as triacylglycerides (TAG) in the insect's fat body, which is the homolog of the mammalian adipose tissue and liver. To test if lipid stores are affected in flies with disrupted Burs/ LGR2 signalling, we first carried out a time-course analysis of lipid stores in *burs* and *rk* mutant flies. We found, that during adulthood, *burs* and *rk* mutants lose their lipid reserves in a time dependent manner, while controls stay the same over the evaluated time (Figure 4-8 C). We also analysed TAG levels of newly born animals and found that both mutant and control flies start with the same amount of TAG (Figure 4-8 C), showing the observed metabolic phenotype is independent of development. Likewise, adult *burs* knockdown in ee cells resulted in progressive loss of whole body TAG content (Figure 4-8 D). Consistently, lipidTOX staining of fat bodies from mutants, as well as *burs* knockdown flies showed smaller lipid droplets, the main organelles for fat storage, when compared to their controls (Figure 4-8 F, G). These results demonstrated the developmental independency of this lipid phenotype and that Burs/ LGR2 signalling controls lipid metabolism.

Next, we asked whether supplementing the food with more calories would help to maintain TAG levels in flies disrupted for Burs/ LGR2 signalling. For this, we fed *burs* mutants with standard food plus 1M sucrose (high sugar diet, HSD). Interestingly, this couldn't rescue the loss of TAG stores. *Burs* mutant flies still lost their TAG content in a time dependent manner, while control flies increased their lipid levels, displaying an obesity-like phenotype (Figure 4-8 E). This demonstrated, that the metabolic defects observed in flies with disrupted Burs/ LGR2 signalling can't be compensated by increased caloric intake.

We previously showed, that Glut1 in ee cells regulated Burs levels and therefore most likely modulates secretion and retention of the hormone to manage starvation survival. We next asked if lipid levels are changed in flies with an ee specific knockdown of *glut1* and found that these animals displayed reduced TAG levels and smaller lipid droplets when compared to their control counterparts (Figure 4-9 A, B). We were intrigued by those results and wanted to be certain that observed Burs regulation is dependent on Glut1 specifically. To test this, we also knocked down *sugar transporter 2* (*sut2*) specifically from ee cells and found no difference in TAG levels compared to controls (Figure 4-9 C). This suggested that *sut2* in ee cells does not alter lipid metabolism, but the efficiency of the RNAi line should be tested. Additionally, expressing the dominant negative form of Insulin receptor (inr^{DN}) and Dp110 ($dp110^{DN}$) didn't affect TAG levels compared to control flies (Figure 4-9 D), suggesting that Insulin signalling within ee cells is not responsible for the regulation of lipid metabolism. These results suggested, that glucose sensing by Glut1 is responsible for Burs regulation, independently of local Insulin signalling within ee cells.

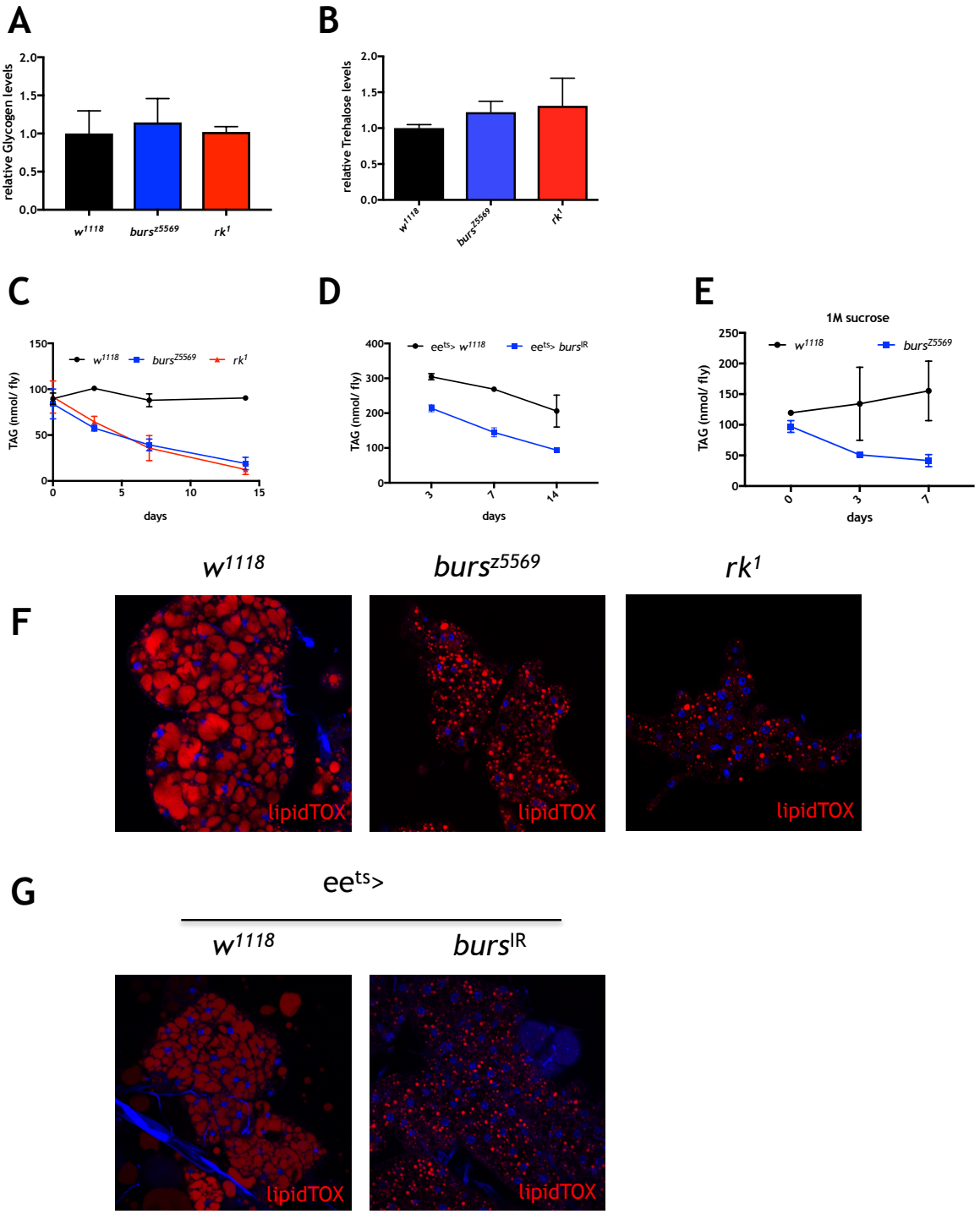

Figure 4-8: Impairment of Burs/ LGR2 signalling resulted in lipid loss.

(A) Whole fly glycogen levels were measured and plotted as relative values to protein content. No significant changes were observed in *burs* and *rk* mutants compared to control flies. (B) Trehalose levels were measured in whole flies using LC-MS analysis. No changes were seen in the genotypes tested. (C and D) Lipid levels of whole flies were measured and reported as relative per fly of indicated genotypes. Note that nb *burs* and *rk* mutants showed the same amount of lipid levels compared to nb controls, showing the independency of development. Both mutants and *burs* targeted knockdown flies showed a time dependent loss of lipids compared to controls. (E) Lipid measurements of *burs* and control flies of indicated ages when fed with 1M sucrose added to standard food. (F and G) Representative confocal images of fat bodies of indicated genotypes stained with lipidTOX. Note that *burs* and *rk* mutants (10d old) and *burs* knockdown flies display smaller lipid droplets, indicating increased lipolysis, compared to controls.

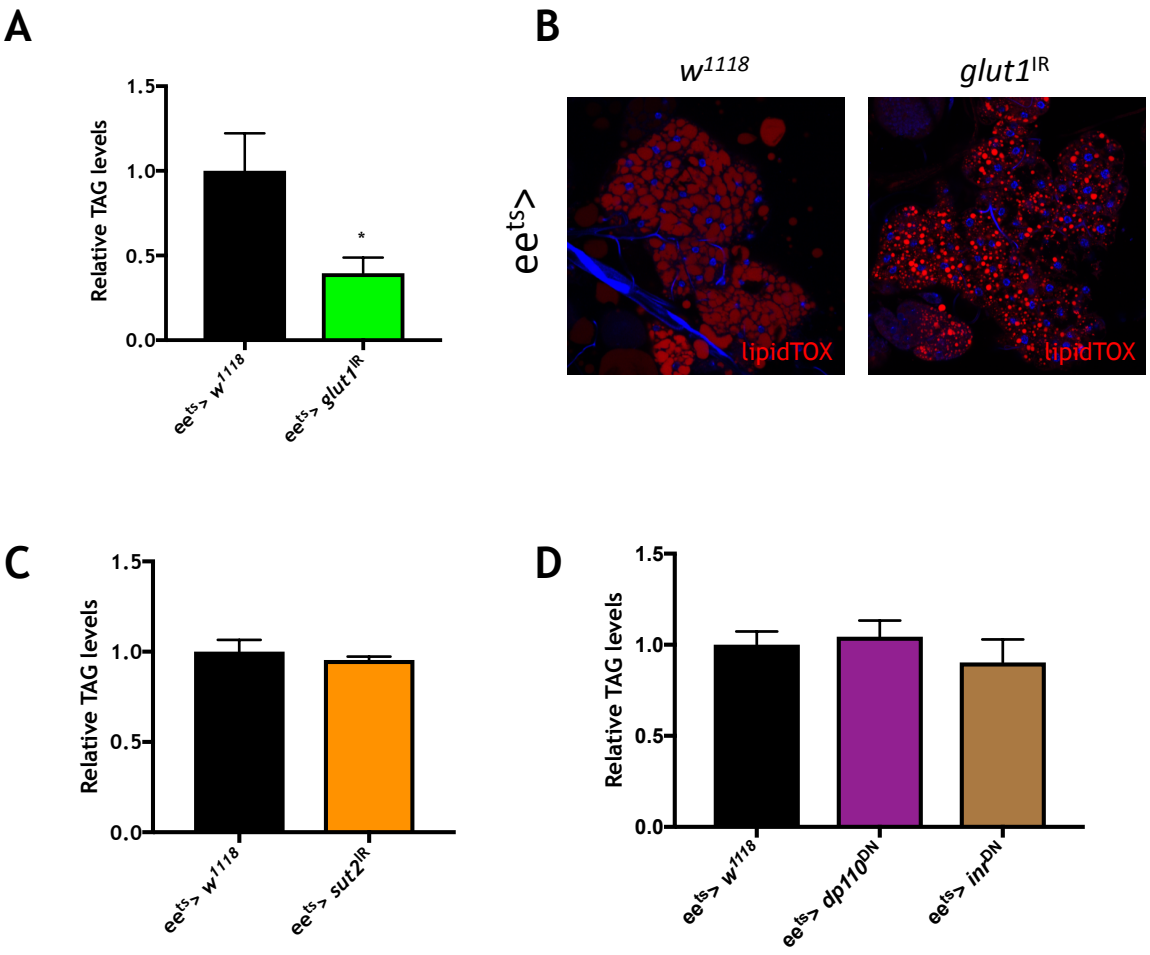

Figure 4-9: Glut1 in ee cells regulates lipid metabolism.

(A) Lipid measurements of adult ee specific *glut1* knockdown resulted in reduced lipids per fly compared to controls. (B) Representative confocal images stained for neutral lipids with lipidTOX in the fat body of flies for indicated genotypes. Note, *glut1* knockdown showed smaller lipid droplets. (C and D) Lipid measurements of flies with indicated genotypes. No significant differences were observed, showing the independency of Sut2 and Insulin signalling within ee cells to regulate lipid metabolism.

Loss of energy reserves could be a consequence of impaired nutrient intake and/or absorption ability of the mutant animals. Therefore, we next analysed food consumption by feeding flies with a red dye (Allura red) for 2h and measured its absorbance from whole fly lysates as a read-out of food intake. We found that *burs* mutants ate constantly and much more compared to controls (Figure 4-10 A). This overfeeding could be confirmed using ee specific *burs* knockdown flies (Figure 4-10 B). These results showed, that the loss of lipids is neither a consequence of decreased feeding activity nor related to developmental defects of the mutants. In fact, flies with a loss of Burs/ LGR2 signalling are hyperphagic, perhaps as a mean to compensate for the loss of energy.

Alternatively, increased feeding behaviour could be the consequence of the inability of the intestine to absorb ingested nutrients properly. Therefore, to check for defects in lipid absorption, we developed a method to measure lipid content excreted by the flies. We collected the excrement of ee specific *burs* knockdown and control flies and measured TAG and FFA within. We observed no differences in the excreted lipids of control and knockdown animals (Figure 4-10 C, D). This data, together with the loss of lipid phenotype, clearly demonstrated that Burs/ LGR2 signalling regulates lipid metabolism, in a fashion that does not involve an effect on nutrient absorption.

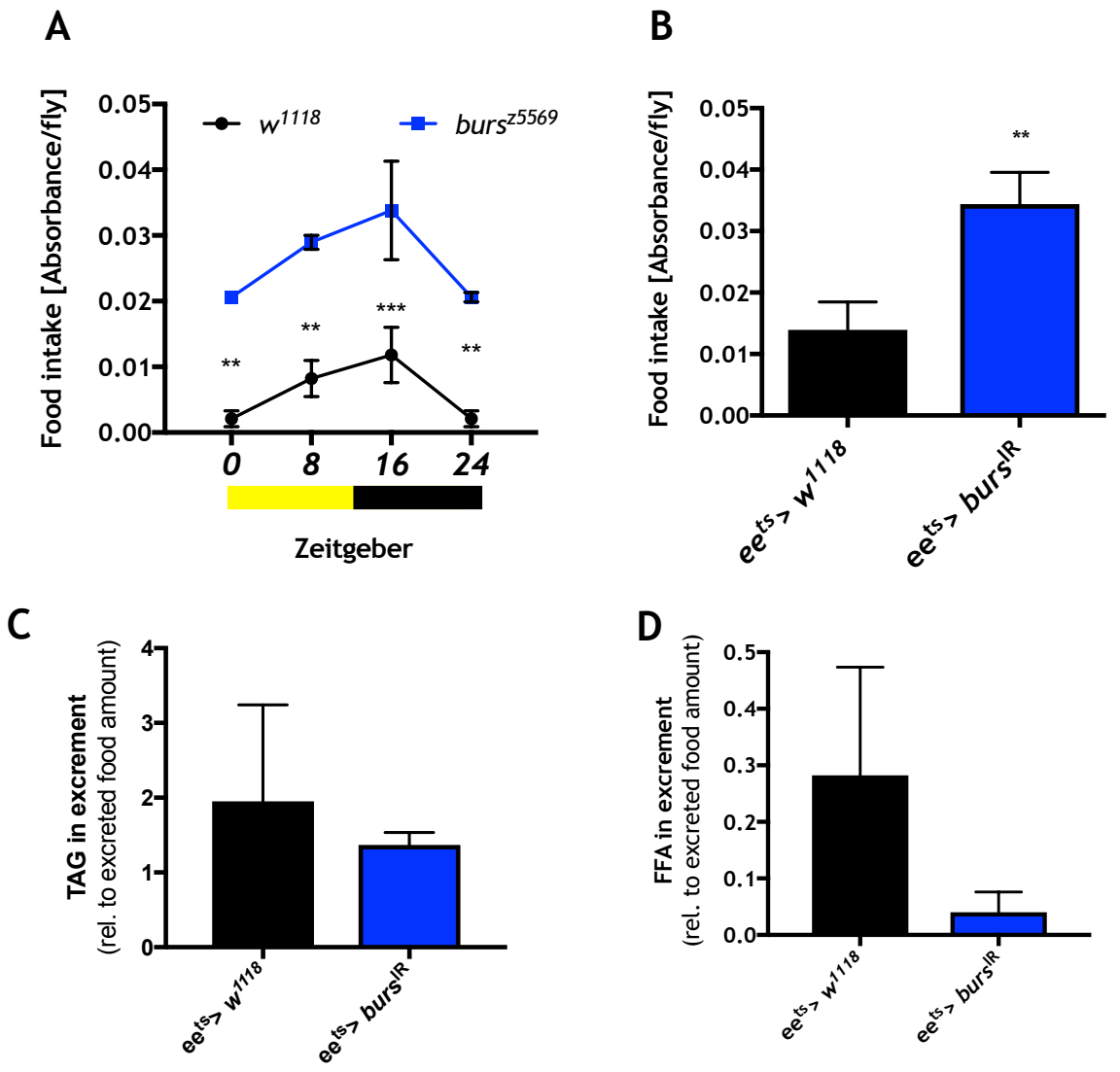

Figure 4-10: Loss of Bursicon signalling resulted in increased food intake.

(A) Flies were fed for 2h with an Allura red containing sucrose solution. Afterwards, absorbance of Allura red in fly lysates was measured. Note that *burs* mutants eat constantly and much more compared to control flies. Zeitgeber (ZT) indicates the time of the day. Please note that ZT0 and ZT24 are the same timepoints. Yellow bar represents light phase, whereas the black bar represents the dark phase of a 12h-12h light-dark cycle. (B) The same experiment as in (A) was done with ee specific *burs* knockdown flies at Zeitgeber 24. (C and D) The excrement of indicated genotypes was collected and TAG and FFA levels within were measured. Values are relative to ingested food intake. TAG = triacylglycerides, FFA = free fatty acids.

Weight loss, due to loss of fat and muscle mass is a common symptom of undiagnosed diabetes, resulting from low or absent circulating Insulin or due to acquired Insulin resistance. Loss of Insulin signalling prevents the uptake of glucose into the cell, leading to an increase in circulating glucose levels, known as hyperglycemia. Therefore, the organism responds with breakdown of lipids and muscle mass to cope with the body's energy demand. Hence, we next tested, if disrupting Burs/ LGR2 signalling is affecting circulating sugar levels. We found lower circulating glucose levels in 3d old *burs* mutants compared to w^{1118} control flies (Figure 4-11 B). To analyse if the observed hypoglycaemia is a consequence of the developmental defects of *burs* mutants, we extracted hemolymph of newly born *burs* mutant and control animals. Circulating glucose levels were unchanged when comparing *burs* mutant and w^{1118} hemolymph (Figure 4-11 A), suggesting that observed glucose reduction emerges during adulthood, and is independent of developmental defects occurring in *burs* mutant animals. Furthermore, we also analysed whole fly glucose levels and detected less glucose per fly in *burs* and *rk* mutant animals compared to w^{1118} control flies (Figure 4-11 C). We confirmed that ee specific *burs* knockdown also led to decreased circulating and whole body glucose when compared to control flies (Figure 4-11 D, E). Observed hypoglycaemia was opposite to what we hypothesised, but the data demonstrated that Burs/ LGR2 signalling affected circulating glucose levels.

We already showed that food intake is not responsible for the loss of energy reserves (Figure 4-10 A, B). However, hypoglycaemia could also just be the consequence of a decrease in sugar absorption ability. To test for potential defects in glucose absorption, we fed the flies with coloured food containing a fluorescent glucose analogue, 2-NBDG, which can't be metabolised and therefore accumulates in tissues. After overnight feeding of flies with coloured food containing 2-NBDG or ethanol as a control, we transferred the flies back onto normal food and measured fluorescent intensity in fly lysates once the food dye wasn't present in the gut anymore. This ensured, that obtained measurements weren't contaminated with residing 2-NBDG in the gut lumen. We found that *burs* mutants absorbed more 2-NBDG, which was proportional with the increase in food intake, suggesting that glucose absorption from the midgut was increased (Figure 4-11 G). Next we performed the same absorption experiment in ee specific *burs* knockdown flies and found no difference of 2-NBDG fluorescent intensity when comparing

them to controls (Figure 4-11 H). Additionally, we also analysed glucose levels within the faeces of ee specific *burs* knockdown flies in the same way as described earlier for excreted lipids. We could not detect any differences in excreted glucose comparing *burs* knockdown and control flies (Figure 4-11 F). These absorption and excretion assays clearly showed that reduced circulating and whole body glucose levels upon *burs* knockdown are not caused by decreased glucose absorption or increased glucose disposal.

Altogether these results provide a new insight in Bursicon/ LGR2 signalling in adult *Drosophila melanogaster* and prove its importance in controlling systemic metabolism independently of the role of the pathway in developmental.

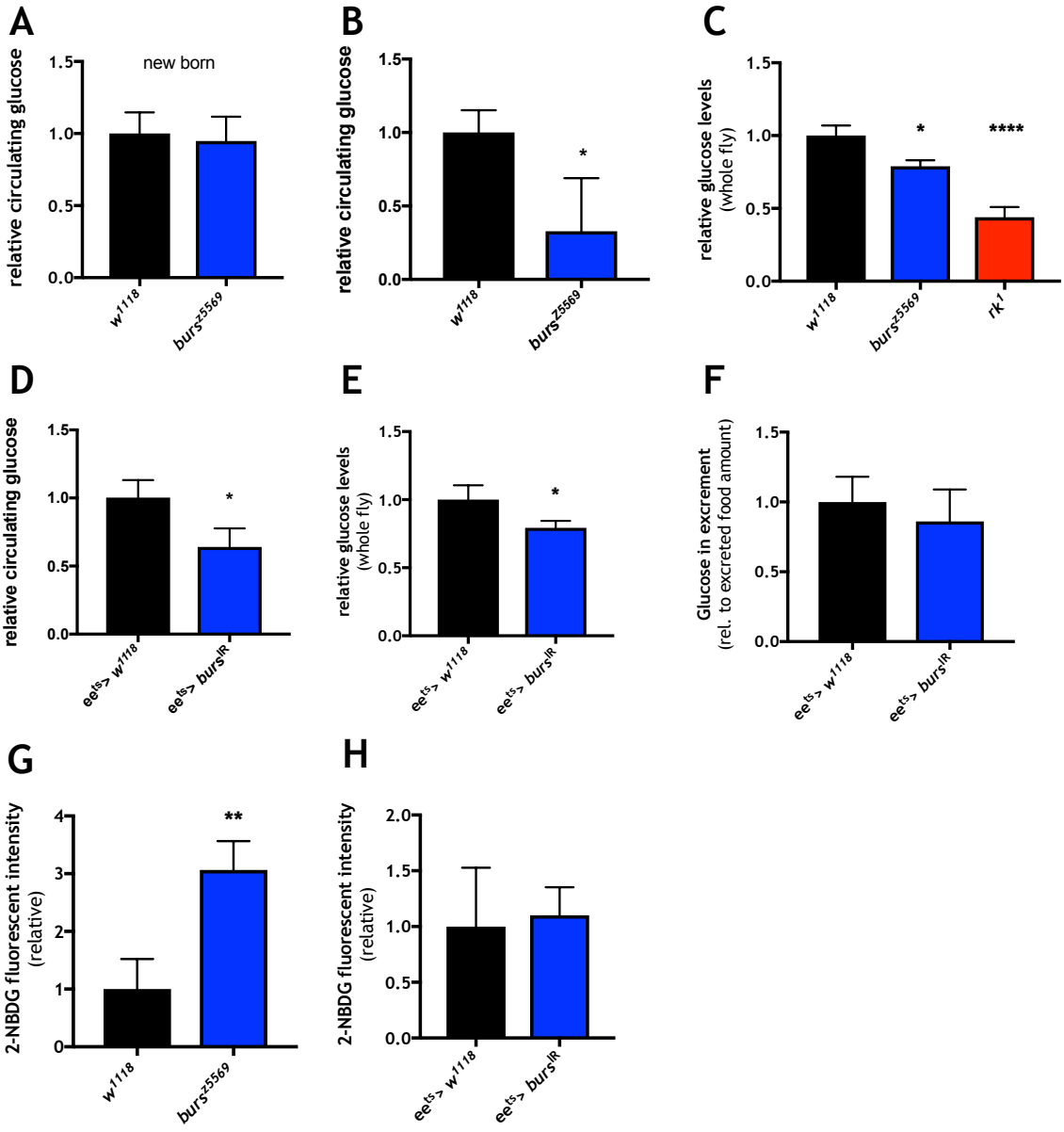

Figure 4-11: Impaired Bursicon signalling resulted in low circulating and whole body glucose levels independent of intestinal absorption or excretion.

(A, B and D) Hemolymph of indicated genotypes was collected and glucose levels within were measured. Note, in (A) circulating glucose levels of newborn (collected within 15-20 min of eclosion) *burs* and control flies are not different. (C and E) Whole fly glucose levels of indicated genotypes were measured. (F) Excrement of ee specific *burs* knockdown and control flies was collected and glucose levels within was measured. (G and H) Flies of indicated genotypes were fed with the non-metabolisable glucose analogue 2-NBDG overnight. After re-feeding on normal food to ensure that no 2-NBDG is anymore present in the gut lumen, flies were lysed and fluorescent intensity was measured.

4.5.1 Burs-β is not involved in the regulation of systemic metabolic homeostasis

The functional Bursicon protein mediating ecdysis is a heterodimeric complex consisting of 2 cysteine-knot proteins, Burs-α and -β (Luo et al., 2005; Mendive et al., 2005). In contrast to the developmental heterodimeric complex, we previously demonstrated, that Burs-β is dispensable for adult midgut homeostasis (Scopelliti et al., 2016). Therefore, to rigorously test for developmental involvement and to further strengthen our hypothesis that Burs-α alone is responsible for observed adult phenotypes, we compared lipid content of *burs-β* mutant and control flies. First we dissected fat bodies of *burs-β* mutants and control flies and stained with the neutral lipid stain lipidTOX and found no apparent differences in lipid droplet size within the fat body (Figure 4-12 A), suggesting that lipid metabolism is not affected upon loss of Burs-β. Next, we assessed whole body TAG levels, which also didn't reveal any differences between control and *burs-β* mutant flies (Figure 4-12 B). These results clearly showed that the role of developmental Burs/ LGR2 signalling is unrelated to its metabolic function on adult flies and further strengthen our previous conclusions that Burs-β is dispensable for adult specific roles of the signalling pathway (Scopelliti et al., 2016).

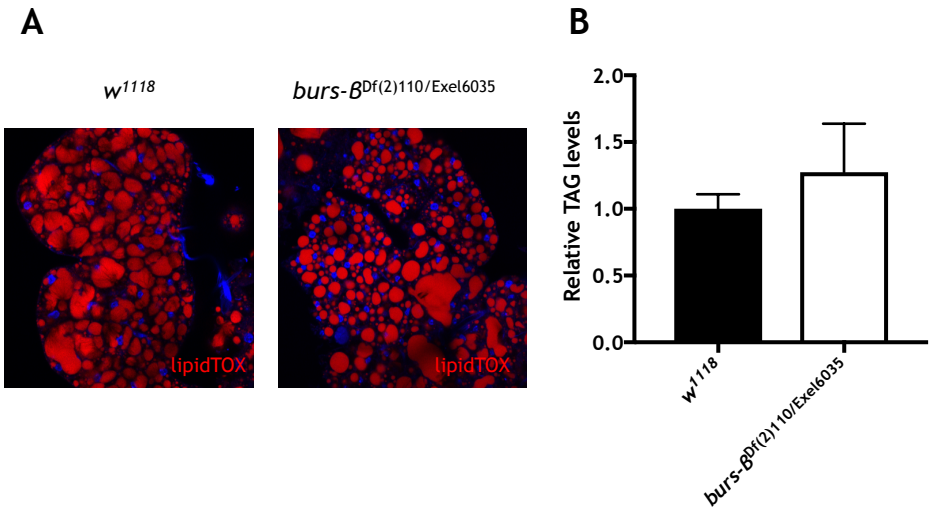

Figure 4-12: Burs-β does not regulate metabolic homeostasis in the adult fly.

(A) Representative confocal images of lipidTOX stained fat bodies of w^{1118} control and *burs-β* mutant flies. Note, no apparent difference in lipid droplet size and number was observed. (B) Lipid levels were measured in control and *burs-β* mutant flies. Again, no differences in TAG levels were observed.

4.5.2 The loss of energy reserves is independent of locomotor activity

We already showed that observed metabolic phenotype in flies with disrupted Burs/ LGR2 signalling was not caused by the inability to absorb nutrients. Another possible way of losing energy storage is by excessive activity.

To assess the activity of flies, we video-tracked the movement of control flies and *burs* and *rk* mutants and quantified their locomotor activity. The results showed that both mutants move less than controls (Figure 4-13 A, B). Those results were expected as the lack of Burs/ LGR2 signalling during development leads to major defects in the legs and wings of *burs* and *rk* mutant flies. To exclude the possibility that the mutants are less active due to their inability to walk properly, we have also video-tracked flies with an ee specific *burs* knockdown. We found no significant differences in locomotor activity comparing 3, 7 and 14d old *burs* knockdown and control flies. However, we observed a clear trend towards less activity in *burs* knockdown flies at 7 and 14 days of age was observed (Figure 4-13 C). This data suggested that increased locomotor activity is not responsible for the loss of energy reserves in mutant and knockdown animals.

But the video-tracking experiments were performed on normal food, resulting in reduction of movement in *burs* and *rk* mutant, as well as *burs* knockdown flies due to their increased feeding behaviour. Therefore, the experiments should be repeated on agar only containing vials to exclude feeding activity. Furthermore, flies should be tracked over a 24h time period, to exclude any circadian influence on the fly's activity.

Altogether, these results suggested that Bursicon/ LGR2 signalling is important for regulating energy metabolism, which is independent of animal feeding behaviour and locomotor activity.

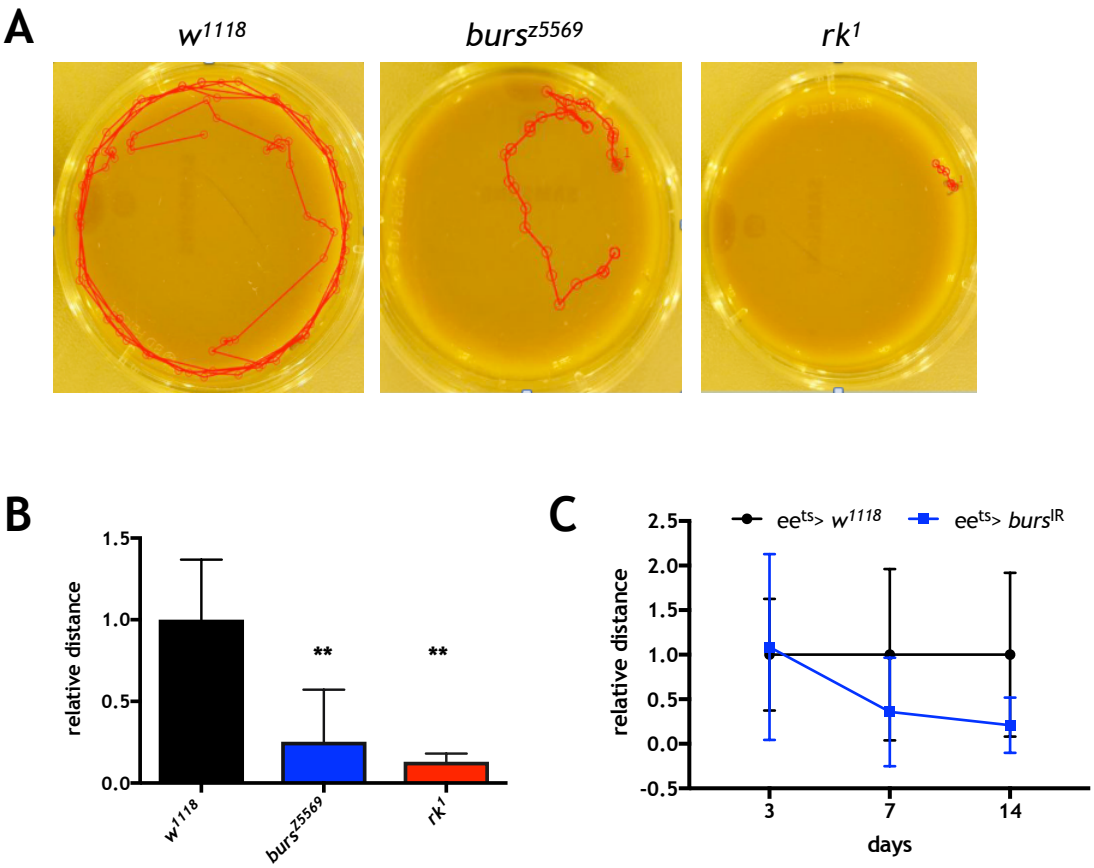

Figure 4-13: Locomotor activity does not seem to be responsible for loss of lipids in flies with impaired Burs/ LGR2 signalling.

(A) Representative pictures of locomotor activity assay from flies of indicated phenotypes. (B and C) Quantification of locomotor activity of (B) 3d old mutant and control flies and (C) ee specific *burs* knockdown and control flies of indicated ages. (n ≥ 4).

4.5.3 Investigating the mitochondrial contribution to the metabolic phenotype observed in flies with impaired Burs/ LGR2 signalling

Within cells, nutrients need to be converted into cellular energy known as ATP. Most of the ATP is produced by oxidative phosphorylation (OXPHOS) of sugars, amino acids and lipids in the mitochondria. Therefore, the more mitochondria within a cell, the more ATP a cell can produce. Since mitochondria, known as the powerhouses of the cell, are key organelles in the conversion of nutrients into energy, we hypothesised that Bursicon/ LGR2 signalling leads to increased mitochondrial number and/or activity, which is responsible for the overuse of energy.

To investigate mitochondrial number and activity we analysed mitochondrial DNA (mtDNA) content and mitochondrial respiration between control flies and mutants, as well as between ee specific *burs* knockdown and control flies. We performed RT-qPCR analysis of whole body DNA extracts to measure mtDNA content. We found a striking 5-fold increase of mitochondrial DNA in whole *burs* and *rk* mutants compared to control flies (Figure 4-14 A), but couldn't detect any differences in 14d old *burs* targeted knockdown flies compared to their control counterparts (Figure 4-14 B). To assess mitochondrial activity, we extracted mitochondria from whole flies and compared O_2-consumption rate of *bursicon* and control mitochondria in collaboration with Dr. Björn Kruspig (Dr. Daniel Murphy laboratory). We found a consistent 15% increase of O_2-consumption in mitochondria extracted from *burs* mutants compared to w^{1118} control mitochondria (Figure 4-14 C). Mitochondrial extracts of *burs* knockdown flies, which were aged for 14d, showed close to significant increase in O_2-consumption when compared to control mitochondria ($p = 0.0595$; Figure 4-14 D).

Those results showed differences between the mitochondrial phenotypes of whole *burs* and *rk* mutants, and *burs* targeted knockdown flies. This would suggest, that observed differences between mutants and *burs* knockdown flies are due to loss of Burs/ LGR2 signalling during development. On the other hand, observed phenotypic differences could be also due to the timepoints chosen to perform the measurements (3d for mutants and 14d for ee specific *burs* knockdown flies). For that reason, to rigorously check if Burs/ LGR2 signalling is affecting mitochondria

number or function, the above experiments done with 14d old ee specific *burs* knockdown flies should be repeated using flies at an earlier stage at their life, to exclude an adaptation to the severe energy loss.

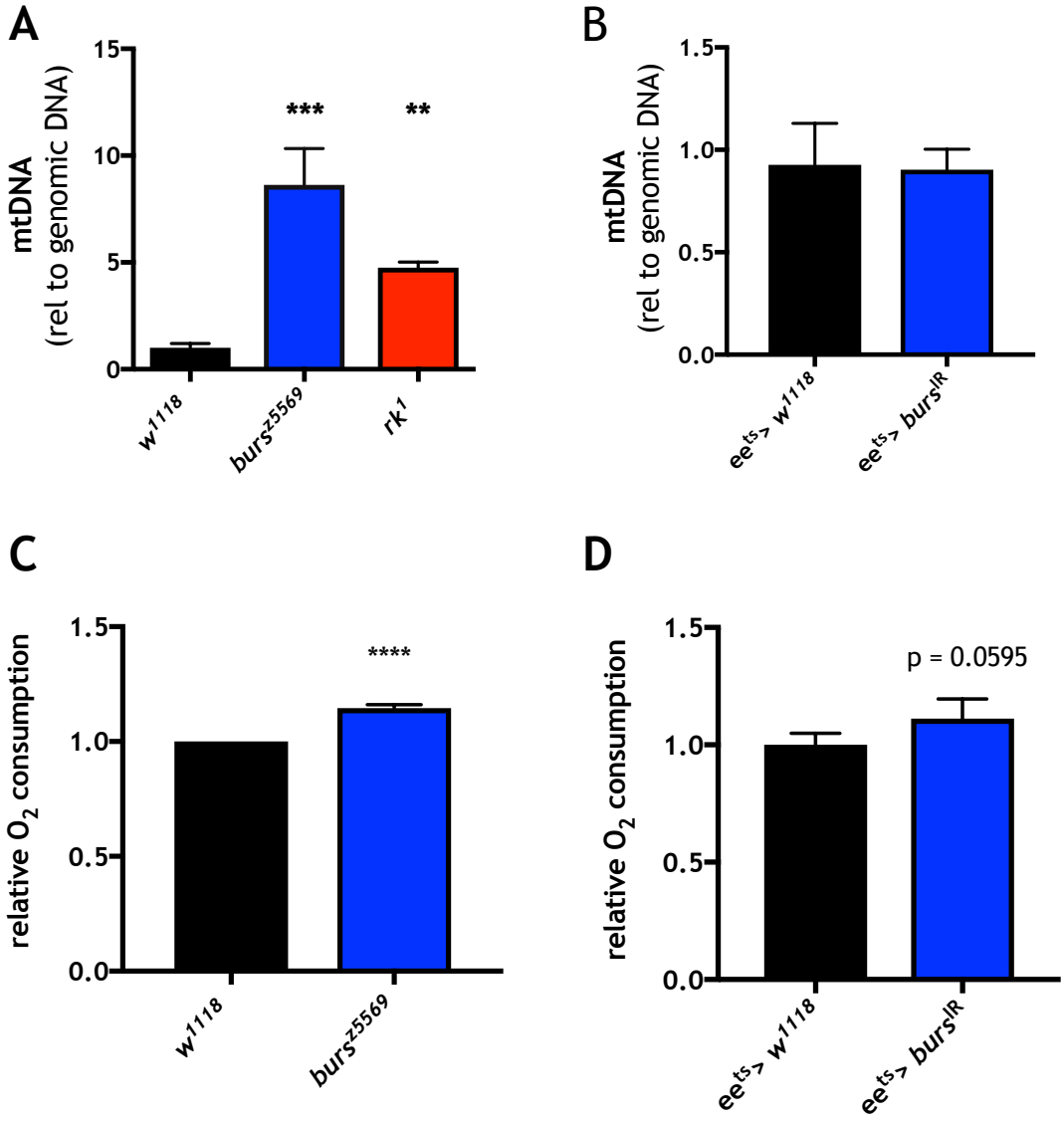

Figure 4-14: Bursicon as a possible regulator of mitochondrial number and activity.

(A and B) Mitochondrial DNA content was measured by RT-qPCR analysis of whole fly DNA extracts using primers specific for mtDNA and genomic DNA of indicated genotypes. (C and D) O_2-consumption was measured in collaboration with Dr. Björn Kruspig. Mitochondria were extracted by differential centrifugation from flies of indicated genotypes and O_2-consumption using a succinate buffer was analysed with an electrode. ($n \geq 3$).

4.6 Overexpression of Bursicon doesn't affect metabolism

Contrary to the increased cell proliferation in adult midguts resulting from ee specific *burs* or VM specific *rk* knockdown, midguts of flies overexpressing *bursicon* have impaired ISC proliferation upon damage and aging (Scopelliti et al., 2016; Scopelliti et al., 2014).

We next asked, whether a similar gain if function phenotype could be observed for the role of Burs in systemic metabolism. To do so, we first checked starvation survival and TAG levels in flies overexpressing *burs*. We started by specifically overexpressing *burs* within adult ee cells and found no effect on survival upon starvation (Figure 4-15 A). Those results indicated, that overexpression of *burs* doesn't have gain of function effects on metabolism.

It would be possible that solely overexpressing *burs* within ee cells might not significantly increase Burs protein within the hemolymph due to posttranslational regulation. For that reason, we overexpressed *burs* within the fat body, a secretory tissue in *Drosophila*. Preliminary data confirmed that *burs* overexpression in fat body cells increased Burs protein content in the hemolymph when comparing to hemolymph from control flies (Figure 4-7 C). Next, we analysed TAG content and survival upon starvation of fat body specific *burs* overexpression and control flies. We couldn't detect any differences in TAG levels or survival upon starvation when overexpressing *burs* in fat body cells compared to control flies (Figure 4-15 B, C).

This data suggested, that *burs* overexpression by itself does not induce an obesity-like phenotype and therefore has no effect on survival upon starvation.

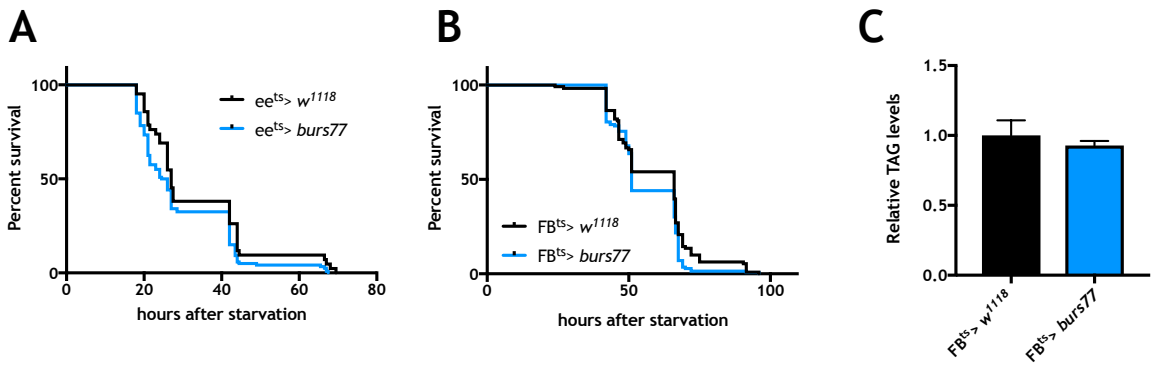

Figure 4-15: Bursicon overexpression didn't result in a metabolic gain of function phenotype.

(A and B) Starvation survival of control flies and flies overexpressing *burs* either (A) in ee cells or (B) in the fat body. No difference in starvation sensitivity was observed. (C) TAG levels were analysed from whole flies overexpressing *burs* within the fat body and their controls. *burs* overexpression didn't increase TAG levels.

4.7 Conclusion

The results presented in this chapter clearly demonstrate a novel role of Bursicon/ LGR2 signalling in whole organismal metabolic regulation.

We demonstrated, that Burs didn't mediate metabolic regulation via its receptor within the VM. This suggested, that Burs is acting in an endocrine manner, which could be validated by differential Burs protein levels in the midgut and in the hemolymph of fed and starved flies. These experiments showed, that Burs is secreted in the fed state and retained when flies are starved, which is possibly dependent on ee specific Glut1 expression.

We found that Burs expressed in ee cells is responsible for maintaining glucose and lipid levels and therefore starvation responses, which is unrelated to activity, food ingestion and absorption of the flies. We would hypothesise that Burs/ LGR2 signalling works by restraining energy overuse and therefore protects the animal from depleting its energy resources. So far, presented results are not conclusive if Burs/ LGR2 signalling is regulating mitochondrial activity, which needs to be investigated in more depth.

In the next chapter, we will analyse where *rk* is expressed and which rk^{+ive} organ is responsible for the observed metabolic phenotypes. Lastly, we will discuss mechanistic insights downstream of Burs/ LGR2 signalling.

5 *rickets* expression pattern and mechanistic insight downstream Bursicon/ LGR2 signalling

5.1 Short Summary

Intestinal derived hormones, many of which act on their neuronal receptors are known regulators of metabolism in mammals.

Here, we uncover a novel gut-neuron communication regulating systemic metabolic homeostasis in adult *Drosophila* mediated by the enteroendocrine derived Bursicon and its neuronal receptor LGR2. Impairment of Burs/ neuronal LGR2 signalling resulted in enormous loss of stored energy reserves, independent of feeding and activity.

5.2 Introduction

Drosophila LGR2 was shown to activate cAMP signalling within the VM to promote intestinal stem cells quiescence (Scopelliti et al., 2014). Here, we demonstrated that, besides the local role of Burs/ LGR2 signalling in the *Drosophila* midgut, Burs is also released into the hemolymph in fed conditions, whereas it is retained in the ee cells upon fasting. This pointed to an endocrine role for Burs to regulate metabolism. Furthermore, LGR2 in the VM was not responsible for observed metabolic phenotypes in *burs* and *rk* mutants, and ee specific *burs* knockdown flies. Thus suggesting that the receptor for Burs, LGR2, is expressed outside of the midgut to mediate metabolic homeostasis.

Drosophila LGR2 is the homolog to the mammalian LGR4, 5 and 6, which function as R-spondin receptors and therefore mediate Wnt signalling (Carmon et al., 2011; de Lau et al., 2011). R-spondins are secreted factors and therefore have a systemic function. In mammals 4 R-spondins are known, R-spondin 1-4 (Rspo1-4) (Chen et al., 2002; Kim et al., 2006), which have important roles in development and stem cell homeostasis (Schuijers and Clevers, 2012). Loss of R-spondin 1 results in abnormal development of ovaries in mice (Chadi et al., 2016), and R-spondin 3 loss is embryonic lethal (Aoki et al., 2007).

LGR4 and LGR5 were also found to be necessary for development, as knockout mice are embryonic/ neonatal lethal (Mazerbourg et al., 2004; Morita et al., 2004). Interestingly, it was shown that homozygous mutant LGR6 mice, created by knock-in of marker genes within the LGR6 gene locus, don't display any obvious phenotypes, and were viable and fertile (Snippert et al., 2010).

After thorough investigation of the *burs* and *rk* mutant, as well as the ee specific *burs* knockdown phenotype, we will show that *rickets* is expressed in several tissues and demonstrate that *rk*$^{+ive}$ neurons are mediating the metabolic phenotype of *rk* loss of function mutants in adult flies.

5.2.1 Expression pattern of mammalian LGRs

LGR4 positivity, using a LGR4 specific antibody, was found in the human mammary ducts and reproductive system, especially within primordial and primary follicles (Yi et al., 2013), thus supporting the known critical role of LGR4 in reproduction (Styrkarsdottir et al., 2013). Furthermore, also murine and human pancreas displayed LGR4 positivity (Yi et al., 2013). Co-staining for Insulin and LGR4 revealed LGR4 expression in all murine pancreatic ß cells (Yi et al., 2013), whereas no LGR5 or LGR6 expression was detected (Hsu et al., 1998). Furthermore, human colon cancer tissues display high LGR4 expression (Yi et al., 2013).

LGR5 is expressed in different tissues, such as intestine, muscle, placenta, spinal cord and brain, and serves in many of them as a biomarker for stem cells (Barker and Clevers, 2010; Barker et al., 2007; Hsu et al., 1998; Schuijers and Clevers, 2012). Wnt signalling was found to be a major regulator of intestinal crypt proliferation (Korinek et al., 1998). Therefore, finding Wnt targets was necessary to establish markers for stem cells. Lineage tracing experiments revealed LGR5 to be an intestinal and pancreatic stem cells marker, among others (Barker et al., 2010; Barker et al., 2007; Sato et al., 2009). Activation of Wnt signalling by deletion of APC within LGR5^{+ive} cells led to rapid transformation of those stem cells, giving rise to cancerous intestinal and gastric tissue (Barker et al., 2010; Barker et al., 2009; Schuijers and Clevers, 2012). LGR5 was also expressed in post-mitotic amacrine cells within the eye, thus representing the first neuronal and non-stem cell lineage domain of LGR5 expression (Sukhdeo et al., 2014).

LGR6 expression was found in brain, mammary gland, lungs and skin cells (Leushacke and Barker, 2012; Snippert et al., 2010). During development, LGR6 is mainly expressed in hair peg cells (Snippert et al., 2010). Deleting LGR6 at embryonic stage E17.5, where expression was only found in hair peg cells, and following the lineage using a lacZ antibody revealed widespread staining throughout the skin, whereas later induction of LGR6 deletion showed lesser lineage tracing (Snippert et al., 2010). Interestingly, in many human colon cancer samples LGR6 is highly mutated (Sjoblom et al., 2006) and the promoter region hypermethylated (Mokarram et al., 2009; Schuebel et al., 2007), suggesting a tumour suppressor function for LGR6. Mutations for LGR6 were also found in ovarian and pancreatic cancers (Forbes et al., 2011).

Additionally, all 3 LGRs are expressed within the skin, but only mice with a conditional knockout of LGR4 had impaired hair follicle development (Mohri et al., 2008).

5.3 Expression pattern of *rickets* throughout the adult fly

Since we observed that *rk* in the visceral muscle is not involved in the metabolic function of Bursicon, we hypothesised that hemolymph secreted Burs acts on its receptor LGR2 in an organ distant to the midgut to regulate systemic metabolism.

According to the public database 'Flybase', *rk* shows low expression throughout multiple adult tissues (Figure 5-1 A). We have also performed RT-qPCR analysis to assess *rickets* expression in various tissues and developmental stages. Stage 3 larvae and dark pupae were used as positive controls, which showed expected high *rk* expression (Figure 5-1 B). We also dissected diverse adult tissues of wild type flies and found *rk* expression enriched within adult crops, heads and ovaries, with relatively lower, but still significant gene expression, in midguts and tubules (Figure 5-1 B).

We expressed a nuclear Red Stinger reporter (nRS) or CD8-GFP under the control of a *rk*-specific Gal4 driver to analyse endogenous *rk* expression patterns of the receptor at a cellular level as discrete expression pattern are often missed from whole genome enrichment analysis. First, we wanted to examine if *rk* was expressed within the fat body cells, due to their importance in storing and utilising

energy in response to changes in nutrients. To test this, we dissected cuticles of flies expressing nRS in a *rk* dependent manner and found no expression of *rk* within fat body cells, but interestingly we saw *rk*$^{+ive}$ tracheal cells (akin to mammalian vasculature) entering the fat body (Figure 5-1 C). Using this reporter line, we next analysed *rk* expression within the adult brain, due to the high mRNA levels observed in dissected heads. Dissected brains showed many *rk*$^{+ive}$ neurons and confirmed that *rk* is expressed in tracheal cells around the brain as well (Figure 5-1 D).

These results showed that *rk* is expressed in various tissues throughout adult *Drosophila*, supporting our hypothesis regarding the presence of endocrine Burs/ LGR2 signalling.

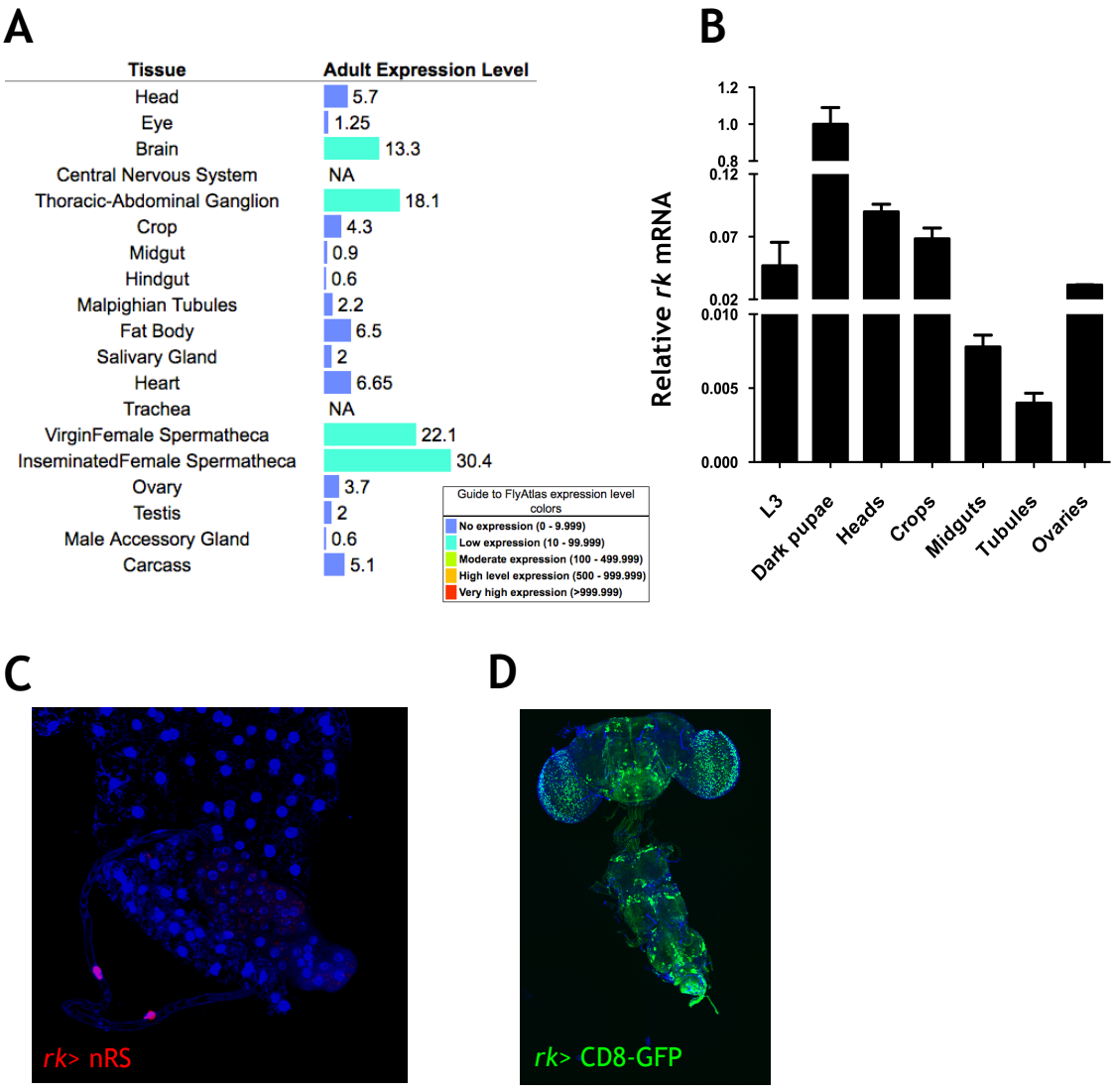

Figure 5-1: *rk* expression in adult tissues.

(A) FlyAtlas anatomical *rk* mRNA expression data. (B) Dissected tissues of control animals were analysed for *rk* expression by RT-qPCR. L3 and DP served as positive controls. L3 = larvae in stage 3; DP = dark pupae. (C and D) Representative confocal maximum projection images of (C) fat body and trachea in flies expressing a nuclear Red Stinger (nRS) and (D) in the adult CNS of flies expressing CD8-GFP in a *rickets* dependent manner.

5.3.1 Investigating the role of tracheal- and fat body-expressed LGR2

As we could clearly see *rk* positivity in tracheal cells associated to various tissues, we next tested whether this source of the receptor was responsible to mediate Burs-dependent energy homeostasis. To test this, we specifically knocked down *rickets* in all tracheal branches using *breathless*-Gal4 (Dicer2; *Btl*-Gal4; *gal80*^{ts}; referred to as *Btl*^{ts}>) and in the terminal tracheal branches using *Drosophila Serum Response Factor*-Gal4 (Dicer2; *dsrf*-Gal4; *gal80*^{ts}; referred to as *dsrf*^{ts}>). Neither approach showed differences in lipid (Figure 5-2 A, E), or whole fly glucose levels (Figure 5-2 D, F) compared to control animals. Lastly, we have also subjected animals with a knockdown of *rk* in DSRF^{+ive} cells to starvation sensitivity tests and found a slight increase in starvation sensitivity in the *rk* knockdown when compared to control flies (Figure 5-2 C). However, this starvation sensitivity is not comparable to what we have observed when knocking down *burs* from ee cells. Critically, we failed to detect a rescue of TAG levels when we overexpressed *rk* in terminal tracheal branches (using *dsrf*^{ts}>) in a *rk* mutant background (Figure 5-2 B). Altogether, these results suggested, that *rk*$^{+ive}$ tracheal cells are not responsible for regulating metabolic homeostasis as seen in ee specific *burs* knockdown flies.

To exclude the possibility of the reporter line not fully recapitulating endogenous *rickets* expression pattern, we next analysed a potential role of *rk* within the fat body. To test this, we knocked down *rk* specifically from the adult fat body (using Dicer2; *Lsp2*-Gal4; *gal80*^{ts}, referred to as Lsp2^{ts}>) and measured starvation sensitivity, but couldn't detect any differences between Lsp2^{ts}> *rk*^{lR} and control flies (Figure 5-2 H). Most importantly, we didn't observe a rescue of lipid content by overexpressing *rk* in fat body cells in a *rk* mutant background (Figure 5-2 G), suggesting that even if expressed in the fat body, *rk* from this tissue has no effect on lipid metabolism.

The above results showed that, even though *rk* is expressed in tracheal cells, that source of the receptor does not influence metabolism in a similar manner as observed in *burs* and *rk* mutants and upon adult ee specific *burs* knockdown. It would be interesting in future experiments to dissect the role of *rk* within the trachea.

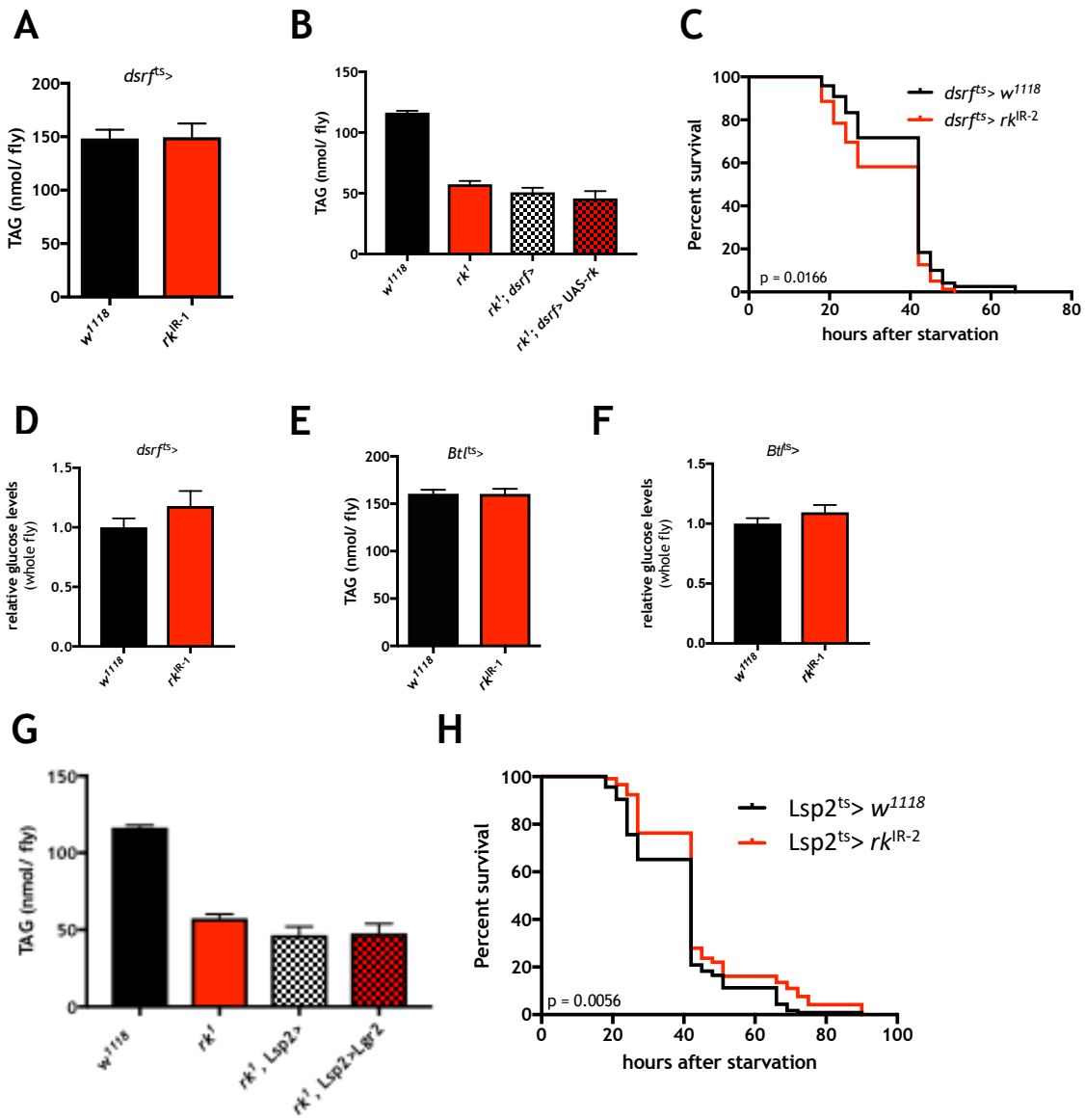

Figure 5-2: Knockdown of *rickets* in the trachea or fat body doesn't affect metabolism.

(A and E) Lipid measurements of whole fly lysates of indicated genotypes. (B and G) Lipid measurements of fly lysates of indicated genotypes. Note that *rk* was overexpressed throughout development. (C and H) Starvation sensitivity tests with flies of indicated genotypes. Slight difference was observed, but this was not comparable to starvation sensitivity observed in *burs* knockdown flies. (D and F) Whole fly glucose levels of indicated genotypes. No statistical significance was observed.

5.3.2 Neuronal LGR2 modulated glucose and lipid metabolism

By analysing the expression pattern of *rk* using the *rk* specific reporter line, we observed high expression of *rk* in the adult *Drosophila* CNS, consisting of brain and ventral nerve cord (VNC) (Figure 5-1 D). We hypothesises that rk^{+ive} neurons may modulate systemic metabolism through binding to ee specific Burs.

We next specifically knocked down *rk* in adult neurons (using Dicer2; *nSyb*-Gal4; $gal80^{ts}$, referred to as $neurons^{ts}$>) to examine its role in metabolism. Our results showed, that flies subjected to pan-neuronal *rk* knockdown were hypersensitive to starvation (Figure 5-3 A), and displayed a similar TAG loss (Figure 5-3 B) and reduction in circulating glucose levels (Figure 5-3 C) to the ones observed when knocking down *burs* in ee cells. This data suggested that the hormone Burs might act via its neuronal receptor LGR2 to mediate metabolic homeostasis.

To clarify that the RNA interference used against *rk* is targeting the *rk* transcript, we performed RT-qPCR analysis from brains of flies with a neuronal knockdown of *rk*. This experiment confirmed that the *rk* transcript is reduced (Figure 5-3 E). However, gene expression knockdown was only partial, likely due to non-neuronal endogenous gene expression and/or contamination of dissected brains with rk^{+ive} tracheal cells.

To rigorously test whether neurons mediate the systemic metabolic phenotype of *rk* mutant animals, we overexpressed *rickets* specifically within neurons using an *elav*-gal4 driver in a *rk* mutant background. This led to a significant rescue of TAG levels compared to *rk* mutants alone (Figure 5-3 D) and demonstrated that Burs/ neuronal LGR2 signalling is responsible for mediating adult systemic metabolic homeostasis.

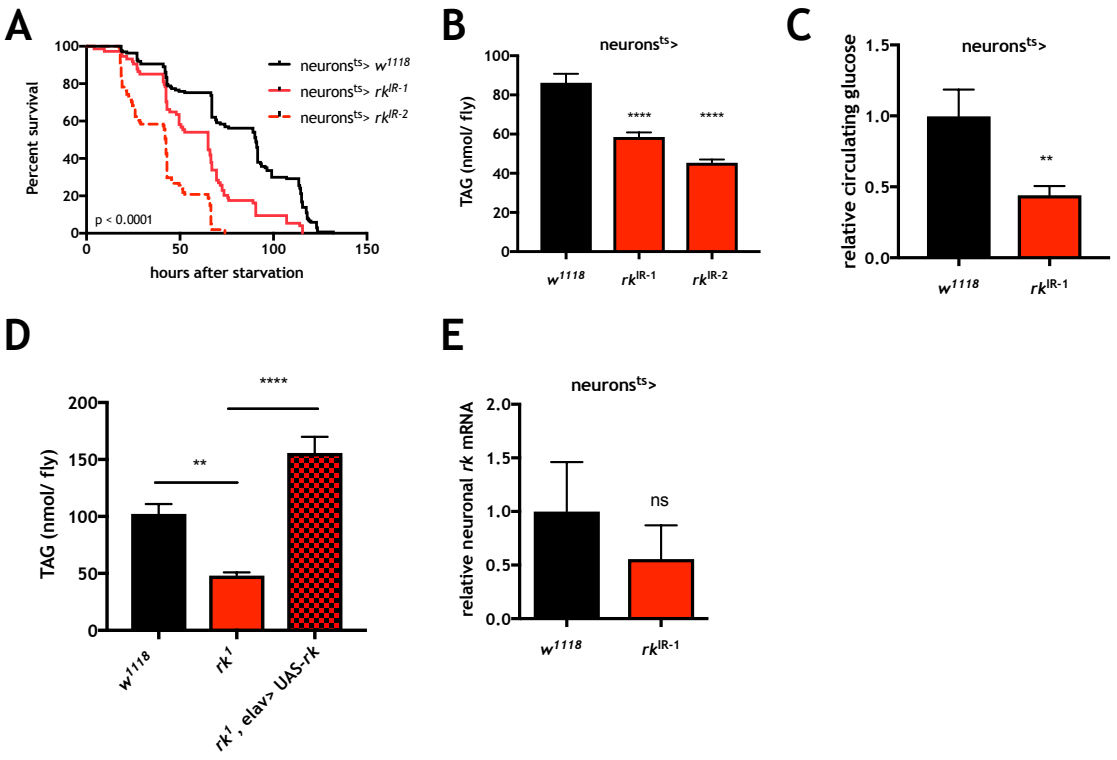

Figure 5-3: Neuronal LGR2 regulated metabolism.

Neuronal knockdown of *rk* resulted in (A) starvation sensitivity, (B) low lipid levels and (C) reduced circulating glucose levels. (D) Lipid levels in 3d old *rk* mutant flies were rescued when *rk* was overexpressed in neurons throughout development. (E) Brains were dissected from 14d old flies of indicated genotypes and RT-qPCR analysis were performed for *rk*. Values are relative to *rpl32* and controls were set to 1.

Next, we analysed feeding and nutrient absorption in adult animals with a neuron specific *rk* knockdown. First, we analysed food intake via coloured food ingestion and found hyperphagic behaviour in neuronal *rk* knockdown flies (Figure 5-4 A). Furthermore, we found no significant difference in the amount of absorbed non-metabolisable glucose analogue 2-NBDG between *rk* knockdown and control flies (Figure 5-4 B). Lastly, we collected the excretion of flies and measured glucose, FFA and TAG levels within. For glucose and FFA levels we found no significant differences comparing *rk* knockdown and control flies (Figure 5-4 C, D), while TAG levels were undetectable in both genotypes. This data clearly showed that ee specific *burs* knockdown and neuronal *rk* knockdown resulted in the same metabolic phenotypes, independent of feeding behaviour and nutrient absorption.

Altogether, these results demonstrated that the hormone Bursicon mediates systemic metabolic homeostasis through its neuronal receptor LGR2.

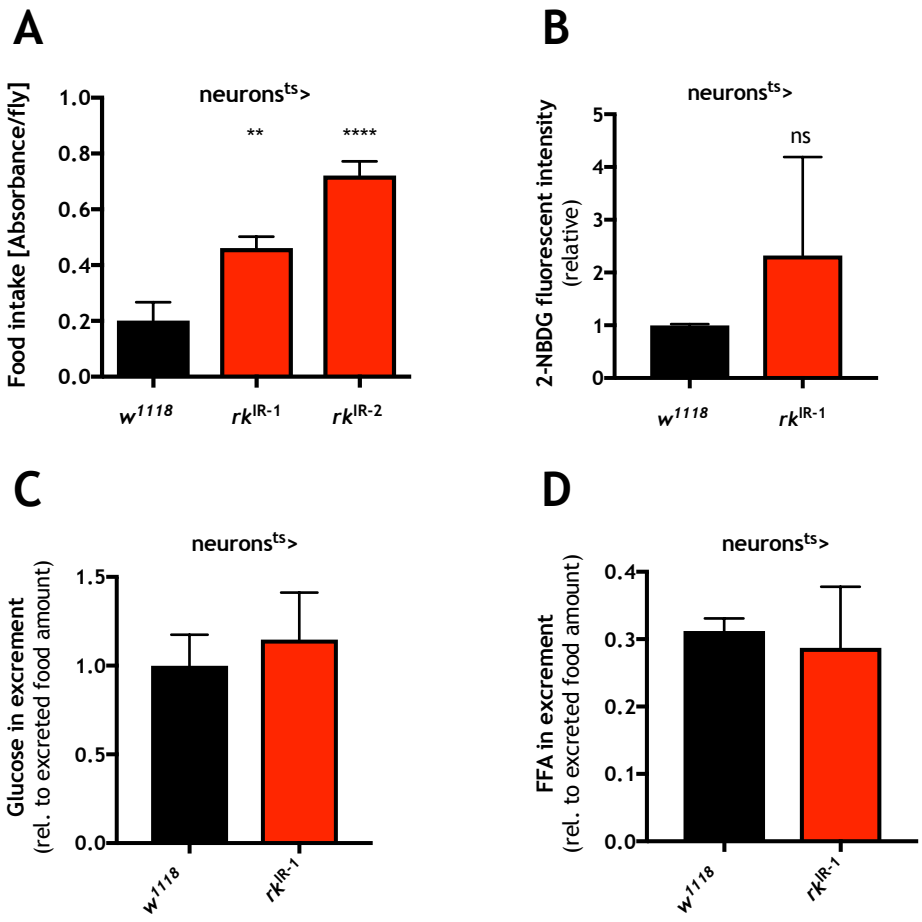

Figure 5-4: Knockdown of *rk* in neurons doesn't result in problems with feeding and intestinal absorption and excretion.

(A) Flies were fed for 2h (ZT 10-12) with an Allura red containing sucrose solution. Afterwards, absorbance of the dye in fly lysates was measured. (B) Flies of indicated genotypes were fed with the non-metabolisable glucose analogue 2-NBDG over night. After re-feeding on normal food to ensure that no 2-NBDG is residing in the gut lumen, flies were lysed and fluorescent intensity was measured. (C and D) Excrement of flies with neuronal *rk* knockdown and controls was collected and glucose and FFA levels within were measured.

5.4 Unbiased approach to uncover downstream mechanisms of endocrine Burs/ LGR2 signalling

After in depth phenotypic characterisation of phenotypes resulting from adult specific disruption of Burs/ neuronal LGR2 signalling, we wanted to unravel involved downstream pathway(s). For this we decided to undertake 2 unbiased approaches. First we performed liquid chromatography mass spectrometry (LC-MS) analysis of dissected midguts, heads (brain enriched) and cuticles (fat body enriched) of 3d old *burs* and *rk* mutant as well as control flies in triplicates. LC-MS analysis were performed in collaboration with Dr. Saverio Tardito.

Secondly, we analysed the transcriptome of midguts, brains and cuticles (fat body enriched) from 14d old adult animals with (1) an ee specific knockdown of *bursicon* and its respective control and (2) neuronal specific knockdown of *rickets* and its control in collaboration with Billy Clark and Ann Hedley. Experiments were done in biological quadruplicates. We first analysed the RNA sequencing (RNAseq) data from different tissues within each genetic background. This created 2 different sets of genes for each tissue dissected. Then we narrowed down our list of candidate genes by selecting genes, which were equally deregulated in both genetic knockdowns within each tissue (Figure 5-5). This resulted in 306 significantly deregulated genes within the midgut, 494 genes within the brain and 503 genes within the cuticle. There were 38 genes significantly regulated in the same direction within all 3 analysed tissues, 97 when comparing the midgut and the brain, 98 comparing the midgut and the cuticle and 117 comparing the brain and the cuticle (Figure 5-6 A).

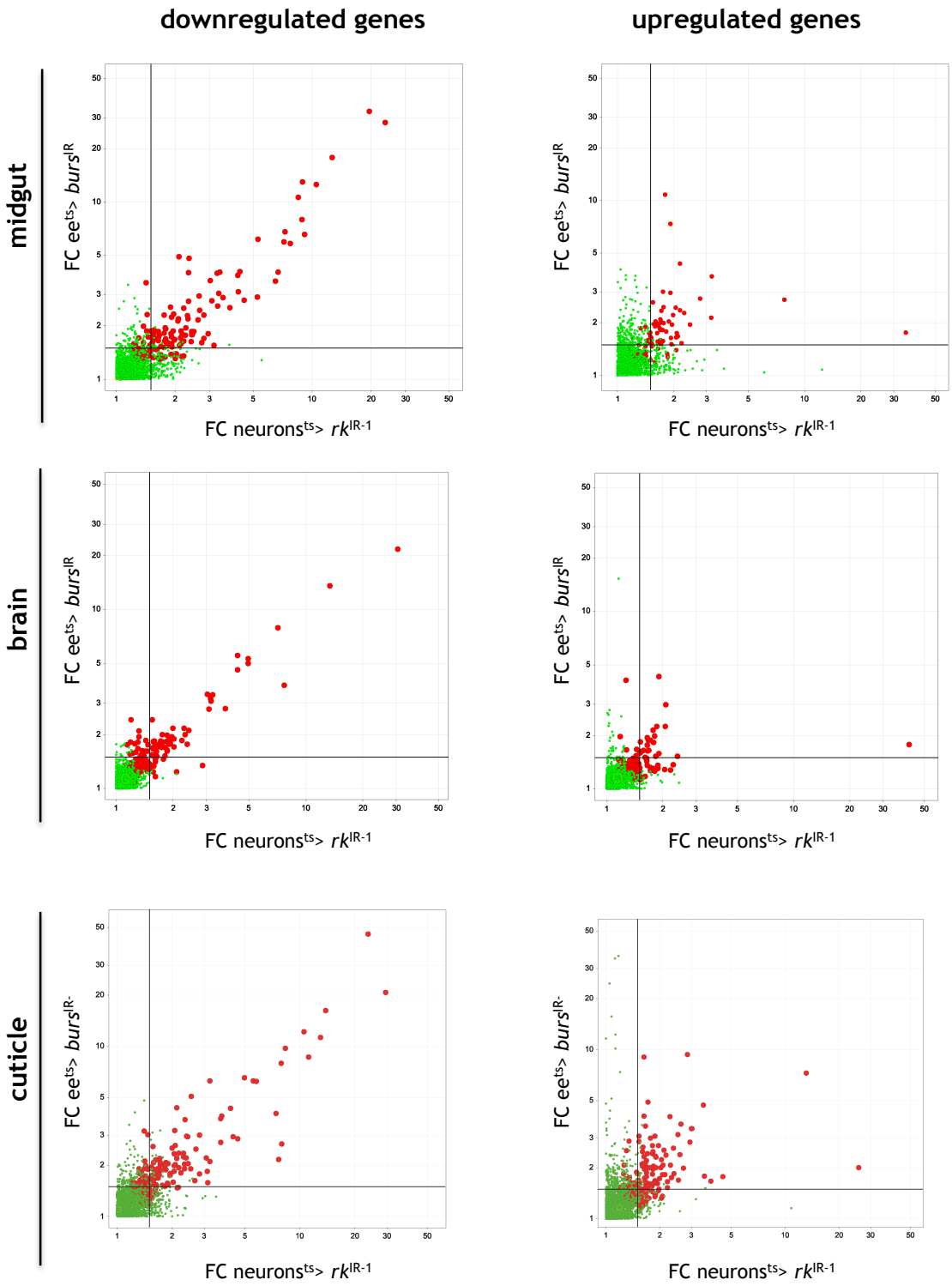

Figure 5-5: RNAseq analysis from dissected midguts, brains and cuticles of knockdown flies compared to their individual control.

14d old flies with an ee specific *burs* knockdown and their controls, and neuronal *rk* knockdown and their controls were dissected, RNA extracted and analysed by RNAseq with the help of Billy Clark and Ann Hedley. Significantly regulated genes of both knockdown conditions were compared to their individual controls and afterwards compared against each other. Green dots represent genes, which were not significantly changed or not changed in the same direction in both knockdown conditions. Red dots represent genes, which were significantly regulated in the same direction in knockdown tissues compared to their individual controls. Graphs were generated with the help of Matthew Davidson, using the program Vortex by Dotmatics.

When analysing genes significantly deregulated in the same way in all tissues and amongst both knockdown conditions, we found *adenylyl cyclase 35C* (*ac13E*) significantly downregulated (Figure 5-6 B). *Ac13E* is known to use ATP to produce cAMP. It has been previously shown, that Bursicon signalling activates cAMP levels in the visceral muscle (Scopelliti et al., 2014). This would support the idea, that Bursicon/ neuronal LGR2 signalling also regulates cAMP within different adult tissues.

Additionally, we have also found *cox I*, *II* and *III*, important for cytochrome c oxidase activity significantly downregulated (Figure 5-6 B), suggesting reduced mitochondrial activity in the tested 14d old adult tissues of both knockdown conditions.

After broad analysis, we went on to further investigate transcriptional and metabolic regulation in different tissues occurring when Burs/ neuronal LGR2 signalling is disrupted.

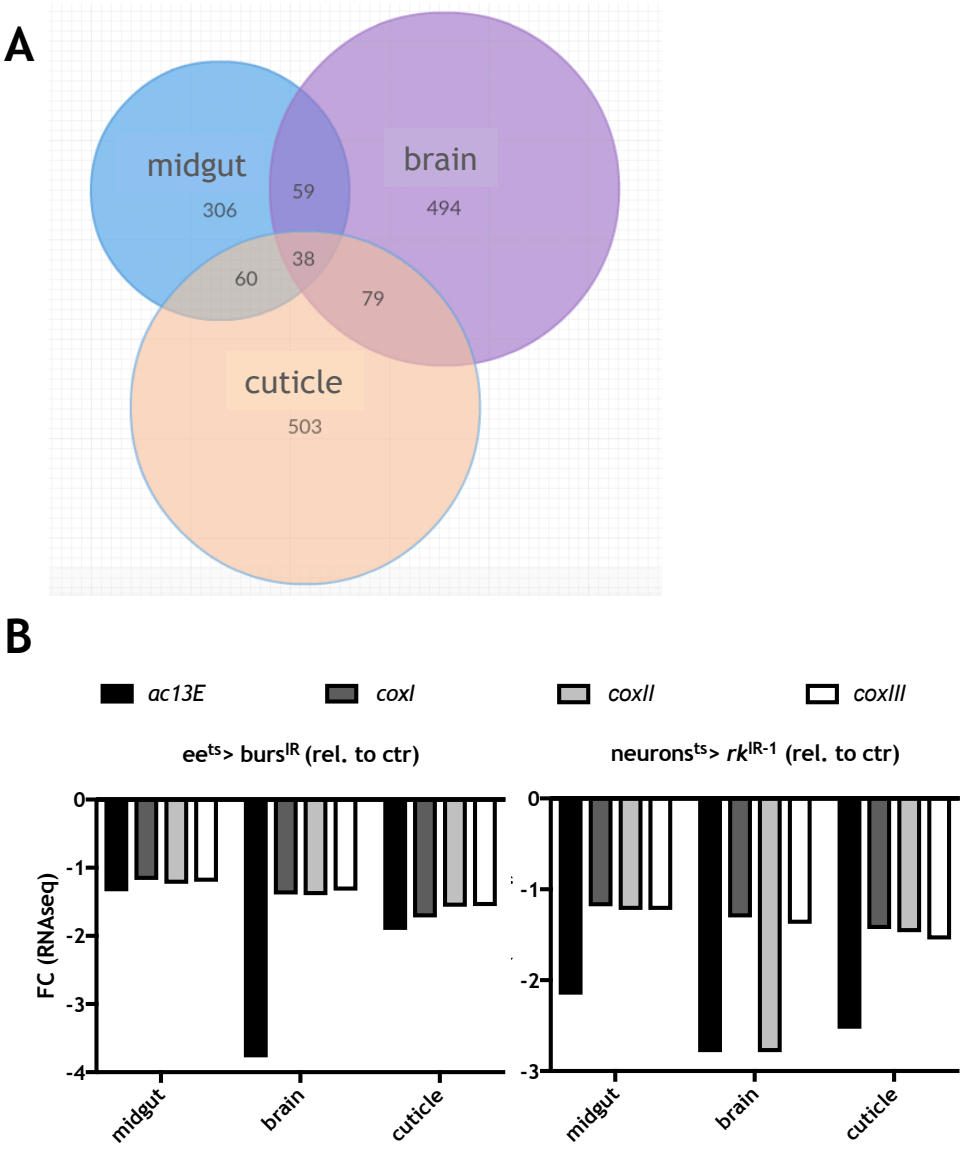

Figure 5-6: RNAseq analysis from dissected tissues of knockdown compared to control flies.

(A) Venn diagram showing genes significantly regulated in the same direction in both knockdown conditions compared to their individual control for each tissue. (B) Indicated genes were significantly downregulated in all 3 tissues when comparing knockdown to control condition. FC = fold change.

5.4.1 Analysing metabolites and transcriptome of midguts from animals with impaired Burs/ LGR2 signalling

We decided to first analyse the RNAseq of dissected midguts by first comparing ee specific *burs* knockdown and their control, to uncover clues of how Bursicon might be regulated. We found nearly 1400 genes differentially regulated, of which around 700 are each significantly up- and downregulated.

Using the functional annotation cluster within the DAVID software, we found the KEGG pathways 'starch and sucrose metabolism' as well as 'other glycan degradation' being significantly enriched within the upregulated genes (Table 5-1), suggesting carbohydrate metabolism is increased. Among all significantly upregulated genes, we have also found that 'lipase' and 'lipase activity' are significantly enriched (Table 5-1), suggesting active breakdown of lipids. Interestingly, we also noticed *amontillado* (*amon*) being significantly upregulated (fold change (FC) = 1.69, p-value = 1.53 E-6). We have shown earlier, that the protease Amon could be a possible regulator of Burs secretion (Figure 4-7 C). This could suggest that upon *burs* knockdown, the fly midgut actively upregulates *amon* expression to efficiently cleave the Burs protein left, but this would need to be examined.

As expected and as a positive control of the midgut RNAseq, we found *burs* to be significantly downregulated (FC = -1.67; p-value = 0.003, Figure 5-7). Furthermore, we found a significant enrichment in Rab protein signal transduction, Arp2/3 protein complex and vesicle-mediated transport among the downregulated genes (Table 5-2), suggesting deregulated directional trafficking within cellular compartments.

We also observed genes encoding for mitophagy and autophagy enriched in the significantly downregulated genes (Table 5-2), which could suggest a deregulation of recycling of macromolecules within the cell possibly to help increase cell surface for nutrient uptake.

Overall these results suggest, that upon ee specific *burs* knockdown, flies actively enhance mechanisms to increase food absorption, which is in line with previous results.

Gene Enrichment for	p value	Benjamini
starch and sucrose metabolism	6.0E -4	3.0 E-2
other glycan degradation	2.7E -3	4.4 E-2
Lipase	9.2E -6	4.1 E-3
lipase activity	2.9E -2	4.1 E-1

Table 5-1: Gene enrichment analysis for all significantly upregulated genes in ee^{ts}> *burs*^{IR} midguts compared to controls.

Gene Enrichment for	p value	Benjamini
Rab protein signal transduction	1.8E -8	2.3 E-5
Arp2/3 protein complex	3.8 E-4	1.1 E-2
vesicle-mediated transport	3.6E -5	1.1 E-2
Mitophagy	2.9E -5	1.2 E-2
Autophagy	1.3E -4	2.1 E-2

Table 5-2: Gene enrichment analysis for all significantly downregulated genes in ee^{ts}> *burs*^{IR} midguts compared to controls.

5.4.1.1 Burs/ neuronal LGR2 signalling feeds back to the midgut

Next we analysed the RNAseq results from midguts with an ee specific *burs* knockdown and neuronal *rk* knockdown compared to their individual controls and created a heatmap, showing all genes regulated in the same way in both knockdown conditions (Figure 5-7).

Earlier we demonstrated an increase in food ingestion and absorption in *burs* and *rk* mutants, as well as in *burs* and *rk* knockdown flies (Figure 4-10, Figure 4-11 F, Figure 5-4), suggesting an active upregulation of lipases and transporters to support their high-energy demand, which could be confirmed by RNAseq analysis. We again used the functional annotation cluster within the DAVID software and found among all significantly upregulated genes enrichment for carbohydrate metabolic process and lipase, furthermore demonstrating an increased uptake of nutrients. RT-qPCR analysis could confirm increased mRNA levels for genes regulating carbohydrate and lipid uptake and metabolism in midguts of ee specific *burs* knockdown and neuronal *rk* knockdown flies (Figure 5-8). This clearly demonstrated, that loss of Burs/ neuronal LGR2 signalling leads to increased nutrient uptake.

Gene Enrichment for	p value	Benjamini
carbohydrate metabolic process	5.3 E-2	9.7 E-1
Lipase	3.3 E-2	9.1 E-1

Table 5-3: Gene enrichment analysis of significantly upregulated genes in *burs* and rk targeted knockdown midguts compared to their individual control.

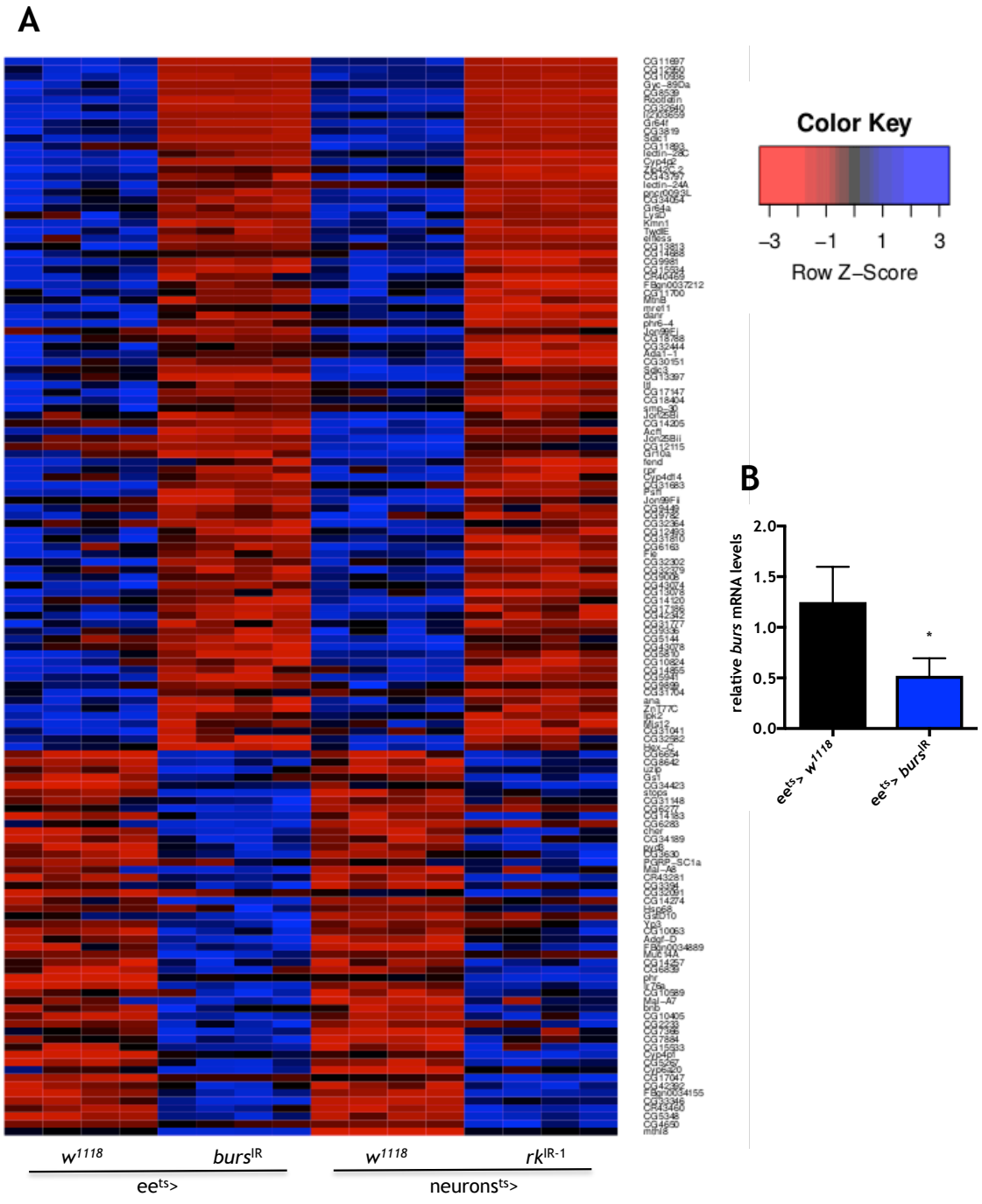

Figure 5-7: Heatmap of significantly regulated genes (FC ≥ 2) in dissected midguts.

(A) RNAseq data from dissected midguts of flies with indicated genotypes was normalised by read counts and heatmap was generated (Ann Hedley). Blue represents upregulation and red downregulation. (B) RT-qPCR for *burs* from midguts of flies with *burs* targeted knockdown. Values are relative to *rpl39* and control values were set to 1.

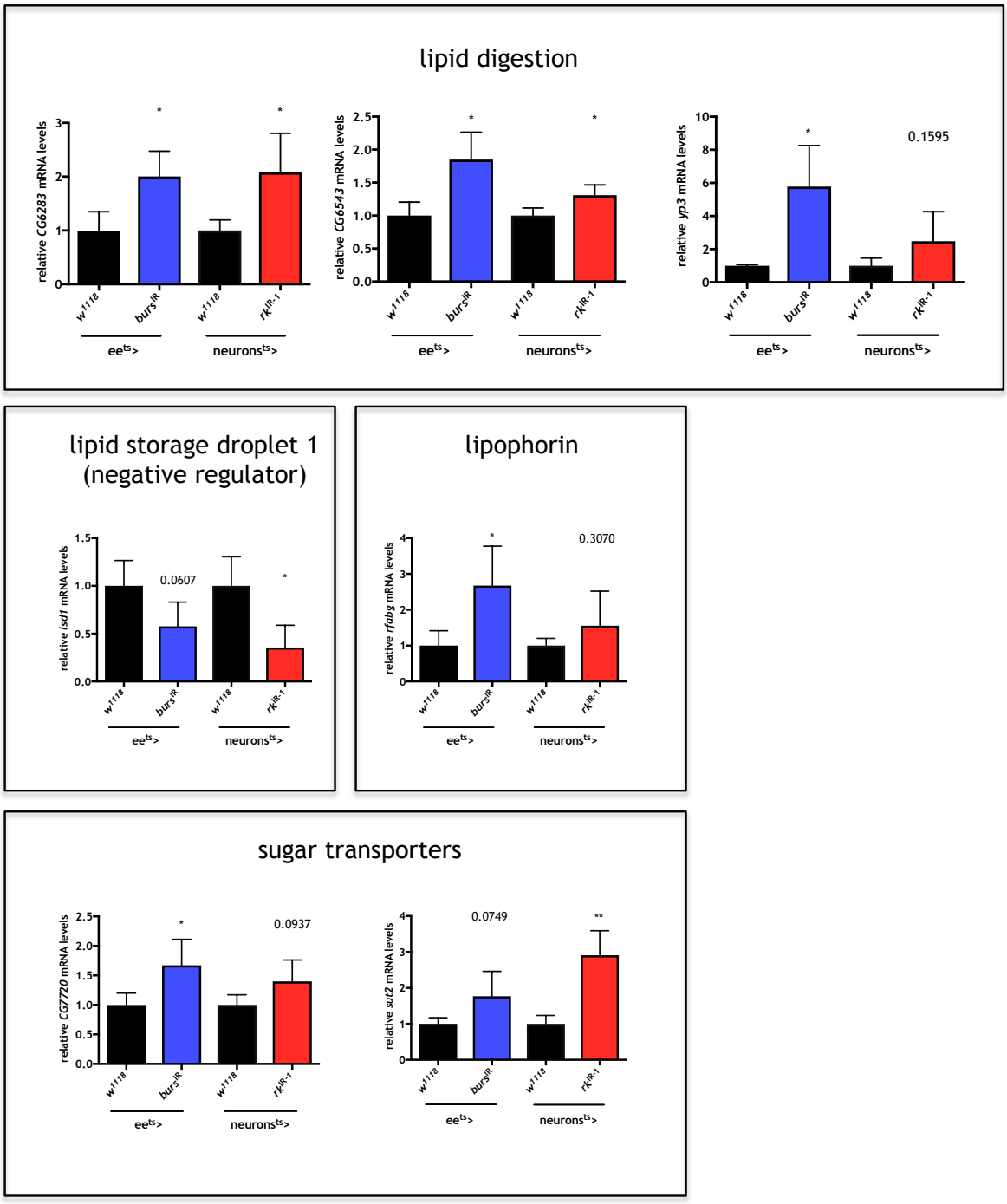

Figure 5-8: Midguts of targeted knockdown flies displayed increased lipid and sugar processing and transport.

RT-qPCR analysis of RNAseq samples from midguts of targeted knockdown flies. Values are relative to *rpl39*, and controls were set to 1.

LC-MS analysis of *burs* and *rk* mutant and control midguts showed no obvious defects in glycolysis and TCA cycle (Figure 5-10), which are important pathways to generate cellular energy in form of ATP. Additionally, energy status and charge, displayed by AMP/ATP and (ATP + 0.5 ADP) / (ATP + ADP + AMP) ratio respectively were not changed in *burs* and *rk* mutants compared to controls (Figure 5-9 A, B). The cell generates reactive oxygen species (ROS), which can damage the cells, while producing ATP within mitochondria. An important scavenger for ROS is the reduced form of glutathione (GSH) and the ratio between oxidised (GSSG) and reduced glutathione is a well-established marker of oxidative stress. We found that GSSG/GSH ratio was unaffected in dissected mutant compared to control midguts (Figure 5-9 C). These results suggest, that the cellular health of the midgut is not affected.

The midgut RNAseq and LC-MS data show that Burs/ neuronal LGR2 signalling is affecting the midgut via a feedback mechanism to regulate nutrient uptake, but has no effect on cellular health of the midgut.

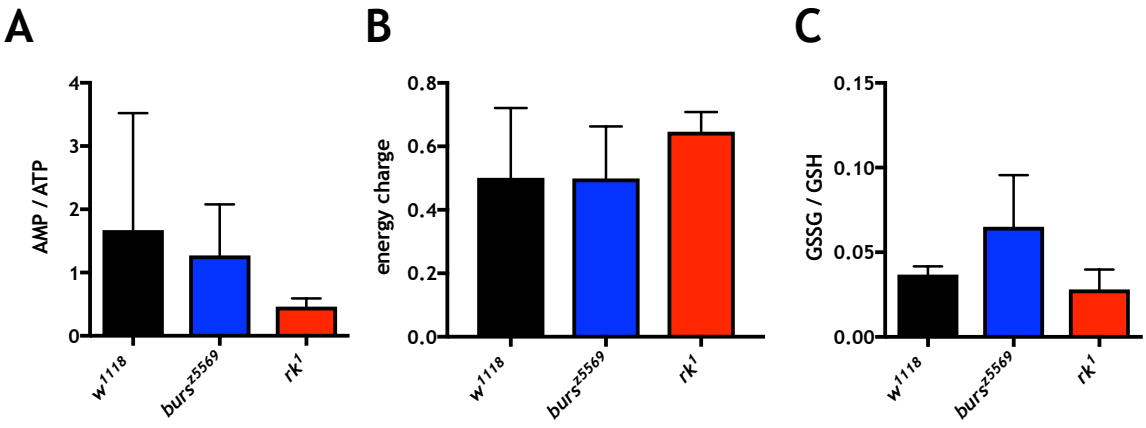

Figure 5-9: Energetic and oxidative states were not affected in *burs* and *rk* mutant midguts.

Steady state metabolites were extracted and analysed by LC-MS. Ratios of (A) AMP/ATP (energy status), (B) (ATP + 0.5 ADP) / (ATP + ADP + AMP) (energy charge) and (C) GSSG/GSH (oxidative stress) were plotted.

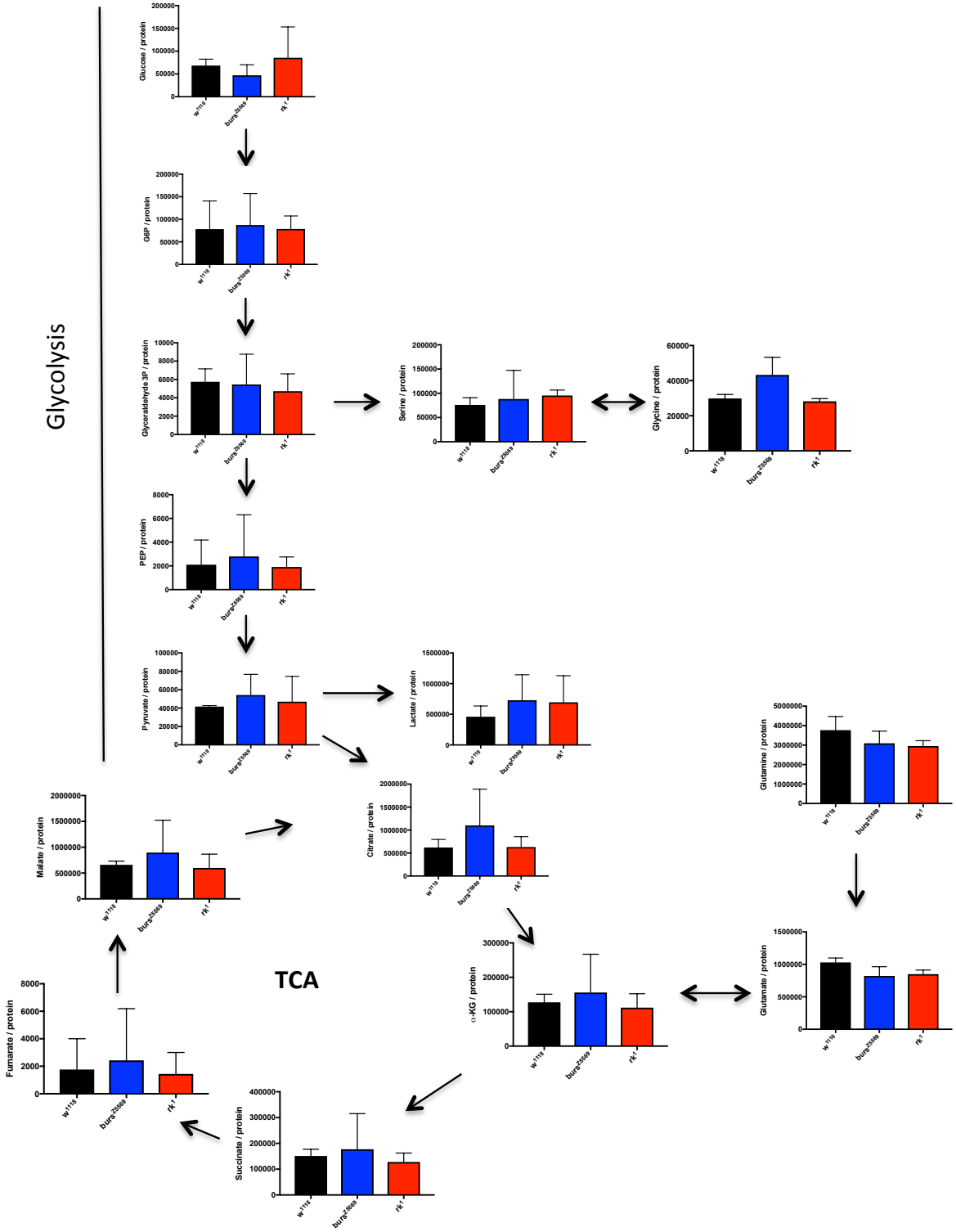

Figure 5-10: LC-MS analysis of metabolites from midguts of *w*^{1118}, *burs* and *rk* mutant flies.

Steady-state metabolites were extracted by us and analysed by Dr. Saverio Tardito using LC-MS. Peak area for each metabolite was divided by protein amount of the sample.

5.4.2 Metabolic analysis of mutant heads and transcriptomic analysis of brains from adult specific knockdown flies

Knowing that neuronal LGR2 is important to regulate energy homeostasis, we wanted to analyse the metabolites and the transcriptome of the heads/ brains from flies with disrupted Burs/ LGR2 signalling, trying to uncover downstream mechanisms.

We started by analysing the RNAseq results of dissected brains by first assessing the differentially expressed genes of brains from knockdown flies versus their individual controls. Next, we selected the significantly deregulated gene sets shared by both knockdown conditions (Figure 5-5) and created a heatmap (Figure 5-11).

Transcriptomic analysis of brains from ee specific *burs* and neuron specific *rk* knockdown animals displayed an enrichment for Arginine and Proline metabolism in all significantly downregulated genes compared to control brains, suggesting that biosynthesis of those amino acids is reduced. In agreement with those results, we found amongst the significantly upregulated genes an enrichment in amino acid transmembrane transporter activity, suggesting an increase of amino acid uptake or shuttling between organelles within the brain. Next we checked our metabolomics studies of *burs* and *rk* mutant and control heads for changes in amino acid levels. We didn't detect a clear deregulation in metabolites of Arginine and Proline metabolism or other amino acids, except Methionine was lower in both mutants compared to w^{1118} heads (Figure 5-12). Lower Methionine levels could suggest overall reduced protein translation, due to Methionine being the starting amino acid for proteins. This hypothesis was further strengthened by the RNAseq data showing significant gene enrichment for cytoplasmatic translation in all downregulated genes in knockdown compared to control brains. Reduced protein translation could be due to the starvation-like phenotype of animals with impaired Burs/ LGR2 signalling.

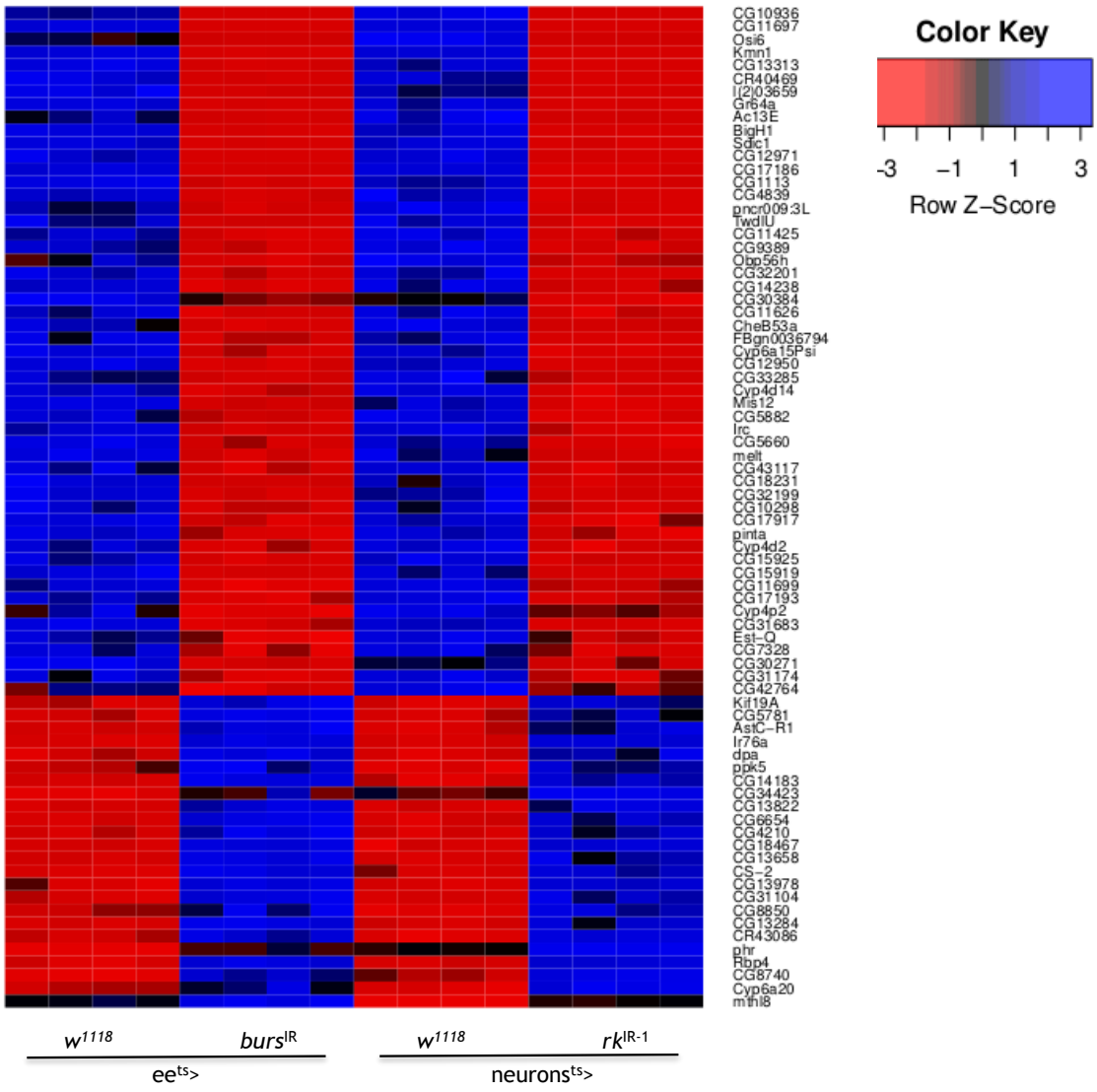

Figure 5-11: Heatmap of RNAseq data from dissected brains.

RNAseq data from dissected brains of flies with indicated genotypes was normalised by reads and a heatmap was generated (Ann Hedley). Blue represents upregulation and red downregulation.

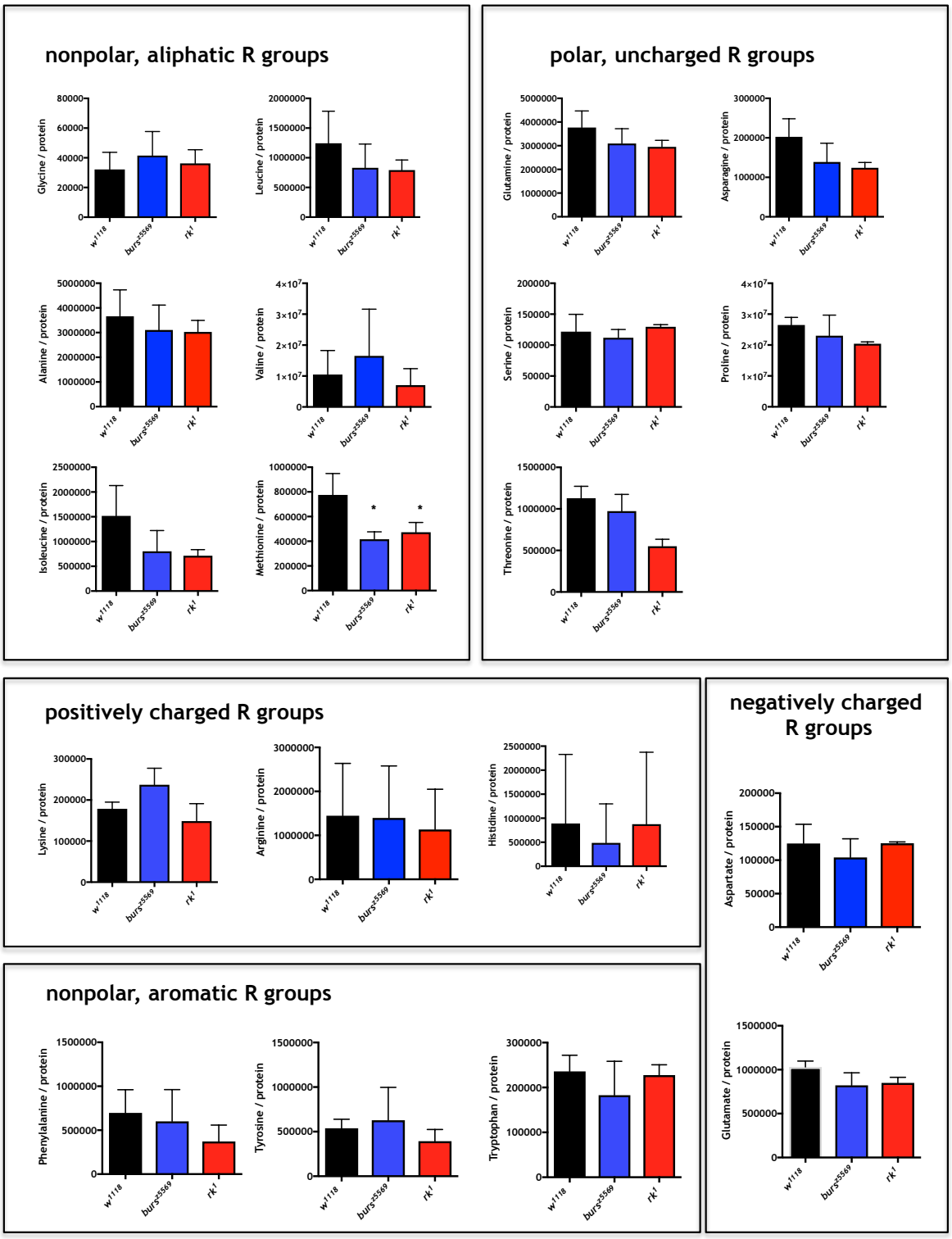

Figure 5-12: All amino acids, but methionine, are unchanged in *burs* and *rk* mutant heads.

Steady-state metabolites were extracted and analysed by LC-MS. Values of peak area were normalised by protein. Please note that only methionine is significantly lower in *burs* and *rk* mutant heads compared to controls.

In line with the starvation-like phenotype seen in *burs* and *rk* knockdown flies, we found an enrichment for the KEGG pathway 'other glycan degradation' among the significantly upregulated genes, suggesting an active breakdown of glycans to use for energy production.

Gene Enrichment for	p value	Benjamini
amino acid transmembrane transporter activity	7.8 E-4	2.1 E-1
other glycan degradation	2.3 E-2	5.5 E-1

Table 5-4: Gene enrichment analysis from all significantly upregulated genes in brains from flies with a targeted knockdown for *burs* and *rk* compared to controls.

Gene Enrichment for	p value	Benjamini
Arginine and Proline metabolism	3.1 E-4	1.9 E-2
cytoplasmatic translation	1.3 E-5	6.1 E-3

Table 5-5: Gene enrichment analysis from all significantly downregulated genes in brains from flies with a targeted knockdown for *burs* and *rk* compared to controls.

The loss of TAG in ee specific *burs* and neuron specific *rk* knockdown flies could be due to increased lipid breakdown and usage or a problem in de novo lipid synthesis. Interestingly, we found *lpr2*, a *Drosophila* lipophorin receptor, which is important for uptake of neutral lipids, significantly upregulated (Figure 5-13). Also *peroxin 3* (*pex3*), indispensible for biosynthesis and integrity of peroxisomes, was amongst the significantly upregulated genes (Figure 5-13). Peroxisomes are important for the breakdown of very long chain fatty acids to feed the electron transport chain to generate ATP. Upregulation of *lpr2* and *pex3* suggest an active increase of lipid uptake and breakdown within the brains of knockdown flies.

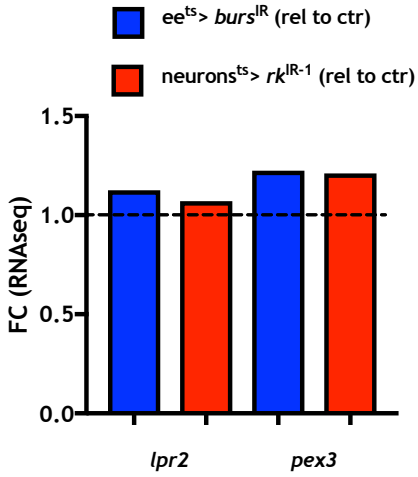

Figure 5-13: Significant upregulation of *lpr2* and *pex3* transcripts in brains of knockdown animals.

Fold change (FC) relative to respective control of RNAseq data from brains are displayed.

LC-MS analysis of mutant and control heads didn't reveal any clear differences in glycolysis and TCA cycle intermediates (Figure 5-15), suggesting that there is no loss of enzyme activity within this pathway. Also, energy status (AMP/ATP) and charge ((ATP + 0.5 ADP) / (ATP + ADP + AMP)) (Figure 5-14 A, B), as well as oxidative state analysed by GSSG/GSH ratio (Figure 5-14 C) were unaffected. *rk* mutant heads showed a higher GSSG/GSH ratio indicative of oxidative stress, which wasn't observed in *burs* mutant heads, suggesting that this discrepancy is not due to impaired Burs/ LGR2 signalling, but rather a consequence of genetic differences between them. This data suggests, that cells within the brain of flies with impaired Burs/ LGR2 signalling are healthy.

Altogether, these results suggested that flies with an ee specific *burs* and neuron specific *rk* knockdown try to cope with the overuse of energy by upregulating pathways, which help to break down macromolecules and by downregulating synthetic pathways within the brain without affecting their cellular health.

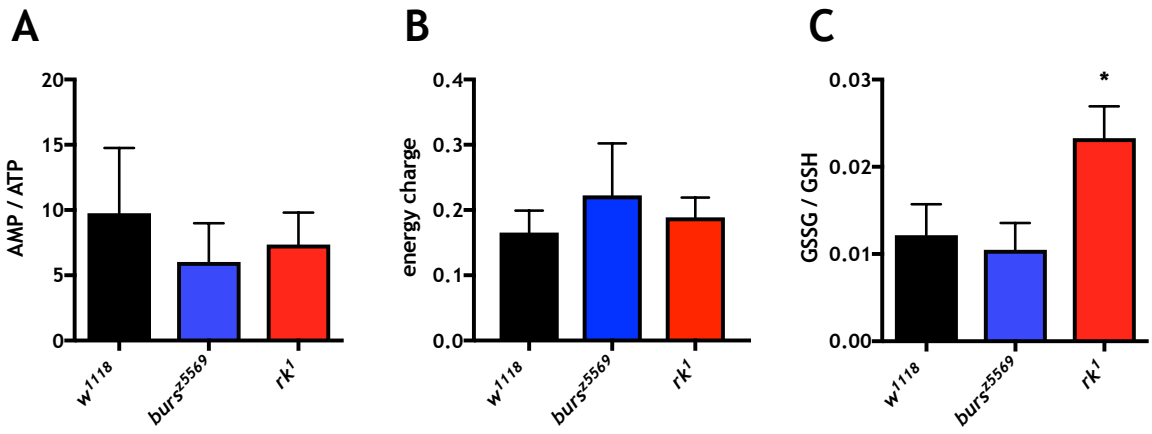

Figure 5-14: Energetic and oxidative states were not affected in *burs* and *rk* mutant heads.

Steady state metabolites were extracted and analysed by LC-MS. Ratios of (A) AMP/ATP (energy status), (B) (ATP + 0.5 ADP) / (ATP + ADP + AMP) (energy charge) and (C) GSSG/GSH (oxidative stress) were plotted.

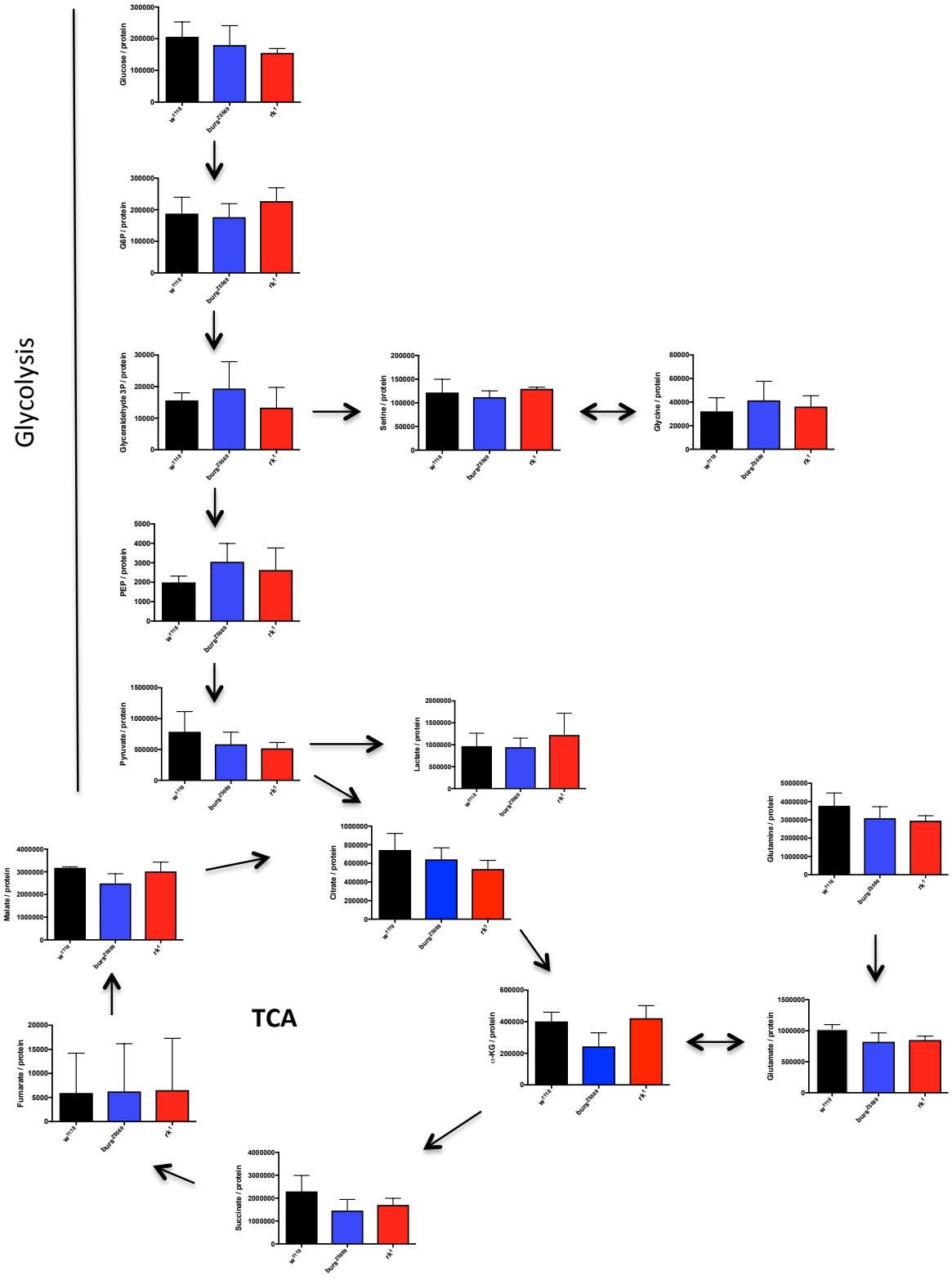

Figure 5-15: LC-MS analysis of metabolites from heads of *w*^{1118}, *burs* and *rk* mutant flies.

Steady-state metabolites were extracted by us and analysed by Dr. Saverio Tardito using LC-MS. Peak area for each metabolite was divided by protein amount of the sample.

5.4.3 Analysis of metabolites and the transcriptome of cuticles with impaired Burs/ LGR2 signalling

Lipids and carbohydrates are mainly stored within the *Drosophila* fat body. For that reason, we have also performed metabolomics (w^{1118}, *burs* and *rk* mutants) and transcriptomics (ee^{ts}> $burs^{IR}$ and $neuron^{ts}$> rk^{IR}) of cuticles, which are enriched for fat body cells.

As done for RNAseq data from midguts and brains, we first compared gene transcripts between knockdown and their individual controls. Thereafter, we compared both knockdown data sets with each other (Figure 5-5) and generated a heatmap showing all cuticle/ fat body genes, which are significantly regulated in the same direction in both knockdown conditions (Figure 5-16).

Transcriptomic analysis of cuticles from ee specific *burs* and neuronal *rk* knockdown flies showed a significant enrichment of 'lipid biosynthesis' and 'fatty acid biosynthesis' among the downregulated genes, suggesting an active reduction in *de novo* lipid synthesis. Furthermore, we have also found one of the fly's perilipins *lipid storage droplet 1* (*lsd1*; Figure 5-17 A), which serves as a protector of lipid droplets from lipase mediated lipid mobilisation, among the downregulated genes.

Additionally, we found a significant enrichment for 'glycolysis/gluconeogenesis' and 'carbon metabolism' among the significantly upregulated genes, suggesting an active use of carbohydrates to generate energy. Among those genes were *glycogen phosphorylase* (*glyp*) and *phosphoglucomutase* (*pgm*; Figure 5-17 B), suggesting an increased breakdown of glycogen, important for energy generation, in cuticles of 14d old adult specific knockdown animals.

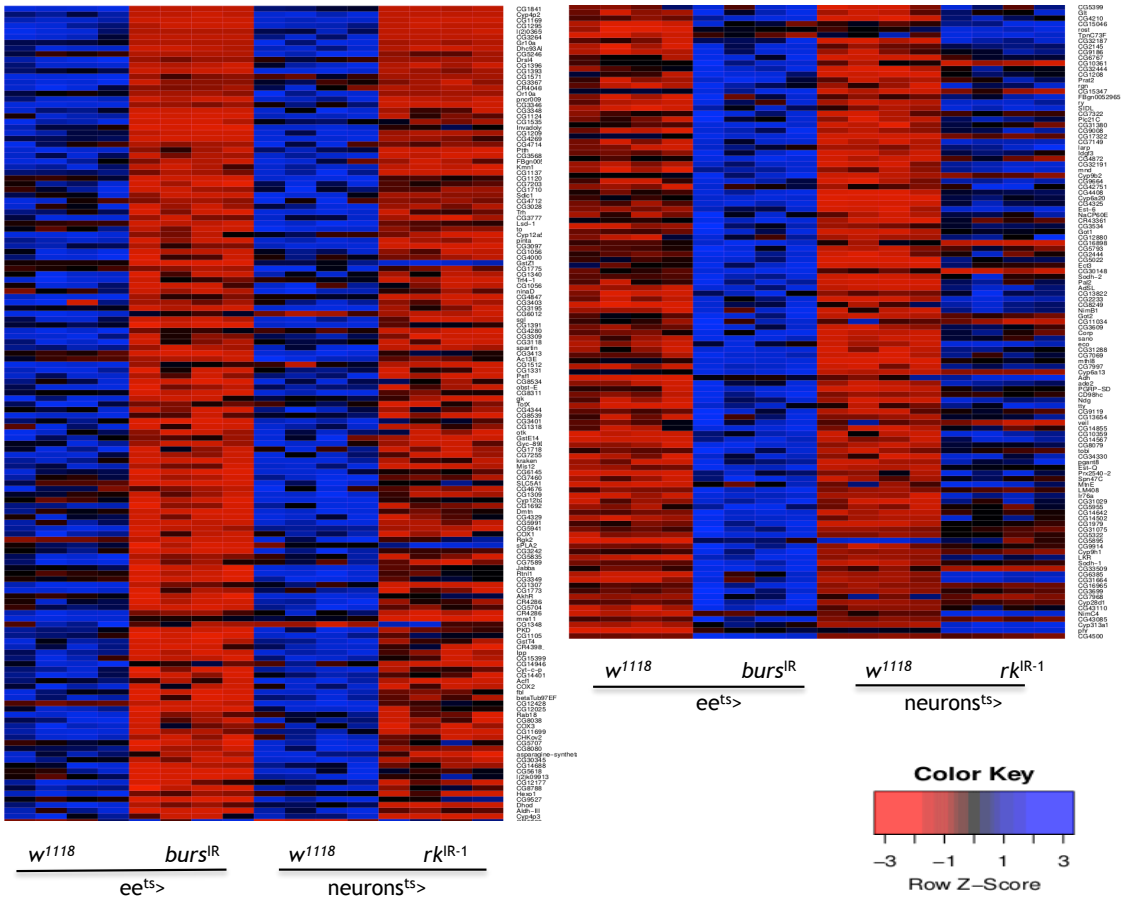

Figure 5-16: Heatmap of RNAseq data from dissected cuticles.

Cuticles from indicated genotypes were dissected, RNA extracted and further analysed by RNAseq. A heatmap of all genes, deregulated in the same manner in targeted knockdown compared to their respective controls. Blue indicates upregulation, red downregulation of transcripts.

Gene Enrichment for	p value	Benjamini
glycolysis/gluconeogenesis	2.4 E-5	6.3 E-4
carbon metabolism	1.7 E-3	2.2 E-2
oxidation-reduction process	5.1 E-10	2.3 E-7
oxidoreductase activity	1.1 E-7	2.8 E-5

Table 5-6: Gene enrichment analysis of all significantly upregulated genes in cuticles from flies with a targeted knockdown for *burs* and *rk* compared to controls.

Gene Enrichment for	p value	Benjamini
lipid biosynthesis	2.2 E-4	7.8 E-3
fatty acid biosynthesis	2.3 E-3	5.2 E-2
Hemolymph juvenile hormone binding	2.3 E-3	5.9 E-1

Table 5-7: Gene enrichment analysis of all significantly downregulated genes in cuticles from flies with a targeted knockdown for *burs* and *rk* compared to controls.

We also found gene enrichment for 'oxidation-reduction process' and 'oxidoreductase activity' amongst all significantly upregulated genes of the RNAseq from cuticles of knockdown flies compared to their controls. This could hypothetical lead to oxidative stress, but this was not supported by LC-MS analysis of *burs* and *rk* mutant cuticles revealing no significant changes in NAD^+/NADH ratio compared to control cuticles (Figure 5-19).

LC-MS analysis of *burs* and *rk* mutant and control midguts showed no obvious defects in glycolysis and TCA cycle (Figure 5-18), which are important pathways to generate cellular energy in form of ATP. Interestingly, when analysing the data of dissected cuticle metabolites, we found that AMP/ATP ratios are much lower in mutant cuticles compared to controls (Figure 5-19), which is due to low AMP and unchanged ATP levels. Also, we detected lower amounts of ADP, GMP, GDP and IMP (the precursor of AMP and GMP), but couldn't see differences of ATP and GTP peak area (Figure 5-19). This could be suggestive of a problem with *de novo* purine synthesis or salvage pathway. To further investigate a possible role of purine metabolism in Burs/ neuronal LGR2 signalling, we analysed the RNAseq data of cuticles and found among the significant upregulated genes, *adenine phosphoribosyltransferase* (*aprt*; Figure 5-17 C), which uses adenine to produce

AMP, and *adenylosuccinate lyase* (*adsl*; Figure 5-17 C), which converts IMP to AMP. Furthermore, RNAseq data showed a transcriptional upregulation of *phosphoribosylamidotransferase 2* (*prat2*; Figure 5-17 C), which encodes for the enzyme responsible for the first and rate-limiting step of *de novo* purine synthesis. Additionally, also transcripts further downstream in the pathway *adenine 2* (*ade2*, encoding a phosphoribosylformylglycinamidine synthase, Figure 5-17 C) and *adenosine kinase* (*adenok*; Figure 5-17 C) were upregulated. These results showed that purine synthesis or salvage pathway is deregulated. However, without LC-MS tracing experiments, western blotting for proteins within the pathway or enzyme activity tests, we cannot identify, how the pathway is deregulated.

Interestingly, among all the significantly downregulated genes, we have also discovered a gene enrichment for 'hemolymph juvenile hormone binding'. This could suggest that juvenile hormone signalling might be involved downstream Burs/ neuronal LGR2.

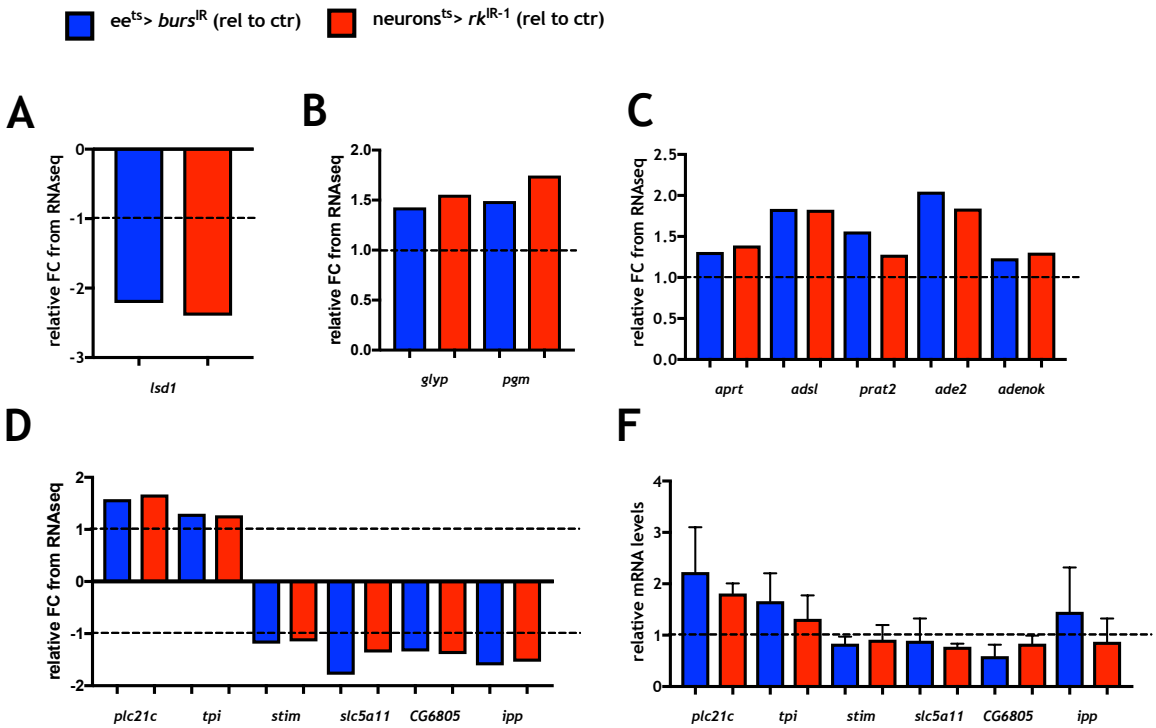

Figure 5-17: RNAseq and RT-qPCRs of cuticles from targeted knockdown flies.

(A-D) Fold change (FC) relative to respective control of RNAseq data from cuticles are displayed. (F) RT-qPCR analysis of cuticle samples from flies of indicated genotypes compared to their respective control. Shown mRNA levels are normalised per *sdha* mRNA levels and values are represented as relative to 1 for the respective controls.

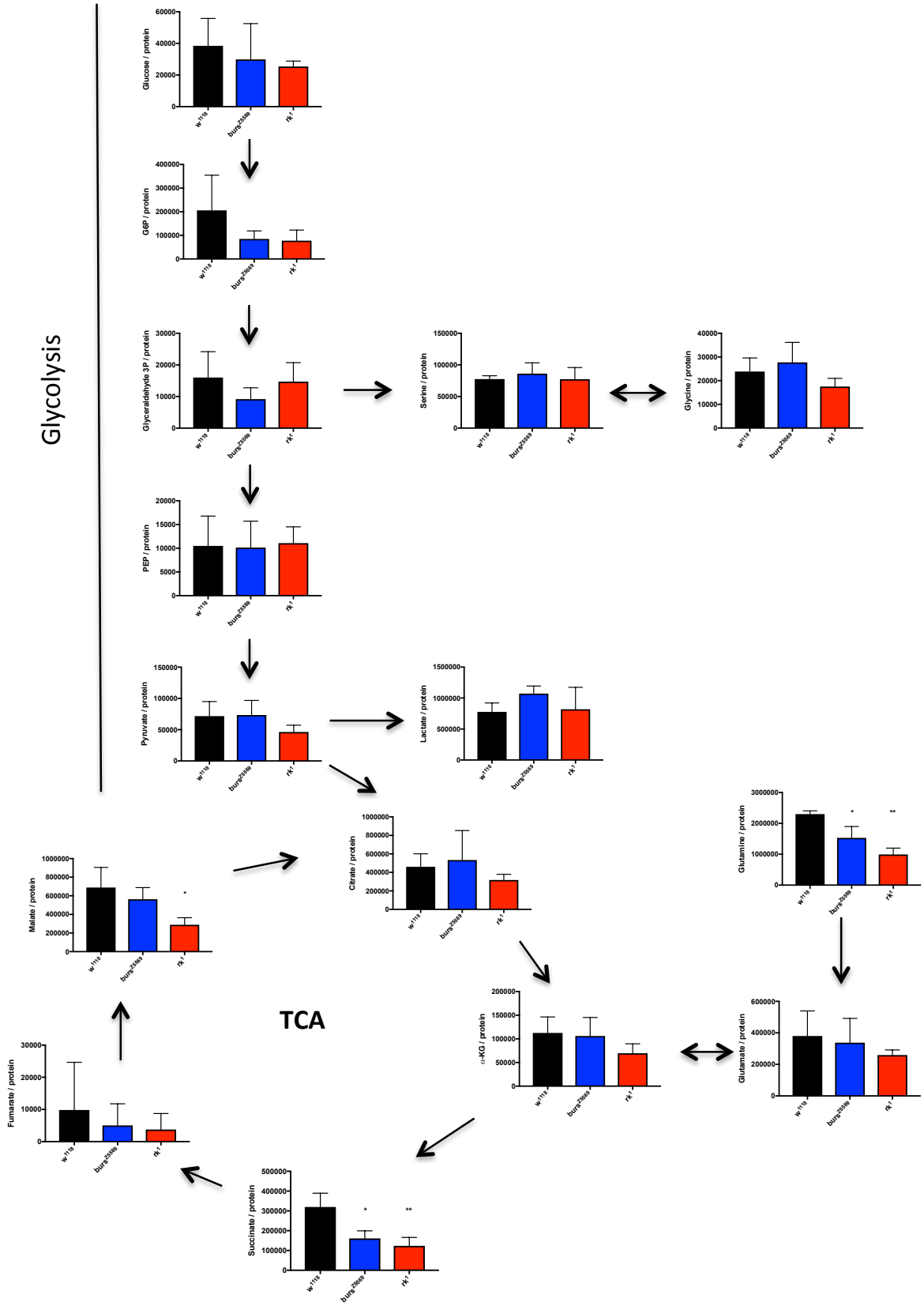

Figure 5-18: LC-MS analysis of glycolysis and TCA intermediates from cuticles of *w*^{1118}, *burs* and *rk* mutant flies.

Steady-state metabolites were extracted by us and analysed by Dr. Saverio Tardito using LC-MS. Peak area for each metabolite was divided by protein amount of the sample.

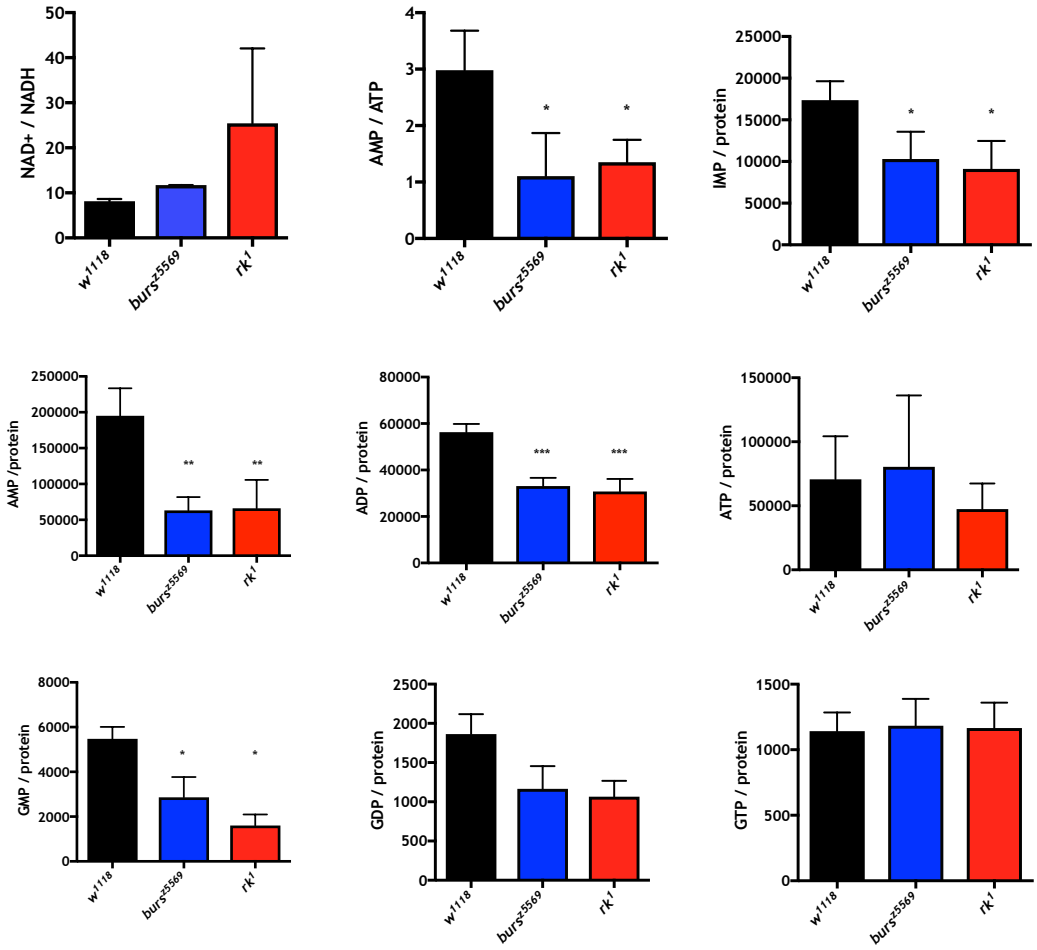

Figure 5-19: LC-MS analysis from cuticles of control and *burs* and *rk* mutant flies.

Cuticles of flies with indicated genotypes were dissected and analysed by LC-MS. Data represents values from biological triplicates.

Another interesting observation we made, whilst analysing the RNAseq data from cuticles of targeted knockdown flies, is that many genes, important for the Phospholipase C (PLC)/ inositol 1,4,5-trisphosphate (IP3) pathway, are deregulated (Figure 5-17 D). This could suggest that Bursicon/ neuronal LGR2 signalling affects the PLC/IP3 pathway. PLC modulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) and therefore leads to the generation of the second messengers, diacylglycerol (DAG) and IP3 (Nishizuka, 1995). IP3 in turn binds to the IP3 receptor on the endoplasmic reticulum to release Ca^{2+} into the cytoplasm to regulate many fundamental cellular functions, like cell proliferation and smooth muscle contraction (Berridge, 1987; Lin et al., 2016; Michell, 1975; Somlyo and Somlyo, 1994). DAG instead activates protein kinase C and D (PKC, PKD), and serves as a precursor for downstream metabolites (Nishizuka, 1995). DAG and IP3 production is stimulated by (1) receptor tyrosine kinases, which can be activated by growth factors, like Insulin, and (2) G-protein coupled receptors, which can be activated by hormones, like neurotransmitters. It has also been shown, that the ER and mitochondria can build structural links able to regulate metabolism. Furthermore, it is reported that Ca^{2+} can regulate cAMP levels (Cooper and Tabbasum, 2014; Omori and Kotera, 2007) and on the other hand cAMP regulates Ca^{2+} channels and pumps, therefore controlling the flow and levels of Ca^{2+} within the cytoplasm (Vandecaetsbeek et al., 2011). In the RNAseq data, we found *phospholipase C at 21C* (*plc21C*; Figure 5-17 F), important for cytosolic synthesis of IP3, as well as *triose phosphate isomerase* (*tpi*; Figure 5-17 F), also important for inositol phosphate metabolism and ATP production, significantly upregulated. Furthermore, we observed transcriptional downregulation of *stromal interaction molecule* (*stim*; Figure 5-17 F), important to regulate calcium levels in the cytosol, the inositol transporter *sodium/solute co-transporter-like 5A11* (*slc5A11*; Figure 5-17 F), a predicted inositol triphosphate phosphatase, *CG6805* (Figure 5-17) and *inositol polyphosphate 1-phosphatase* (*ipp*; Figure 5-17 F), producing inositol. This deregulation of the PLC/IP3 pathway could be confirmed for most genes via RT-qPCR analysis (Figure 5-17 F). This result led us to hypothesise, that the PLC/IP3 pathway within the cuticles could be a downstream modulator of Burs/ neuronal LGR2 signalling regulating metabolism, which will be discussed in more detail later in this thesis chapter.

5.5 Mechanistic insights downstream of Bursicon/ neuronal LGR2 signalling

We found that the endocrine hormone Burs acts on its neuronal receptor LGR2 to mediate glucose and lipid metabolism in *Drosophila*. The central nervous system (CNS) plays a critical role in regulating metabolic homeostasis via multiple regulatory pathways (Myers and Olson, 2012). Known regulators of metabolism are Insulin (Dilp1-7 in *Drosophila*) and Glucagon (AKH in *Drosophila*). Also many gastrointestinal hormones are known to mediate metabolic homeostasis by acting on the CNS. Hormones like Glucagon-like peptide 1 (GLP-1, Pdf in *Drosophila*) and Cholecystokinin (CCK, Dsk in *Drosophila*) are released into the blood stream after a meal to act on the CNS (Sobrino Crespo et al., 2014). We observed the same regulation of Bursicon in *Drosophila*. In fed condition, Burs is released into the hemolymph, while upon starvation the hormone is retained in the ee cells of the midgut (Figure 4-4).

We next wanted to identify the mechanism mediating this novel role of Bursicon/ neuronal LGR2 signalling.

5.5.1 Investigating the involvement of Insulin signalling in Burs/ neuronal LGR2 signalling

Insulin is a mayor anabolic hormone, which in mammals is produced and released by pancreatic ß-cells - known as Insulin-producing cells (IPCs) in *Drosophila* and situated in the CNS - to regulate carbohydrate, lipid and protein metabolism. When circulating glucose levels are high, for example after food ingestion, Insulin gets released into the blood stream to induce uptake of glucose into tissues (Sonksen and Sonksen, 2000). *Drosophila* expresses 7 Insulin-like peptides (Dilp1-7) (Brogiolo et al., 2001; Cao and Brown, 2001). Dilp2, 3 and 5 are produced in the median neurosecretory cells in the pars intercerebralis of the adult fly brain (Broughton et al., 2005). It has been shown, that *dilp2*, *3* and *5* mutants or ablating IPCs genetically (Broughton et al., 2005; Haselton et al., 2010), as well as dietary restriction (Clancy et al., 2002) can extend life span. Furthermore, ablation of IPCs leads to hyperglycemia (Rulifson et al., 2002).

Because Insulin plays a major role in metabolism, and disrupting Burs/ neuronal LGR2 signalling leads to low circulating glucose levels and loss of lipid stores, we next wanted to examine if Burs/ LGR2 affects the Insulin pathway in *Drosophila*.

We first analysed *rk* expression within the brain in conjunction with IPCs. To do so, we expressed CD8-GFP in a *rk* dependent manner and co-stained dissected brains for Dilp2 using a Dilp2 specific antibody. We found co-localisation between GFP, showing *rk* positivity and Dilp2 (Figure 5-20 A), suggesting that Dilp2 neurons could be good candidates for modulation by Burs/ neuronal LGR2 signalling.

It has been reported that upon starvation Dilp2 immunoreactivity is increased in IPCs due to retention of the protein (Enell et al., 2010; Geminard et al., 2009). We utilised a GFP-tagged *dilp2* construct (*dilp2-gfp*) and expressed it in *Drosophila* to analyse if neuronal LGR2 mediates Dilp2 secretion or retention. First, we checked if this construct gives rise to a functional Dilp2-GFP protein. To do that, we subjected flies bearing this construct to normal feeding and 24h starvation and stained the brains of those animals with an anti-GFP antibody. Consistent with previously published data, Dilp2-GFP protein levels in brains of starved animals was higher within IPCs than in well-fed animals (Figure 5-20 B). Next, we analysed flies expressing the *dilp2-gfp* construct and simultaneously knocked down *rk* in IPCs to check if the receptor LGR2 in those cells regulates Insulin secretion directly. Staining dissected brains with a GFP antibody to monitor Dilp2 protein didn't show any differences on Dilp2 protein levels within IPCs of IPC^{ts}> rk^{IR} versus controls (Figure 5-20 C). This data suggested that LGR2 is not directly effecting Insulin secretion in IPCs.

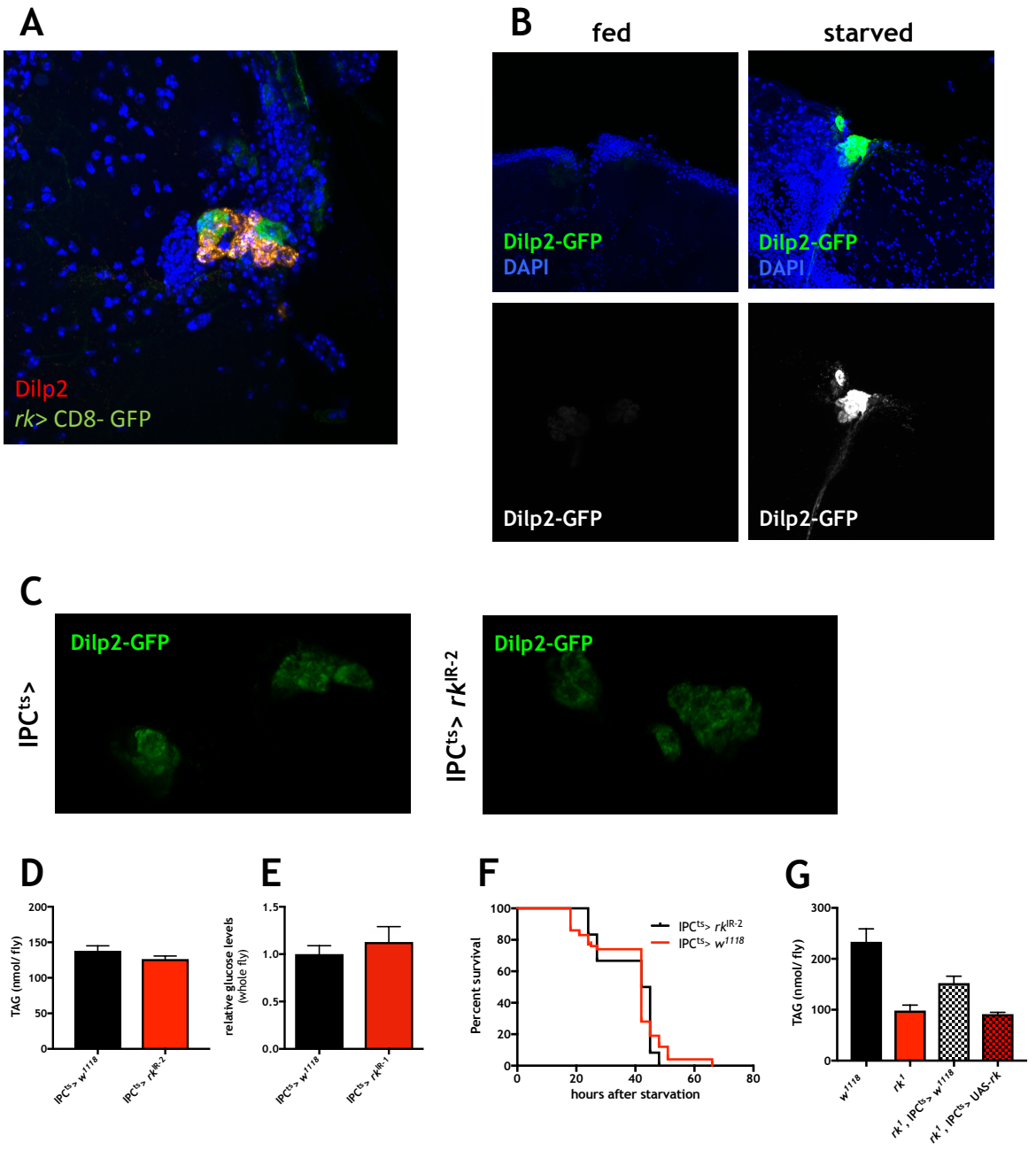

Figure 5-20: *rickets*-Gal4 is expressed in Dilp2^{+ive} cells (IPCs), but *rk* knockdown in those cells has no effect on systemic metabolism.

(A) Representative confocal maximum projection image of brains from flies expressing CD8-GFP in a *rk* dependent manner. Brains were stained with antibodies for Dilp2 (red) and GFP (green). DAPI (blue). (B) Representative confocal maximum projection images of flies expressing the *dilp2-gfp* fosmid subjected to ad-libitum feeding or 24h starvation. Note that Dilp2-GFP intensity is increased upon starvation, due to retention of the protein. (C) Representative confocal images of brains from flies of indicated genotypes, simultaneously expressing the *dilp2-gfp* fosmid. Note, that *rk* knockdown in ISCs doesn't change Dilp2-GFP fluorescent intensity. (D) Lipid measurements in whole fly extracts of indicated genotypes. (E) Whole body glucose levels in flies of indicated genotypes relative to hemolymph volume. (F) Starvation sensitivity tests in flies of indicated genotypes. (G) Lipid measurements in flies of indicated genotypes. Please note, that overexpression of *rk* in a *rk* mutant background doesn't rescue lipid levels.

Next, we tested if LGR2 within IPCs has an effect on metabolic homeostasis as seen in ee specific *burs* knockdown flies. Therefore, we knocked down *rk* specifically within adult IPCs (using Dicer2; *dilp2*-Gal4; *gal80*^{TS}, referred to as IPC$^{ts>}$), but could not detect any difference in lipid levels (Figure 5-20 D), whole fly glucose levels (Figure 5-20 E) and starvation sensitivity (Figure 5-20 F). Furthermore, we did not observe a rescue of lipid levels when re-expressing *rk* within the IPCs in a *rk* mutant background (Figure 5-20 G). Altogether, these results showed that LGR2 within IPCs does not directly regulate metabolic homeostasis.

RNAseq and qRT-PCR analysis showed a significant reduction of *dilp3* and lower, but not significant expression of *dilp5* transcript levels of brains from flies with an ee specific *burs* and neuronal *rk* knockdown (Figure 5-21 A, B) and unchanged expression in *dilp2*. It has been reported that, in response to starvation, *dilp3* and *5* transcripts are reduced, while *dilp2* expression is unchanged (Ikeya et al., 2002). Furthermore, it is reported that starvation leads to transcriptional upregulation of *inr* and *thor* (*4e-bp*), which are targets of Foxo (Junger et al., 2003; Puig et al., 2003). But neither the RNAseq from brains nor RT-qPCR analysis of heads showed significant changes in *inr* and *thor* (Figure 5-21 B), demonstrating that the starvation-like phenotype of knockdown flies doesn't affect Insulin signalling within the brain.

Next, we analysed Insulin signalling within the fat body and found no significant changes in *inr* and *thor* mRNA levels within cuticles via RNAseq and RT-qPCR (Figure 5-21 C). This would suggest, that Insulin signalling within the fat body is also not changed when Burs/ LGR2 signalling is disrupted.

These results implied, that Insulin signalling is not directly regulated by LGR2 in IPCs. Additionally, we also couldn't detect differential Insulin target expression within the brain or fat body.

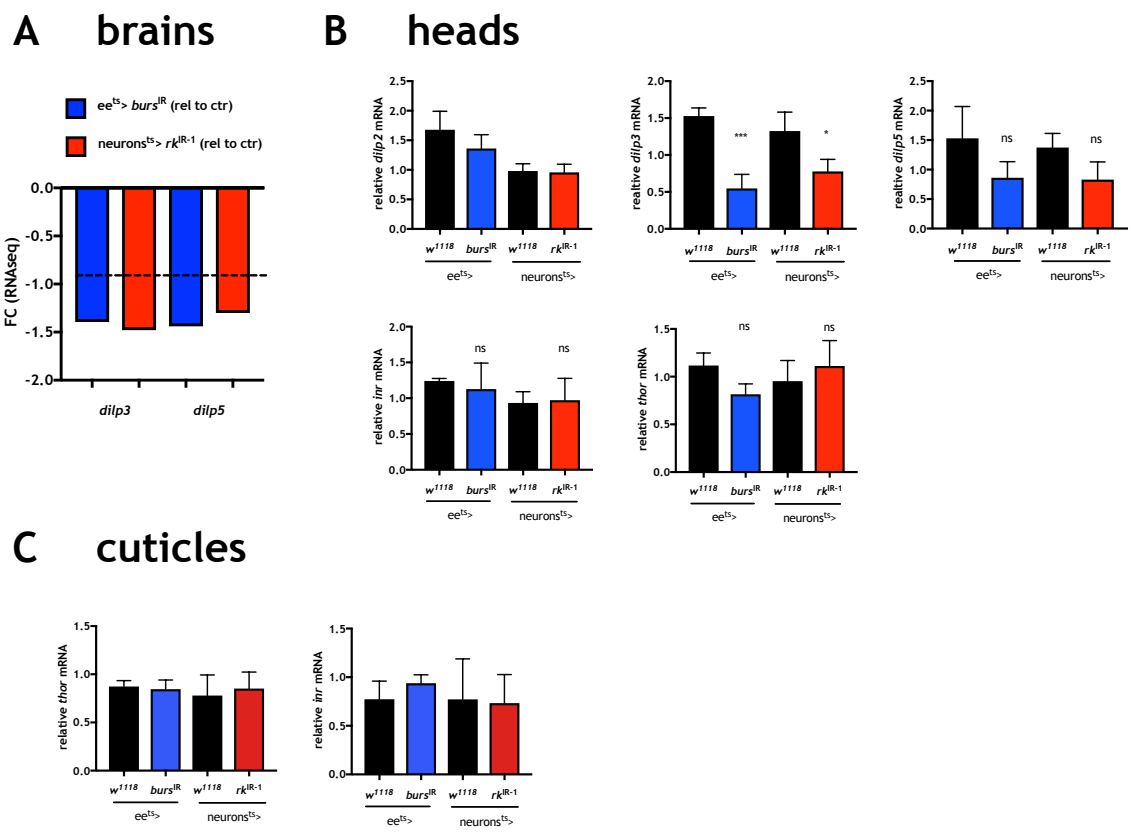

Figure 5-21: Insulin signalling doesn't seem to be affected in heads and fat bodies of flies with impaired Burs/ neuronal LGR2 signalling.

(A) Fold changes (FC) of *dilp3* and *dilp5* from RNAseq analysis from dissected brains of targeted knockdown flies relative to respective controls. (B) RT-qPCR analysis in head of flies with indicated genotypes. mRNA levels were normalised to *act5c*. (C) RT-qPCR analysis in cuticles of flies with indicated genotype. Transcript levels were normalised to *sdha*.

5.5.2 Investigating the involvement of Dsk in Burs/ neuronal LGR2 signalling

Interestingly, Nassel and his group (Soderberg et al., 2012) showed that Insulin-producing cells do not just produce Insulin, but also a hormone called Drosulfakinin (Dsk) (homolog of the mammalian Cholecystokinin (CCK)), which was found to mediate food intake (Nichols et al., 1988). Notably, Dsk is also expressed by several other neurons in addition to IPCs (Nichols, 1992; Nichols and Lim, 1996). We found *rk* expression in many neuronal cells, not just IPCs, making it possible that Dsk is the neuronal regulator of the metabolism downstream of Burs/ neuronal LGR2 signalling. Because Dsk regulates feeding behaviour (Soderberg et al., 2012), we asked whether there might be a connection between LGR2 and Dsk. To test this, we knocked down *dsk* in neurons and found that flies showed decreased levels in TAG, which were comparable to those observed upon neuronal *rk* knockdown (Figure 5-22 A). In *Drosophila* two Dsk receptors are known, CCKLR17-D1 and –D3. We next analysed the expression pattern of *ccklr17-d1* and *-d3* within the brain by expressing RFP with a *ccklr17-d1* and *-d3* specific driver. We found that just the *ccklr17-d3* Gal4 showed positivity in neurons of the adult *Drosophila* brain (Figure 5-22 B, *ccklr17-d1* Gal4 not shown). This led us to hypothesise, that neuronal Dsk/CCKLR17-D3 signalling might be the downstream effector of Burs/ neuronal LGR2. To test if neuronal CCKLR17-D3 controls lipid metabolism, we knocked down *ccklr17-d3* and, as a control, *ccklr17-d1* from all neurons and measured lipid content of the flies. We observed a clear reduction in TAG levels when knocking down *ccklr17-d3,* but not *ccklr17-d1* (Figure 5-22 A), which is in agreement with our hypothesis. This shows that disruption of neuronal Dsk/CCKLR17-D3 signalling phenocopied metabolic effects observed in flies with disrupted Burs/ neuronal LGR2 signalling.

Next, we performed various genetic experiments to assess the epistatic relationship between both pathways, but failed to find genetic interactions (data not shown) suggesting a lack of a functional connection between Dsk and Burs/ neuronal LGR2 signalling.

For now, we conclude, that neuronal DSK/ CCKLR17-D3 is not mediating the metabolic phenotype downstream of Burs/ neuronal LGR2, but it might mediate

the increased feeding behaviour observed upon loss of Burs/ neuronal LGR2, which would have to be tested in follow up experiments.

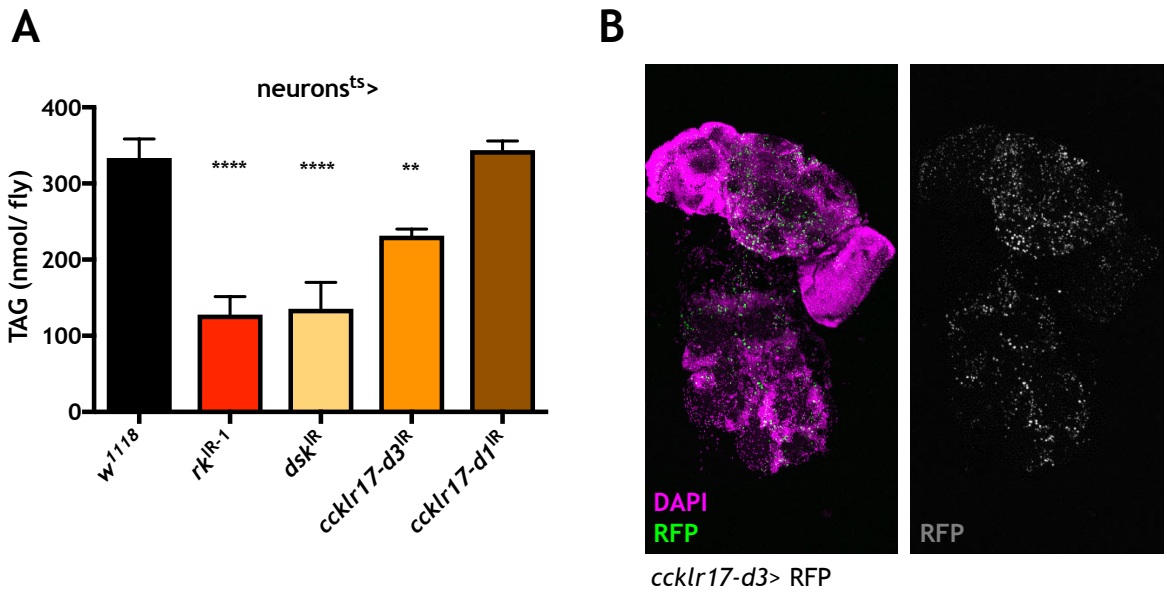

Figure 5-22: Knockdown of *dsk* and *ccklr17-d3*, but not *ccklr17-d1* in neurons resulted in whole body TAG reduction.

(A) Whole fly lipid levels of flies with indicated genotypes were measured and displayed as lipid concentration per fly. (B) Representative confocal maximum projection image of adult brains expressing RFP in a *ccklr17-d3* dependent manner. Note many neurons are positive for *ccklr17-d3* expression.

5.5.3 Investigating the involvement of AKH signalling in Burs/ neuronal LGR2 signalling

The regulation of glucose metabolism is essentially achieved by two central pathways: Insulin signalling, which promotes the transport of glucose from circulation into various tissues to store it, and Glucagon signalling, which acts in the opposite way by promoting the breakdown and release of stored energy sources.

Fasting leads to low circulating glucose levels, which is the signal for pancreatic α-cells to release Glucagon (AKH in *Drosophila*), into the bloodstream. It has been shown in multiple reports that increased AKH signalling leads to the mobilisation of energy stores (Kim and Rulifson, 2004; Lee and Park, 2004). AKH is expressed in the endocrine tissue called corpora cardiaca (CC). It has been shown that IPC axons directly project to the CC directly connecting Insulin and Glucagon producing cells (Ikeya et al., 2002; Lee and Park, 2004; Rulifson et al., 2002). Interestingly, our RNAseq data from the cuticles of ee^{ts}> $burs^{IR}$ and $neuron^{ts}$> rk^{IR} flies indicated that AKH/ AKHR signalling might be affected. There are 2 known pathways downstream of AKH/ AKHR. One is acting via Phospholipase C (PLC21C), mediating release of Ca^{2+} (Baumbach et al., 2014), whereas the other arm of the cascade is mediated by adenylyl cyclase to increase cAMP levels. In our RNAseq data from cuticles we found transcriptional changes in both of those pathways. Therefore, we hypothesised, that AKH/ AKHR signalling in targeted knockdown flies is deregulated, which leads to metabolic changes and energy loss.

To test for an involvement of AKH/ AKHR signalling, we started by analysing if *rk* is expressed within AKH^{+ive} cells. To do so, we expressed GFP under control of a *rk*-specific driver and simultaneously stained with an AKH antibody. We found that AKH positive neurons didn't co-localise with *rk* expressing neurons but they were in very close contact to each other (Figure 5-23 A). Therefore, we tested whether *rk* within neurons regulated AKH signalling indirectly. We knocked down *akh* and *rk* simultaneously from all neurons and checked survival upon starvation compared to single knockdown of *akh* and *rk*, as well as to control flies. As expected, flies bearing neuronal *rk* knockdown showed reduced survival upon starvation when compared to control animals (Figure 5-23 B). Strikingly, we observed a highly significant increase in survival upon starvation of animals bearing combined

neuronal knockdown of *akh* and *rk* (Figure 5-23 B). Altogether, this data suggests that AKH signalling is increased in flies with disrupted Burs/ neuronal LGR2 signalling and therefore reducing AKH levels is able to rescue starvation sensitivity.

These results are very exciting and promising, and more experiments are being conducted to confirm whether AKH signalling is indeed a downstream mediator of Burs/ neuronal LGR2 signalling during systemic metabolic homeostasis.

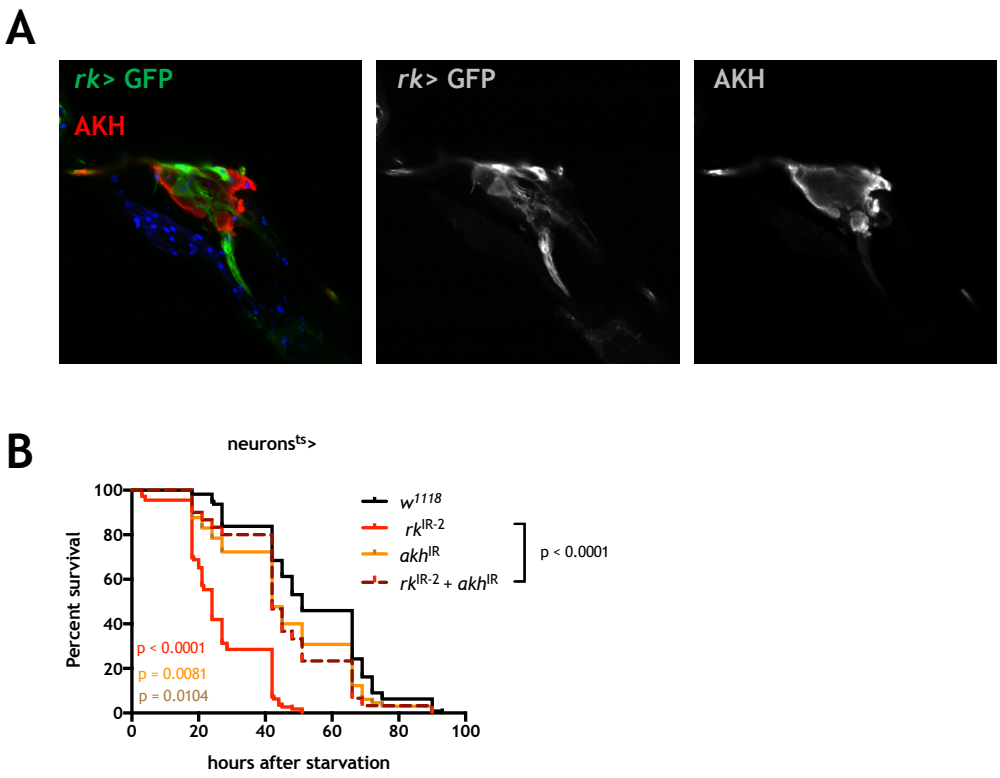

Figure 5-23: AKH is a possible downstream target of Burs/ neuronal LGR2 signalling.

(A) Representative confocal image of the AKH-producing corpora cardiaca. Flies expressed GFP in a *rk* dependent manner and tissues were stained with antibodies for GFP (green) and AKH (red). Note, no co-staining of GFP and AKH is observed. (B) Starvation sensitivity tests with flies of indicated genotypes. Coloured p-values present significance compared to control. Note simultaneous *rk* and *akh* knockdown is significantly more resistant to starvation compared to *rk* knockdown alone.

5.6 Conclusions

Here we demonstrated that the neuronal receptor LGR2 is responsible to maintain systemic metabolic homeostasis. Furthermore, 2 unbiased approaches, LC-MS and RNA sequencing, gave us confidence, that loss of energy reserves observed in flies with disrupted Burs/ neuronal LGR2 is due to increased energy breakdown and not a consequence of a failure in nutrient uptake. We clearly demonstrated that Burs/ neuronal LGR2 signalling is necessary to balance systemic metabolism, which is possibly maintained by counteracting AKH/ AKHR signalling. This hypothesis is being further investigated.

So far, we found a possible underlying downstream mechanism regulating Burs/ neuronal LGR2 signalling, which is a novel regulator of systemic glucose and lipid metabolism in adult *Drosophila*.

6 Discussion

6.1 Main conclusions

During development it is known that the hormone Bursicon consists of a heterodimer of 2 cysteine knot proteins, Burs-α and -ß (Luo et al., 2005). Through the work presented in this Thesis, we could clearly demonstrate, that during adult life, Burs-α is responsible for maintaining local intestinal homeostasis and systemic metabolism. This function of Burs-α is independent of its role in development and does not require association with its partner Burs-ß and development (Scopelliti et al., 2016; Scopelliti et al., 2014).

We showed that *burs-ß* mutant midguts displayed a wild type-like phenotype, whereas *burs-α* mutants exhibited multilayering and increased proliferation of the intestinal epithelium, suggesting *burs-ß* independent regulation of intestinal homeostasis (Scopelliti et al., 2016). We further confirmed this hypothesis by overexpressing *burs-α* within cell types of the midgut, where we normally don't detect endogenous gene expression, which was able to significantly reduce age and damage induced overproliferation (Scopelliti et al., 2016). Furthermore, synthetic Burs-α protein was sufficient to induce cAMP signalling within the visceral muscle in a LGR2 dependent manner (Scopelliti et al., 2016).

In addition to the role of Burs-α (Burs) in maintaining midgut homeostasis, we demonstrated that Burs expressed in ee cells also controls metabolic homeostasis, independently of LGR2 in the visceral muscle, but dependent on neuronal LGR2 expression. We found that impairing Burs/ neuronal LGR2 led to a decrease in lipid storage, reduced circulating glucose levels and starvation resistance, which was independent of feeding behaviour and locomotor activity.

The metabolic phenotypes observed in *Drosophila* with disrupted Burs/ neuronal LGR2 signalling resembled many symptoms of hypermetabolism, which is defined as a physiological state in which the basal metabolic rate of the organism is abnormally increased. Symptoms in humans include increased caloric intake, weight loss, muscle weakness, fatigue and sweating, which can have different causes, like infections, fever, insomnia, hyperthyroidism or prolong steroid therapy.

The results presented in this thesis and many other reports clearly show the importance of using *Drosophila* as a model organism to study inter-organ communication and the regulation of metabolism. Using *Drosophila* had a tremendous impact in understanding many biological pathways and gene functions, which are conserved between flies and mammals. Many of those studies, especially large-scale screens, couldn't have been done that easily and quickly in mammalian models.

6.2 Burs-ß is dispensable for intestinal homeostasis in adult *Drosophila*

Our work provides evidence, that the neuroendocrine hormone Burs-α has a role in the maintenance of ISC quiescence in the adult *Drosophila* midgut without its heterodimeric partner Burs-ß. Here, we showed that, despite the common requirement for Burs-α, Burs-ß and LGR2 during development, this signalling system operates in a different manner during adult tissue homeostasis. Namely, Burs-α, but not Burs-ß is involved in the latter. Critically, our FLIM-FRET experiments clearly showed that cAMP production was mediated by Burs-α via its G-protein-coupled receptor LGR2 in the visceral muscle, which makes it unlikely that an unknown receptor is involved in Burs-α dependent signalling (Scopelliti et al., 2016).

These results challenged the current view of Bursicon-LGR2-cAMP signalling triggered by obligate heterodimers between Burs-α and Burs-ß. *In vitro* competitive binding experiments previously excluded any biological activity of Burs-α or –ß homodimers (Luo et al., 2005), but there is also some evidence, suggesting that Burs-α and –ß homodimers exist *in vitro* and *in vivo* (Honegger et al., 2002). However, their biological functions have remained mostly elusive.

Another report supporting our data comes from Dai and colleagues, showing that during moulting in *Manduca sexta* and *Teleogryllus commodus* some neurons express just one of the 2 different Bursicon subunits (Dai et al., 2008).

Bursicon/ LGR2 signalling during development is undoubtedly mediated via its heterodimeric complex of Burs-α and –ß, but our cAMP *ex vivo* and *in vivo* functional data argues that at least in the adult midgut Burs-α acts likely as a

homodimer. The observed discrepancies between our work and published data might be due to context and developmental stage specific differences. For example, the gut might express cofactors, which could facilitate Burs-α homodimer binding and that may be lacking in cell-based studies (Luo et al., 2005).

Our study represents the first evidence for a Burs-α specific and Burs-ß independent role of the enteroendocrine hormone Bursicon and provides critical insights into the understanding of Bursicon/ LGR2 signalling in adult tissue homeostasis.

6.3 Burs/ neuronal LGR2 regulates systemic metabolism

Burs from ee cells acts on its neuronal receptor LGR2 to maintain metabolic homeostasis and disruption of this signalling led to increased lipid usage even when nutrients were available ad-libitum. Also in mammalian systems many gut hormones, like Ghrelin (Tschop et al., 2000; Wren et al., 2001), Cholecystokinin (CCK) (Liddle et al., 1985; Rehfeld, 2004), Gastric Inhibitory Polypeptide (GIP) (Meier et al., 2002; Woods et al., 1981), Glucagon-like peptide 1 (Baggio et al., 2004; Turton et al., 1996) and 2 (Tang-Christensen et al., 2000) (GLP-1 and 2) to name a few, are known to regulate energy metabolism, most by acting on the brain to regulate appetite and food intake. Pancreatic Polypeptide (PP) is a gut hormone regulated in a circadian rhythm, due to the circadian food intake. It has been shown, that PP is secreted into the bloodstream after a meal (Track et al., 1980), but it is also regulated by other gut hormones; such as Ghrelin, Motilin, Secretin and Somatostatin (Arosio et al., 2003; Funakoshi et al., 1989; Gomez et al., 1997). This is highlighting the complexity of hormone interaction and control of metabolism.

Many gut hormones have been shown to affect food intake and weight in the same direction with weight gain or loss associated to increased or decreased food intake, respectively. In our studies, we observed that impairment of Burs/ neuronal LGR2 signalling resulted in hyperphagic, yet leaner flies.

A recent study in *Drosophila* demonstrated, that loss of Foxo activity in muscles resulted in hyperphagic, but leaner flies, which was due to decreased *de novo*

lipid synthesis from glucose (Zhao and Karpac, 2017). However, the recent report showed several differences compared to our results obtained in flies with disrupted Burs/ LGR2 signalling. For example, they observed reduced intestinal lipid staining when Foxo was inhibited within the muscles (Zhao and Karpac, 2017), which we have not observed (data not sown). This would indicate, that the reasons for decreased lipid levels might be different, but it would still be interesting to analyse *de novo* lipid synthesis and breakdown in flies impaired for Burs/ neuronal LGR2 signalling.

It has been shown, that many gut hormones also function within the CNS as neurotransmitters, like CCK, Ghrelin and GLP-1 and -2. Knowing that Burs and the ee-Gal4 driver we used in our studies are expressed throughout the CNS, we carefully evaluated Burs immunoreactivity within adult brains and found no Burs^{+ive} cells (Figure 6-1), which is in line with reported data showing that Burs^{+ive} neurons undergo apoptosis just after adult emergence (Honegger et al., 2011). To rigorously show that Bursicon expression/secretion is not regulated within the adult CNS, we analysed Burs immunoreactivity after starving flies for 24h. Unlike Burs regulation by starvation in the midgut, we couldn't detect Burs^{+ive} neurons after 24h starvation (Figure 6-1), showing the unlikeliness that Bursicon is expressed in the adult CNS and could be mediating the metabolic phenotypes observed in the knockdown animals.

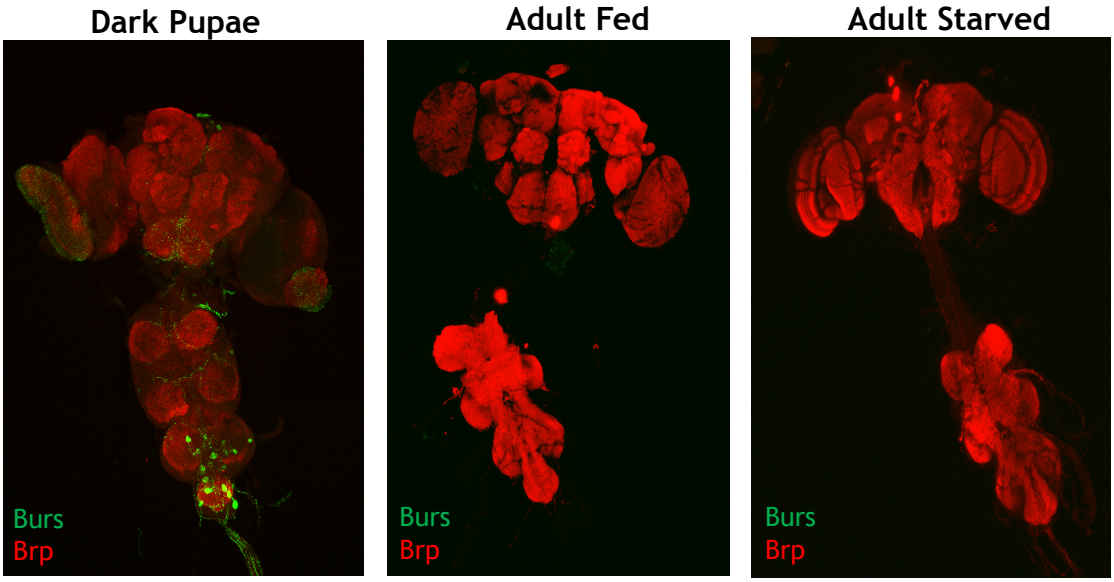

Figure 6-1: No Burs protein detectable in the adult *Drosophila* CNS.

Representative confocal maximum projection images of brains stained with a Burs specific antibody (green) and Brp (red) to label the neuropil. Note, during development (Dark Pupae) many Burs^{+ive} neurons are detectable, but not in the adult CNS (fed and 24h starved).

6.3.1 Burs regulation within ee cells

Our results clearly showed, that Burs from ee cells is secreted into the hemolymph in the fed state and retained when the flies are starved. The same regulation has been reported for mammalian gut hormones, like GLP-1 (Ghatei et al., 1983; Kreymann et al., 1987; Orskov et al., 1994), while Ghrelin is regulated in the opposite fashion (Cummings et al., 2001). Our experiments also demonstrated a Glut1 dependent regulation of Burs within the ee cells, supporting the idea that Glut1 regulates Burs secretion into the hemolypmh, which would have to be further examined, for example by Western Blotting analysis for Burs of hemolymph from ee specific *glut1* knockdown flies.

In mammalian systems it has been reported that diverse nutrient sensors are expressed in endocrine cells of the intestine. Glucose within the lumen of the gastrointestinal tract is responsible for the inhibition of food intake (Savastano et al., 2005) and stimulation of pancreatic secretion of Insulin (Drucker, 2007; Li et al., 2001), amongst others. Interestingly, GIP, GLP-1 and -2 are secreted in response to glucose (Dockray, 2013; Drucker, 2007). Additionally, glucose within the gut lumen leads to the upregulation of glucose transporters within ECs to increase glucose uptake (Dyer et al., 2007). Our results indicated that flies with disrupted Burs signalling within ee cells are likely unable to sense luminal glucose levels, possibly via Glut1. Interestingly, we observed transcriptional upregulation of transporters and enzymes in midguts of targeted *burs* and *rk* knockdown flies (Figure 5-8), and increased nutrient absorption by the intestinal epithelium (Figure 4-10, Figure 5-4), suggesting that ee sensing and EC uptake of nutrients are not coupled and rather indicated that ee specific glucose sensing regulated metabolic homeostasis by mediating use or storage of energy.

In agreement with a Glut1 dependent carbohydrate sensing, we observed that re-feeding flies with a sucrose solution after 24h starvation showed less Burs

immunoreactivity within ee cells (

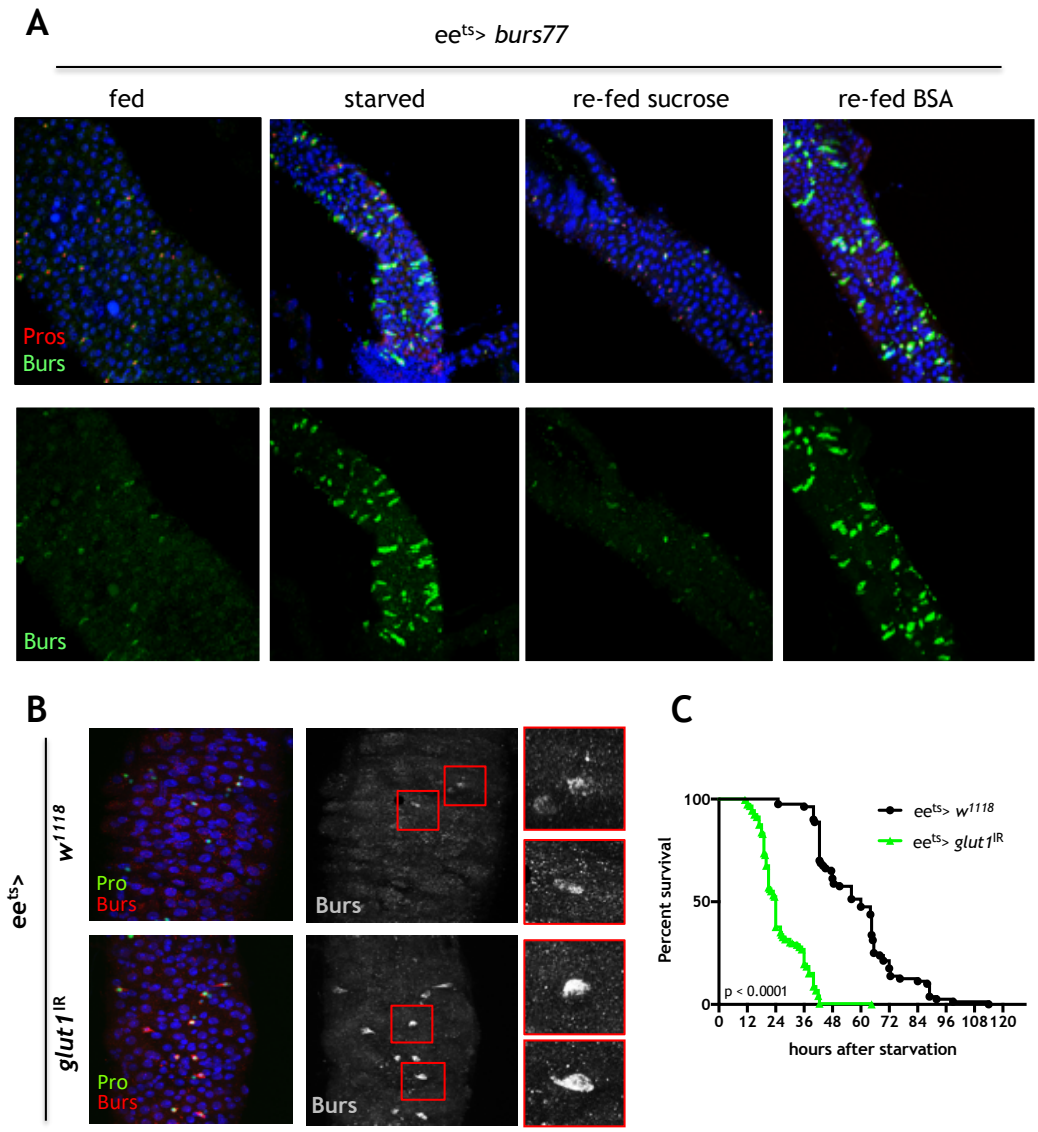

Figure 4-5), suggesting increased secretion. Carbohydrate signalling is mainly regulated by Insulin, but we didn't observe any indication of ee cell autonomous involvement of Insulin signalling. This suggested that Insulin signalling within ee cells doesn't affect Burs regulation and therefore systemic metabolism. But those results don't rule out that systemic Insulin signalling could influence Burs secretion within ee cells, which will be discussed later.

6.3.1.1 How is the release of Burs from ee cells regulated?

Upon starvation we saw more Burs immunoreacticity in ee cells and less Burs hormone in the hemolymph, showing that starvation leads to gut retention of the hormone. Furthermore, upon starvation, midgut ISCs enter quiescence, which can be explained by the increased Burs hormone within ee cells able to control local

signalling through VM LGR2 and therefore inhibiting proliferation. This is in line with observed reduction in intestinal stem cells proliferation when *burs* is overexpressed in ee cells, or other cytotypes within the gut (Scopelliti et al., 2016; Scopelliti et al., 2014). Therefore, knocking down *burs* from ee cells resulted in increased proliferation of ISCs, due to decreased Burs within ee cells, and led to the systemic metabolic switch resulting in loss of lipids, because circulating Burs levels are low.

Furthermore, we found that Burs in ee cells is regulated by Glut1 and luminal sucrose, suggesting that Burs is likely secreted upon intake of carbohydrates. Additionally, future experiments would have to be done to analyse if systemic Insulin signalling is regulating Burs in ee cells. This could be done for example by immunostainings for Burs in Insulin mutant and control midguts and Western Blotting for Burs in hemolymph of Insulin mutant animals. Preliminary data further suggested, that a possible protease, Amontillado, might be involved in Burs secretion. It would be interesting to explore the function of Glut1 and Amon in regulating Burs secretion and retention in future experiments.

The lack of a metabolic gain of function phenotype when overexpressing *burs* in ee cells is not surprising, because Burs is secreted in feeding conditions. So far, we have only analysed TAG levels in fully fed flies and starvation sensitivity, and couldn't detect any changes in *burs* overexpressing animals compared to control ones. It would be interesting to analyse how starvation affects Burs levels in the hemolymph, when *burs* is overexpressed within ee cells. Does that lead to increased, circulating Burs levels? Or is Amon/Glut1 upstream Burs blocking its secretion? Do those flies display more lipids upon 24h starvation? And how does Amon exactly regulate Burs in ee cells? These questions would also need to be addressed in future experiments to dissect the role of Glut1/Amon/Burs regulation within the adult midgut.

6.3.2 Nutrient sensing and gut-derived hormones effect the brain

Our data demonstrated that loss of *burs* from ee cells and the receptor *rk* from neurons are responsible for the observed metabolic switch. Also in mammals it has been reported that gut derived hormones are secreted and act on the brain. Ghrelin is the first intestinal hormone discovered, mainly produced by the stomach

(Kojima et al., 1999), which induces growth hormone secretion from the endocrine pituitary gland and therefore is able to cross the blood-brain barrier (Stengel et al., 2013). Ghrelin stimulated growth hormone release and led to an increase in cellular Ca^{2+} levels by activating the IP3 pathway (Kojima and Kangawa, 2005). Our preliminary results suggested a role of PLC/IP3 and calcium signalling in the fat body downstream of Burs/ neuronal LGR2 due to modulation of neuronal AKH/fat body AKHR (Glucagon-like signal).

Furthermore, it is very important to sense nutritional status within the intestinal lumen to react accordingly. Interestingly, glucose dependent secretion of the incretin GLP-1 modulates Insulin secretion (Ahren, 2004), and the use of transgenic mice deleted for the glucose sensor *gustducin* or *T1R3* displayed abnormalities in GLP-1 and GIP secretion, Insulin and glucose levels (Shirazi-Beechey et al., 2011), showing that nutrient sensors regulate secretion of gut hormones and therefore regulate whole organismal metabolism. Further reports have shown that the human Sodium-Glucose co-transporter 1 (SGLT-1) is expressed by enterocytes and has high affinity to D-Glucose and Galactose and transports them across the membrane, therefore acting as a carbohydrate transporter. Interestingly, another family member, SGLT-3, was not present in ECs and is suggested to be a glucose sensor, rather than a transporter (Diez-Sampedro et al., 2003; Freeman et al., 2006), demonstrating that even though a protein belongs to a certain family, its function can be different and unexpected. Therefore, in *Drosophila*, Glut1 could be a glucose sensor within ee cells rather than a glucose transporter, which would have to be determined.

Interestingly, nutrient sensors can have different affinities to different nutrients and therefore distinguish specific forms of nutrients. In nature, *Drosophila* needs to adapt to changes in food availability and quality to make appropriate choices in food intake (Dahanukar et al., 2007; Slone et al., 2007). It has been shown that decreased circulating sugar levels in flies led to the preference for a calorie-rich diet and mutant flies for taste receptors preferred sugar- over agar-containing food after food deprivation, indicating that taste is not the only measure for food preference, but rather suggests that metabolic need is the driver for those food choices (Dahanukar et al., 2007; Dus et al., 2011; Slone et al., 2007). Similar studies in *trpm5*$^{-/-}$ (*transient receptor potential cation channel subfamily M*

member 5) mutant mice, which are unable to taste sugars, showed a preference for sucrose solution over water-only or sucralose solution (a non-metabolisable sugar), because of its nutritional value and the release of dopamine as a reward in response to the metabolisable sugar sucrose (de Araujo et al., 2008). In agreement, it has been shown that after prolonged starvation, flies prefer metabolisable (sucrose and D-glucose) to non-metabolisable sugars (sucralose and L-glucose), due to the systemic energy need (Dus et al., 2011). These results clearly show that there is a mechanism other than taste in making appropriate food choices. Our data showed, that re-feeding flies with sucrose, a metabolisable carbohydrate, after prolonged starvation leads to reduced Burs immunoreactivity in ee cells, suggesting increased secretion. It would be interesting to determine, if non-metabolisable carbohydrates affect Burs levels in a similar fashion. Furthermore, food choice experiments in normal conditions and after a period of starvation in wild type versus *burs* knockdown animals would reveal the role of Burs as a hormone controlling food intake after ingestion, possibly due to neuronal regulation and reward signalling via LGR2.

Interestingly, it has also been shown that neurons innervate the visceral muscle surrounding the gut and the underlying epithelial layer in *Drosophila* (Cognigni et al., 2011), suggesting organ communication between CNS, intestine and trachea around the gut. Trachea, the mammalian counterpart of lungs and vasculature, and especially the terminal tracheal branches are responsible for gas exchange with each cell of the organism (Fraisl et al., 2009; Ghabrial et al., 2003; Uv et al., 2003). Recent reports further revealed nutritional plasticity in tracheal cells (Linneweber et al., 2014). It has been discovered that enteric neurons, producing *Drosophila* Insulin-like peptide 7 (Dilp7) and Pigment Dispersing Factor (PDF), act as nutrient sensors and mediate the remodelling of the trachea around the gut by increasing or decreasing terminal tracheal branching in response to high or low nutrient availability (Linneweber et al., 2014). Therefore, inhibition of Insulin and PDF signalling within the terminal tracheal branches caused reduction of terminal tracheal branching and organismal lipid levels in larvae and adult *Drosophila* (Linneweber et al., 2014). Our data showed, that many neurons are positive for *rk*, therefore it is possible that Dilp7 and PDF neurons, which are in close proximity to *burs* expressing ee cells (data not shown) express *rk*, which would have to be examined. Possible *rk* expression in Dilp7 and/or PDF neurons could either suggest

a direct signal from the gut to the brain to sense intestinal nutrient availability or a feedback signal from the CNS to the gut to communicate organismal energetic state. To analyse if Dilp7 and/or PDF neurons directly modulate Burs secretion/retention, we would need to examine Burs levels within ee cells in flies with constantly activated or blocked secretion in Dilp7/PDF neurons or in flies which express an apoptotic protein within them to specifically ablate those neurons. Furthermore, if Burs levels are different in either of these conditions, the metabolic phenotype of those flies would have to be determined, by looking at organismal lipid and circulating glucose levels, as well as starvation sensitivity.

Furthermore, we observed *rickets* expression within tracheal cell throughout the adult fly and more experiments need to be done, to reveal the function of LGR2 within the trachea.

6.3.3 Adult *Drosophila* and energy use - the role of Burs/ neuronal LGR2

As demonstrated above, gut hormones are very important to modulate metabolism. In *Drosophila* and other insects, it is necessary to regulate energetic homeostasis to adapt to different metabolic states. The main purpose for female *Drosophila* is to generate progeny, which is very energy consuming, but at the same time, they need to monitor the food availability and adapt to starvation periods appropriately. Therefore, synthesis and breakdown of energy molecules needs to be tightly regulated. Indeed, impaired Burs/ neuronal LGR2 signalling resulted in the decrease of lipid and circulating glucose levels, which flies try to counteract by overfeeding. We showed that this is independent of VM LGR2, but dependent on LGR2 within the neurons. By impairing Bursicon/ neuronal LGR2 the fly's ability to maintain metabolic homeostasis is affected, which led to the uncontrollable loss of energy storage.

It is known that the metabolic rate in *Drosophila* can drastically change in accordance to the need of energy. For example it is known that during extensive flight, and therefore muscle function, glycogen stores are rapidly depleted, whereas during starvation, glycogen and lipids are used in a similar ratio (Wigglesworth, 1949). Glucose is readily and quickly available from the breakdown of glycogen, therefore it is the primary source of energy during flight

(Wigglesworth, 1949). Interestingly, we observed a decreased locomotor activity and an overuse of lipids first, rather than glycogen depletion in fully fed flies with impaired Burs/ neuronal LGR2 signalling. Those results showed, that increased activity is not the cause for the loss of energy reserves. Additionally, the overfeeding and increased absorption of nutrients further indicated that the energy is actively used.

We also observed decreased circulating and whole fly glucose levels in knockdown flies, which could be due to the use of glucose for lipogenesis. It has been shown that ingested glucose is a major source for lipogenesis in the fat body. In the mosquito *Aedes aegypti*, it was shown that half of the glucose is used for lipid synthesis, whereas just 35% is incorporated into glycogen (Zhou et al., 2004). Therefore, it would be important to analyse incorporation of ingested glucose into different metabolites. Heavy carbon-labelled glucose (C^{13} glucose) could be used to analyse glycolytic and TCA cycle flux, and incorporation into newly synthesised lipids to get further insight into the mechanism of Burs/ neuronal LGR2 signalling. From our data, we would hypothesise that the metabolic flux through glycolysis and TCA cycle of ingested nutrients is higher, whereas glucose incorporation into newly synthesised lipids is reduced in ee specific *burs* and neuronal *rk* knockdown flies compared to controls. Due to the observed hyperphagic behaviour of the knockdown animals those experiments would need to be carried out in a very controlled manner, to be able to correct data by the starting amount of labelled glucose ingested.

We obtained evidence that glucose sensing via Glut1 within the ee cells leads to Burs secretion. Burs could be a novel, circulating hormone, communicating to the organism how much glucose/carbohydrates are available. Consistently, knockdown of *burs* would lead to the systemic signal that glucose levels are low and that lipid breakdown through ß-oxidation in the mitochondria needs to be initiated.

6.3.3.1 Mitochondria, ß-oxidation and thermogenesis

Mitochondria are necessary for many cellular functions. Most importantly they provide the vast majority of cellular energy in the form of ATP by using oxygen (Athenstaedt and Daum, 2006), which also provides a useful source of water as a

by-product. Therefore, the more mitochondria a cell contains and the better their activity, the more energy can be provided when oxygen and nutrients are available. Mitochondria also play important roles in cellular survival and apoptosis, biosynthesis of fatty acids, amino acids, purines and steroid hormones (Goffart and Wiesner, 2003; MacDonald et al., 2005) and different cell types can adapt differently to cellular or organismal signals (Garesse and Vallejo, 2001).

No reports have been published showing a direct connection of gut hormones regulating mitochondrial respiration, but many steroids, such as estrogens (Klinge, 2008), cortisol (Mansour and Nass, 1970) and thyroid hormones (Wrutniak-Cabello et al., 2001) have been shown to affect mitochondrial respiration.

Thyroid hormones have major effects on mitochondria, and therefore thermogenesis and metabolism. It has been shown that hyperthyroidism results in a hypermetabolic state due to increased basal metabolic rate, in which the nutrient supply can't keep up with the demand (Sestoft, 1980; Sterling et al., 1980). This phenotype is similar to what we have observed in flies with impaired Burs/ neuronal LGR2 signalling. The thyroid hormone T_3 directly activates mitochondrial respiration and therefore ATP synthesis, by stimulating O_2-consumption (Muller and Seitz, 1981; Sterling, 1979). Interestingly, 3d old *burs* mutant mitochondria showed a consistent 15% increase in O_2-consumption rate (Figure 4-14). T_3 stimulates energy-wasting processes, such as the Cori-cycle, a metabolic pathway involving the recycling of lactate and glucose between the muscle and liver, which accounts for a net loss of 4 ATP (Huang and Lardy, 1981; Muller et al., 1983; Muller and Seitz, 1980). This leads to increased heat production and elevated body temperature in hyperthyroid patients, whereas the opposite occurs in hypothyroid individuals (Himms-Hagen, 1976; Sestoft, 1980).

Due to increased respiration observed in *burs* mutants and the decreased locomotor activity, we suspected that Burs/ LGR2 signalling could control mitochondrial activity, which would lead to changes in body temperature. Therefore, we analysed resistance to cold stress in 3d old whole mutant animals and animals bearing ee specific *burs* and neuronal *rk* knockdown and their respective controls and found a highly significant increase in cold resistance in mutant and knockdown flies compared to their controls (Figure 6-2). This data argues in support of a scenario where impairment of Burs/ neuronal LGR2

signalling would lead to increased metabolic rate resulting in upregulation of body temperature. Adaptation to different temperatures can also be achieved by regulating the amount of specific “anti-freezing” metabolites like trehalose and glycerol (Bale, 2002). Nevertheless, we didn’t detect differences in trehalose levels, a natural occurring “anti-freeze” carbohydrate (Elbein et al., 2003) in *burs* and *rk* mutant flies compared to controls (Figure 4-8 B). The levels of free glycerol, the backbone of TAGs, would have to be determined in future experiments, but we would hypothesise that glycerol levels are most likely not changed or even lower in *burs*/*rk* knockdown flies due to increased lipid loss.

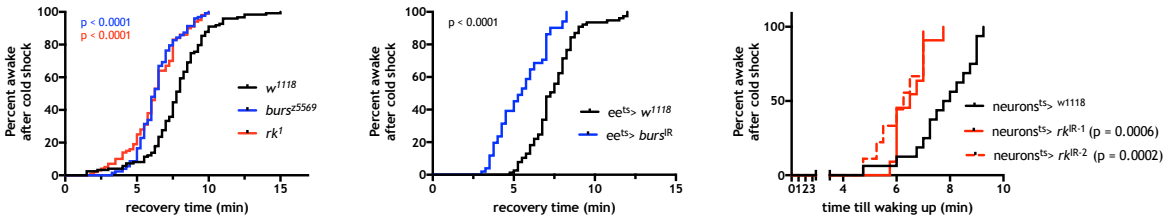

Figure 6-2: Adult flies with an impairment of Burs/ neuronal LGR2 signalling resulted in increased resistance to cold stress.

3d old flies of indicated genotypes were subjected to ice water for 10 min and recovery time was recorded. Note, that impairment of Burs/ neuronal LGR2 signalling significantly increased cold stress resistance.

6.3.3.2 Hormonal control of insect physiology and its connection to Burs/ LGR2 signalling

The major steroid hormone in *Drosophila* is Ecdysone, which gets hydroxylated to 20-hydroxyecdysone (20E). 20E plays a major role during development and metamorphosis. Ecdysone levels drop just before eclosion, which leads to neuronal Burs secretion to mediate post-eclosion events (Arakane et al., 2008; Di Cara and King-Jones, 2013). Another important mediator of metamorphosis is Juvenile Hormone (JH). JH is responsible for the growth of the larvae, while inhibiting metamorphosis (Jindra et al., 2013). 20E and JH have opposing effects during development (Riddiford, 1993) and also during adulthood (Belles and Piulachs, 2015; Gruntenko and Rauschenbach, 2008).

We found that loss of Burs/ LGR2 signalling resulted in smaller ovaries and reduced fecundity (Figure 6-3 A, B) and it is known that ovaries are comprised of high amounts of lipids, which are the main source of energy in the developing egg (Beenakkers et al., 1985). This could suggest, that Burs/ LGR2 signalling may be important to maintain egg production through preservation of energy reserves and hypothesised that JH might be the mediator of this function of Burs/ LGR2.

JH is produced by the corpora allata (CA). Ablation of CA cells shows, among others, reduced fecundity (Yamamoto et al., 2013). Thomsen could demonstrate, that JH affects O_2-consumption and ovarian growth (Thomsen, 1949). In addition, Grutenko and colleagues showed that ablation of the CA in adult *Drosophila* (by using *Aug21*-Gal4 driving UAS-*grim*) impaired JH metabolism and reduced egg laying, which could be restored by supplying the flies with the JH analogue Methoprene (Gruntenko et al., 2012). We determined if *rk* expression within the CA cells has an effect on fecundity. To do this, we specifically knocked down *rk* in CA cells (using *Aug21*-Gal4; *gal80*^{TS}, referred to as CA$^{ts>}$) and counted the amount of eggs laid by each female every day. We noticed that CA specific knockdown of *rk* led to reduced fecundity compared to control females (Figure 6-3 A), suggesting that Burs/ LGR2 signalling may be regulating JH metabolism directly or indirectly. Also, preliminary data showed that *burs* mutant flies fed with Methoprene produced more eggs compared to *burs* mutants fed control diet (Figure 6-3 C). To rigorously show that JH is the mediator of this effect, we would

need to do rescue experiments in CA specific *rk* knockdown and ee specific *burs* knockdown animals by supplying the food with Methoprene and analyse fecundity. It would have to be further examined, if *rk* within the CA cells also has an effect on glucose and/or lipid metabolism or whether this is an exclusive role of the signalling in female fecundity.

The actions and physiological roles of thyroid hormones (THs) and JH and 20E are very similar suggesting at least a partial functional conservation of those signalling pathways and downstream effects. It has been shown that Thyroid hormones applied to insects mimic many aspects of JH action (Davey, 2000; Davey and Gordon, 1996).

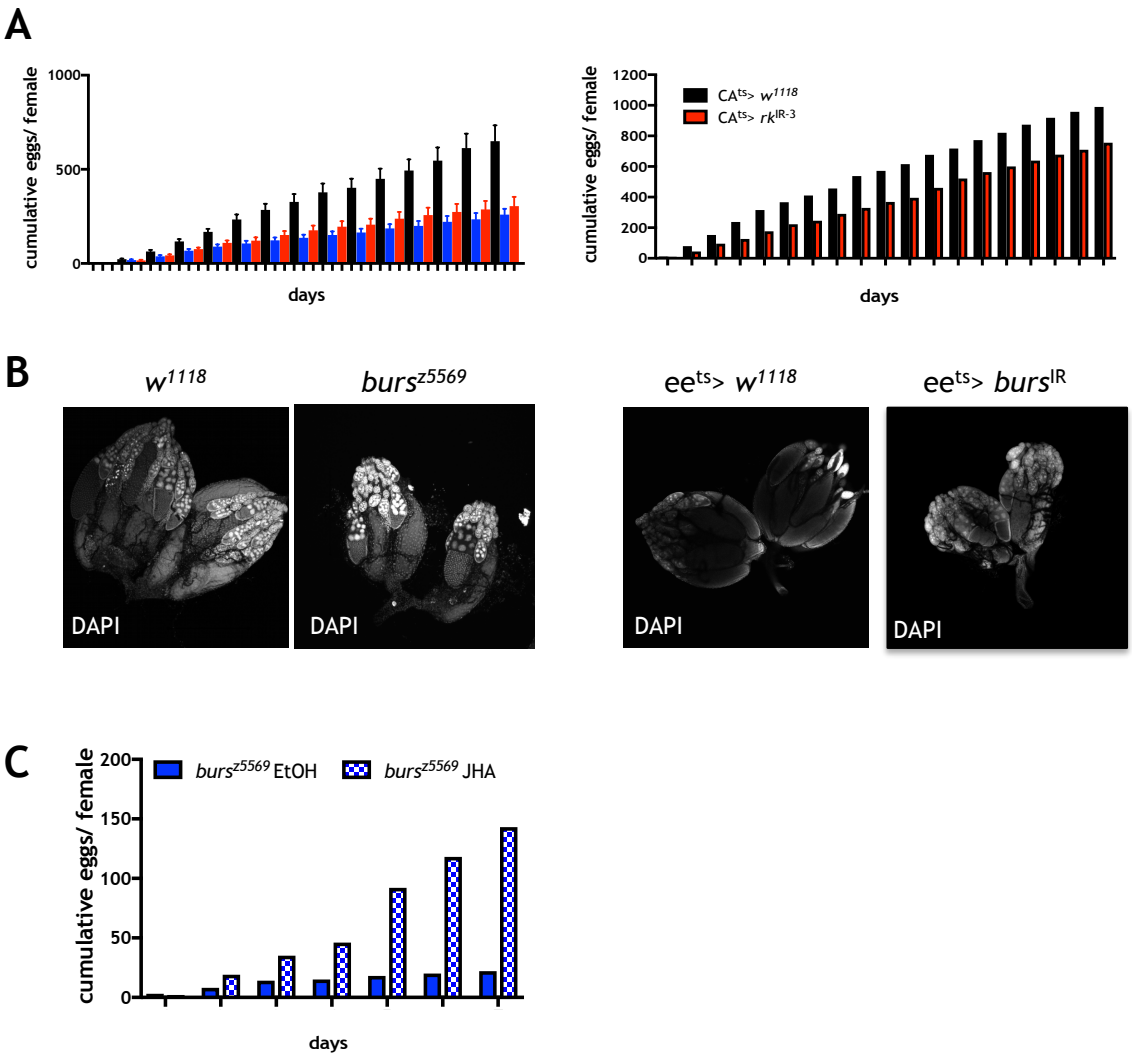

Figure 6-3: JH as a possible mediator of Burs/ LGR2 signalling.

(A) Female egg production of indicated genotypes was counted every day and plotted as cumulative oviposition per female over the evaluated time. (B) Representative confocal maximum projection images showing female ovaries of indicated genotypes. Nuclei were labelled with DAPI.

Furthermore, a recent study in *Pyrrhocoris apterus* (firebug), feeding on dry linden seeds, found, that metabolic active *P. apterus* displayed a higher body temperature compared to metabolic inactive firebugs (Slama and Lukas, 2016). Heat production is believed to be the by-product of lipid breakdown to produce metabolic water, which is dependent on JH (Slama and Lukas, 2016). Firebugs produce water metabolically, due to their dry and no water containing diet (Slama and Lukas, 2016). Our data showed that impairment of Burs/ neuronal LGR2 signalling resulted in increased cold stress resistance and reduced fecundity. It would be interesting to examine in the future if these phenotypes are due to *rk* expression in the CA mediating JH metabolism, possibly to regulate organismal water balance.

An interesting point substantiating that hypothesis is, that Burs/ LGR2 signalling is important for cuticle tanning and hardening after eclosion (Mills, 1967; Luo et al., 2005; Davis et al., 2007). This exoskeleton is the main protection barrier against predators and desiccation, which could suggest that flies with disrupted Burs/neuronal LGR2 signalling burn their lipids in order to produce metabolic water to protect the flies from desiccation, which in turn leads to increased heat production. Furthermore, *Drosophila* feed mostly on rotten fruits, which display high concentration of sugars and we have seen, that ee specific knockdown of *glut1* led to the same metabolic phenotypes observed in ee specific *burs* knockdown flies. Additionally, we also showed, that Burs protein levels are

mediated by carbohydrates and not by protein (

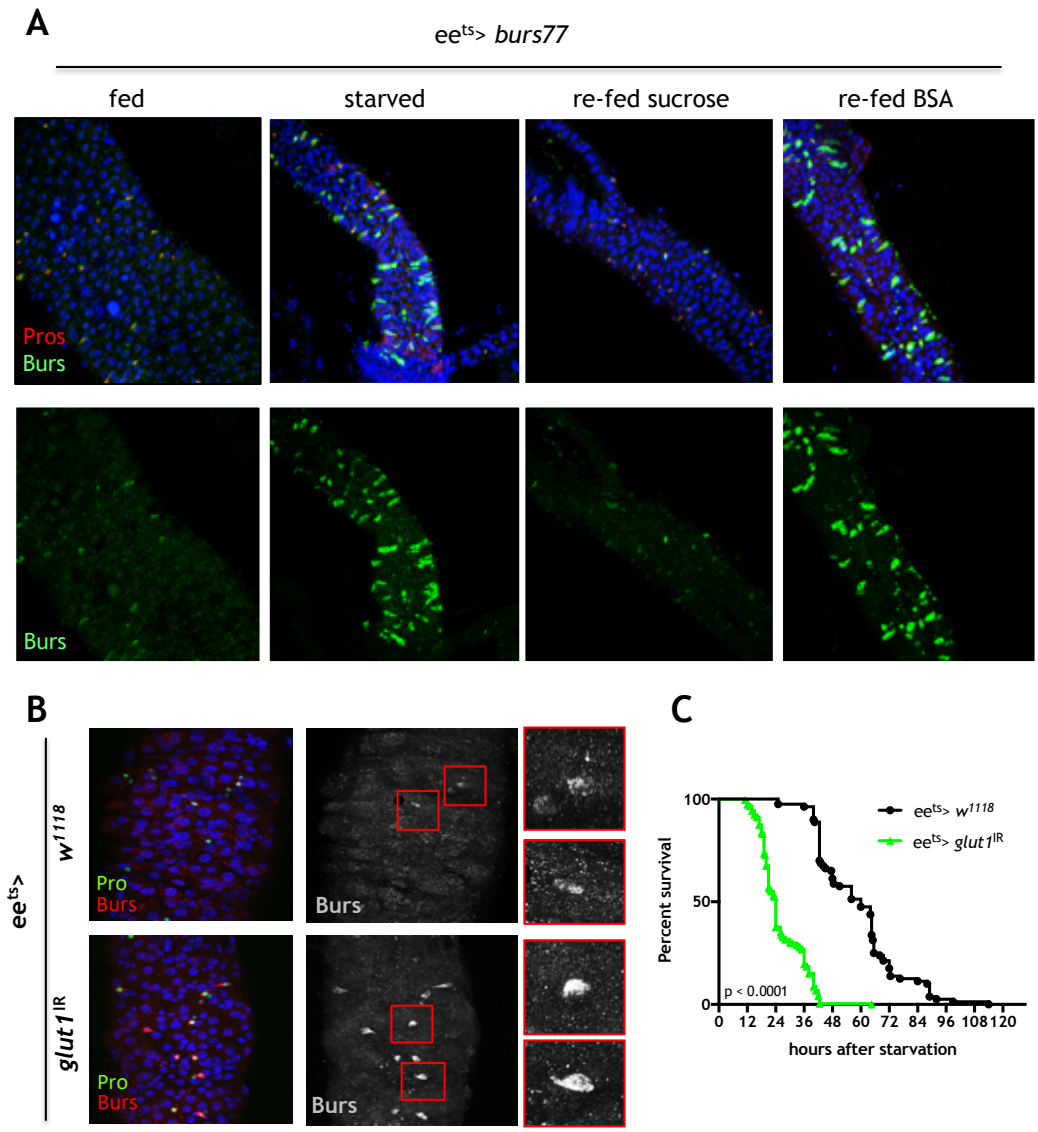

Figure 4-5). Together with the resistance to cold stress and published data, this could imply, that sugar and water consumption is coupled, thus flies with disrupted Burs/ neuronal LGR2 signalling need to produce metabolic water by burning lipids.

In the future, more experiments would need to be done to test the hypothesis, that Burs/ LGR2 signalling is responsible to maintain water balance. We would like to start by analysing resistance to desiccation in *burs*/*rk* targeted knockdown flies and also check the fly's behaviour and metabolic state when fed with dry food, like sucrose crystals. If those results are promising, more sophisticated and collaborative experiments can be done to help understand the hypermetabolic phenotype of loss of Burs/ LGR2 signalling, for example measuring body heat with a thermal camera, O_2-consumtion/ CO_2 production rate in whole flies and amount

of hydrocarbons, which are cuticular lipids known to protect from desiccation (Gibbs, 1998). It has been reported, that *cytochrome p450* (*cyp*) genes are important for synthesising those cuticular hydrocarbons (Qiu et al., 2012), and our RNAseq data showed many of those *cyp* genes deregulated in all 3 tissues in knockdown compared to control flies (Figure 6-4). Furthermore, those genes are also important for ecdysone, JH and fatty acid synthesis (Chung et al., 2009; Gilbert, 2004; Helvig et al., 2004).

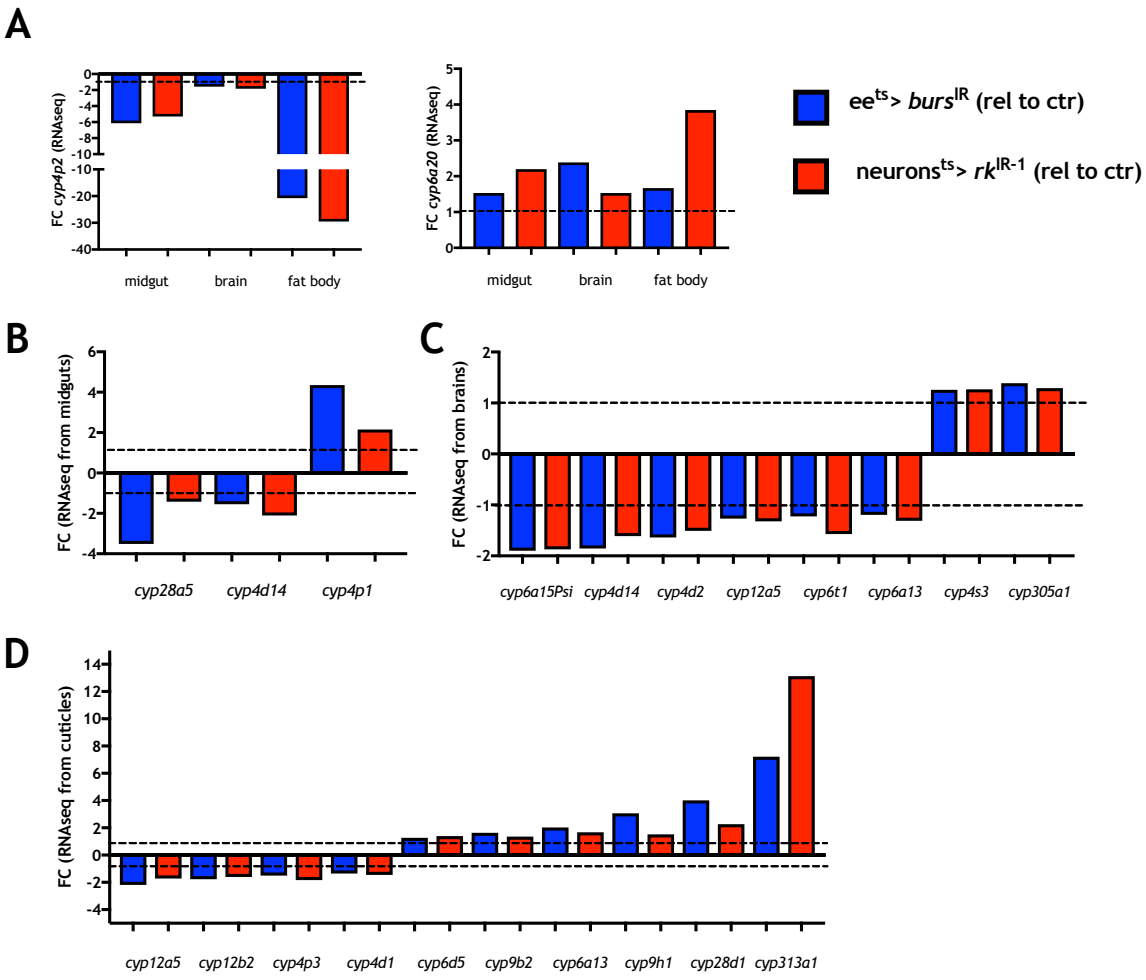

Figure 6-4: RNAseq data revealed many deregulated *cytochrome p450* (*cyp*) genes.

FC of RNAseq data for indicated genotypes relative to its respective control. (A) Deregulated *cyp* genes common to midgut, brain and fat body. (B) Deregulated *cyp* genes in the midgut. (C) Deregulated *cyp* genes in the brain. (D) Deregulated *cyp* genes in the fat body.

6.4 Physiological function of Burs/ LGR2

Animals in the wild are consistently exposed to environmental cues to which they need to react to mount adequate systemic and cellular responses (Owusu-Ansah and Perrimon, 2015). Many hormones and neuronal pathways are involved in the regulation of stress and maintenance of systemic homeostasis (Breen and Karsch, 2006; Lapot et al., 2007; Schank et al., 2012). *Drosophila* has proven to be a great model to understand such complex processes (Hull-Thompson et al., 2009; Karpac et al., 2009; Karpac and Jasper, 2009; Padmanabha and Baker, 2014).

We observed *rk* expression in various adult tissues including the visceral muscle, CNS and trachea. Previously, we reported a local function of Burs/ VM LGR2 signalling regulating intestinal homeostasis (Scopelliti et al., 2014). Here, we provide data for the role of endocrine Burs/ neuronal LGR2 in controlling systemic metabolic homeostasis. It would be interesting, to examine further roles of Burs/ LGR2 signalling in the future.

Our loss of function data suggests that Burs is secreted in response to nutrients to induce energy storage. Upon starvation, systemic Burs signalling needs to be halted, as energy sources are used. On the other hand, local Burs signalling in the midgut needs to be activated as ISCs go into quiescence upon starvation.

Furthermore, we saw that Burs immunoreactivity was enhanced upon starvation and reduced to normal fed ad-libitum levels when re-fed with sucrose alone. In the wild, *Drosophila* feed on rotten fruits, containing high concentration of sugars and it is possible that water- and sugar-sensing is regulated simultaneously. By ingesting sugars from rotten fruits, the fly also "drinks" water, therefore Burs is released into the hemolymph, but if sugar/water can't be sensed for example when flies are re-fed with a no-sugar-containing BSA solution, Burs is retained in the ee cells, which results in the hypermetabolic phenotype.

Our preliminary data suggests that neuronal *rk* loss leads to increased AKH secretion, which in turn results in breakdown of lipids via its AKH receptor in the fat body. AKH/ AKHR signalling was shown to activate calcium signalling via the PLC/IP3 pathway in the fat body mediating the activation of Hormone-sensitive lipase (Hsl) resulting in lipid mobilisation. Preliminary results are indicative of a

role for PLC/IP3 signalling downstream of Burs/ neuronal LGR2, which will be followed up in future experiments. In Figure 6-5 we propose our current working model, where under fed ad-libitum condition Burs from ee cells is secreted into the hemolymph to bind its neuronal receptor LGR2. This results in the retention of AKH, possibly via JH, to increase lipid storage or decrease lipid usage.

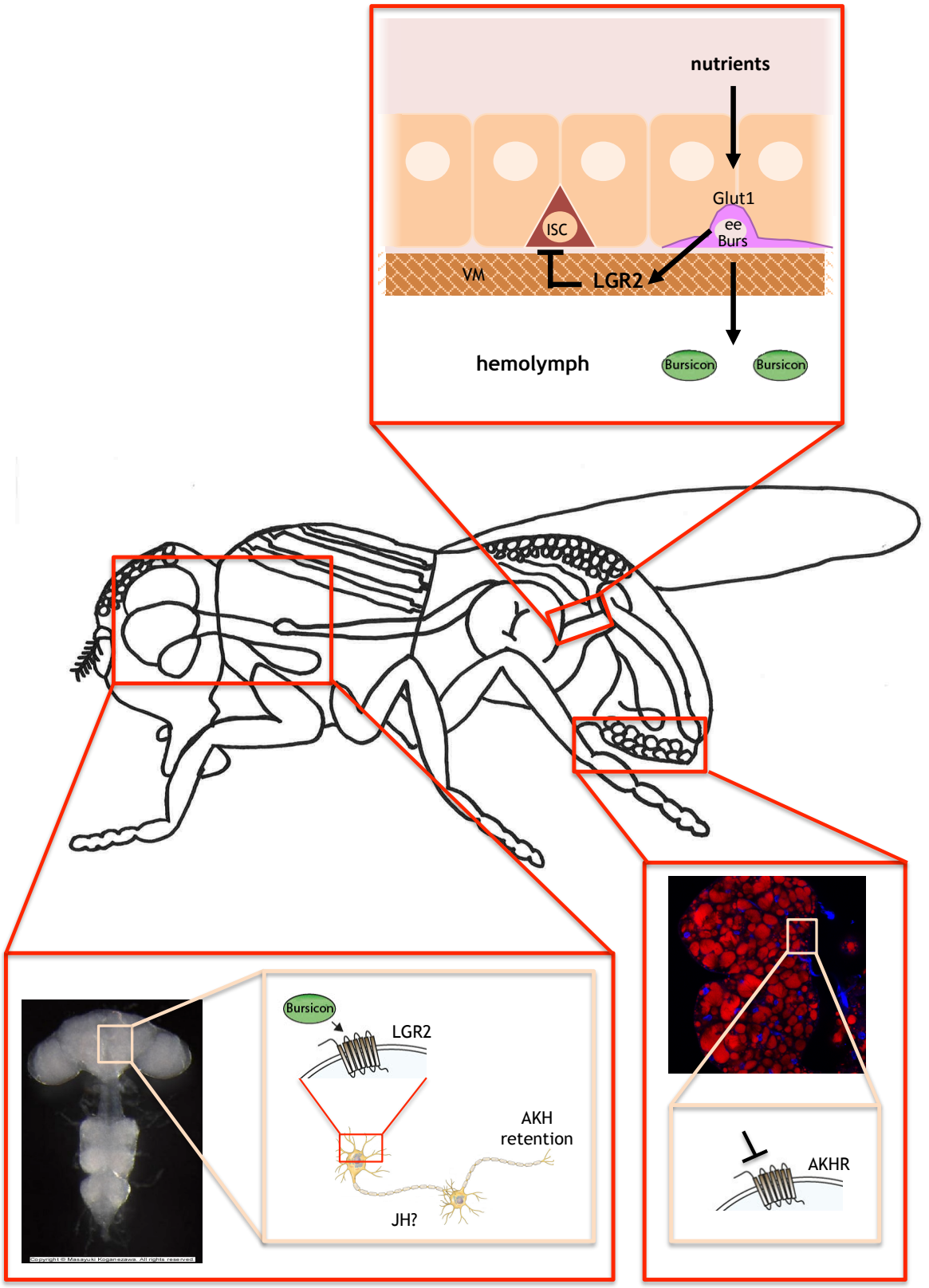

Figure 6-5: Bursicon/ neuronal LGR2 mediates energy homeostasis via AKH/ fat body AKHR - Working model.

Top: Local regulation of intestinal stem cell quiescence by Bursicon/ VM LGR2 (Scopelliti et al., 2014). Systemic regulation of Burs secretion into the hemolymph by nutrients via Glut1. **Bottom left:** Circulating enteroendocrine derived Burs binds to its neuronal receptor LGR2 to mediate AKH retention via an unknown factor, possibly JH. **Bottom right:** AKH is retained in neurons and therefore can't bind to its receptor AKHR on fat body cells to mediate lipid breakdown.

6.5 *Drosophila* LGR2 and its mammalian homologs LGR4, 5 and 6

Drosophila LGR2 is closely related to mammalian LGR4, 5 and 6, which function as R-spondin receptors and mediate Wnt signalling (Carmon et al., 2011; de Lau et al., 2011).

LGR5 is a well-characterised stem cell marker, especially within the intestine (Barker and Clevers, 2010; Barker et al., 2007; Hsu et al., 1998; Schuijers and Clevers, 2012). Our previous work showed that LGR2 within the *Drosophila* intestine is not expressed in ISCs, but in the VM surrounding the gut (Scopelliti et al., 2014). This led us to hypothesise, that LGR5 is most likely not the functional equivalent of *Drosophila* LGR2.

LGR6 homozygous mutant mice are viable (Snippert et al., 2010), whereas LGR4 and 5 homozygous mutant mice are embryonic/ neonatal lethal (Mazerbourg et al., 2004; Morita et al., 2004). Furthermore, LGR6 mutant mice didn't display any obvious phenotypes, which led us to hypothesise that LGR6 is most likely also not the functional equivalent of *Drosophila* LGR2.

Interestingly, LGR4 was associated with obesity. Heterozygous mutation of *LGR4* in humans correlated with reduced body weight and homozygous *LGR4* mutant mice showed loss of adipose tissue and increased energy expenditure (Wang et al., 2013). *LGR4* mutant mice were resistant to diet- and Leptin-induced diabetes and showed improved glucose metabolism (Wang et al., 2013). The same research group also suggested that LGR4 in muscles regulates the switch from glucose- to lipid-prone metabolism due to differences in glucose availability (Sun et al., 2015). Their respiratory exchange rate (RER) was different between fasting (fatty acid-prone) and eating stages (glucose-prone) compared to control mice (Sun et al., 2015). During fasting times, *LGR4* mutant mice used primarily fatty acids as an energy source, while during feeding times they displayed a more glucose-prone metabolism, suggesting that *LGR4* mutation increases lipid usage, when glucose is depleted (Sun et al., 2015). We found that loss of LGR2 signalling resulted in a hypermetabolic state, where flies burn through their energy reserves, especially lipids. Interestingly, *burs* mutants were also resistant to high sugar diet (HSD) induced obesity (Figure 4-8 E) and ee specific *burs* knockdown animals showed

increased overall survival rate when fed with HSD compared to control flies (Figure 6-6).

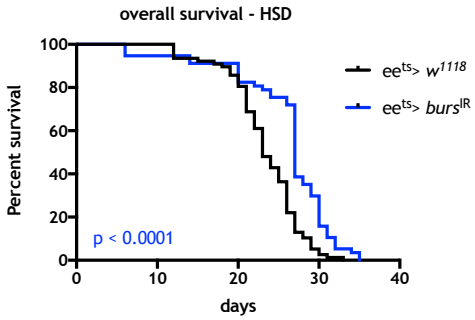

Figure 6-6: Animals with an ee specific *burs* knockdown survive longer on a high sugar diet.

Animals were subjected to standard food supplemented with 1M sucrose and survival was examined. Dead flies were counted.

Furthermore, under fasting conditions LGR4 mutant mice displayed higher expression of PGC-1α and uncoupling protein 3 (UCP3), whereas the glucose transporter Glut4 was downregulated in the muscle, indicating mitochondrial thermogenesis and lipid oxidation, and reduced glucose transport (Sun et al., 2015). Given the current data on LGR4 function, it would be interesting to analyse the muscles in more detail in flies with impaired Burs/ LGR2 signalling. Additionally, LGR4 was suggested to be a regulator of food intake and therefore energy homeostasis (Li et al., 2014). R-spondin 1 and 3 were regulated by Insulin injection and feeding state of the mice, and injections of R-spondin 1 and 3 into the brain led to decreased food intake (Li et al., 2014). Furthermore, only LGR4 was expressed in neurons known to regulate food intake, unlike LGR5 and 6 (Li et al., 2014). Also, our experiments showed that Burs is regulated by ingested nutrients, more specifically sucrose within ee cells and impairment of Burs/ neuronal LGR2 signalling resulted in increased food intake. Additionally, we obtained data suggesting that Burs/ LGR2 signalling mediated mitochondrial respiration activity, but this would have to be further investigated in the future.

In conclusion, we hypothesise that mammalian LGR4 is likely to be the closest functional homolog of *Drosophila* LGR2 due to its many similarities in the metabolic phenotypes of loss of function mutant mice. To test our hypothesis, we would need to analyse metabolic phenotypes in *rk* mutant flies expressing the human forms of LGR4, 5 and 6 globally or in a tissue-specific manner. Furthermore, it would be interesting to analyse further the role of LGR4 in intestinal mouse models, where very little has been reported.

Our work provides new insights into the systemic regulation of Burs/ neuronal LGR2 signalling in maintaining metabolic homeostasis in adult *Drosophila melanogaster*. This Thesis demonstrated nicely the interaction between gut hormones and neurons to mount appropriate systemic responses to nutrient availability. Many signalling pathways and proteins were uncovered in *Drosophila* first and had enormous impact on understanding mammalian physiology and pathology. Revealing the functional mammalian homologs of Burs and LGR2 could help to find therapeutics for human pathologies associated with weight gain.

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Appendices

Scopelliti, A., **Bauer, C.**, Cordero, J. B., & Vidal, M. (2016). Bursicon-alpha subunit modulates dLGR2 activity in the adult Drosophila melanogaster midgut independently to Bursicon-beta. *Cell Cycle, 15*(12), 1538-1544. doi:10.1080/15384101.2015.1121334