Doctoral Thesis

Control of intestinal epithelial homeostasis by Drosophila cyclin D/Cdk4 regulates organismal energy storage

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Control of intestinal epithelial homeostasis by Drosophila
Cyclin D/Cdk4 regulates organismal energy storage

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SUMMARY

Survival of all organisms requires that they correctly coordinate the processes of food breakdown, uptake and storage. How this is achieved in the fruit fly, Drosophila melanogaster, is currently poorly understood. Recent metabolic studies have mostly focused on the control of storage and breakdown of dietary lipids, however how absorption of lipids and other nutrients in the intestine is regulated remains unclear. Very recent studies have begun to elucidate the mechanisms that regulate intestinal epithelial cell homeostasis in Drosophila, but the metabolic relevance and consequences at the whole organism level remain uninvestigated. This doctoral thesis has uncovered a new role for Drosophila Cyclin D and its cyclin dependent kinase 4 (Cdk4) in controlling intestinal cellular composition and homeostasis and consequently influencing the metabolic status of the entire adult fly.

The Drosophila Cyclin D/Cdk4 complex has previously been shown to control the growth of adult tissues. Adult flies that are mutant for Cdk4 or Cyclin D are smaller and lighter. Here it is shown that these flies additionally display substantially reduced levels of stored lipids and glycogen and are consequently starvation sensitive. These phenotypes result from defects in intestinal epithelial homeostasis, which most likely causes absorption defects. Investigation of the intestines in Cdk4 mutant adults revealed reduced numbers of all cell types residing in the gut epithelium and growth defects in the main absorptive cell type, the enterocyte. Moreover, the proliferative rate of intestinal stem cells was dramatically reduced. Restoring Cdk4 specifically in the enterocytes reversed the reduction in nutrient stores and most intestinal phenotypes, including the proliferative rate of stem cells. These results demonstrate that Cdk4 is required for normal growth of enterocytes and consequently for their absorptive functions. Additionally they suggest that enterocytes may contribute to an as-yet-undefined permanent stem cell niche in the epithelium that regulates homeostatic divisions of intestinal stem cells.

In general terms this study has provided insight into how adult flies control their normal intestinal homeostasis and stem cell proliferation by demonstrating that sufficient enterocyte numbers and function are prerequisites for homeostasis. This thesis also
Summary

shows for the first time that disruptions of epithelial homeostasis can have profound effects on energy metabolism and nutrient storage in the whole animal.
ZUSAMMENFASSUNG


Zusammenfassung

sowie Funktion der Enterozyten zu gewährleisten. Weiterhin lassen sie die Annahme zu, dass Enterozyten zu einer bis dato unbekannten permanenten Stammzell-Nische im Epithel beitragen, welche die gleichmässige Teilung der Darm-Stammzellen reguliert.

Diese Arbeit zeigt, dass eine ausreichende Anzahl an Enterozyten vorhanden sein muss, damit in adulten Fliegen die zelluläre Homöostase des Darmepithels und die Stammzellproliferation kontrolliert ablaufen können. Darüber hinaus wird in dieser Studie erstmals beschrieben, dass die Störung der epithelialen Homöostase weitreichende Konsequenzen auf den Metabolismus und Energiehaushalt des gesamten Organismus hat.
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Lastly, I would like to thank my family for being there for me during my studies, especially my mother that always encouraged my further education. I am very thankful that she taught me the value of being determined and independent.

I would like to dedicate this thesis to my grandmother Halima, I know she would have been very proud.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AJ</td>
<td>Adherens Junctions</td>
</tr>
<tr>
<td>AMG</td>
<td>Anterior midgut</td>
</tr>
<tr>
<td>Arm</td>
<td>Armadillo</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>Cdk4</td>
<td>Cyclin dependent kinase 4</td>
</tr>
<tr>
<td>CycD</td>
<td>Cyclin D</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs Large</td>
</tr>
<tr>
<td>dTOR</td>
<td>Drosophila Target of Rapamycin</td>
</tr>
<tr>
<td>EB</td>
<td>Enteroblast</td>
</tr>
<tr>
<td>EC</td>
<td>Enterocyte</td>
</tr>
<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td>PMG</td>
<td>Posterior midgut</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time PCR</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>Tsc1/2</td>
<td>Tumour Sclerosis Complex 1 and 2</td>
</tr>
<tr>
<td>SJ</td>
<td>Septate Junctions</td>
</tr>
<tr>
<td>Wg</td>
<td>Wingless</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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1. INTRODUCTION

1.1. Cyclins in cell cycle control

1.1.1 The eukaryotic cell cycle

The core eukaryotic cell cycle consists of four distinct phases: the synthesis (S) phase, the mitotic (M) phase and the two intervening gap phases, G1 and G2. During S phase the DNA is replicated while during M phase the replicated genetic material is segregated into the two resulting daughter cells. The particular order of the cell cycle, with mitosis always following S phase, is driven by the cyclic accumulation and degradation of cyclins. Cyclins and their associated kinase partners, cyclin dependent kinases (Cdks) are protein heterodimer complexes that are the core engines driving the cell cycle (Budirahardja and Gonczy, 2009), (Lilly and Duronio, 2005). Based on experiments using cultured mammalian cells and depletion and activation of individual Cdks, it has been well characterised that distinct Cdks regulate specific cell cycle phases or transitions (Malumbres and Barbacid, 2009). These studies demonstrated that Cdk4-Cyclin D and Cdk6-Cyclin D are needed to commit a cell to the G1/S phase transition, Cdk2-Cyclin E is required for the initiation of S phase and Cdk2-Cyclin A for S-phase completion and exit (Fig. 1). Certain cyclins are synthesized periodically preceding the particular stage at which their activity is required and degraded thereafter. Concurrently, the activity of the Cdks is tightly regulated and their activation requires the binding of a specific cyclin to the Cdk kinase moiety. Although there is a strict functional specialization among Cdks in some metazoan organisms, considerable redundancy does exist and this also holds true for the specialization of the cyclins (Budirahardja and Gonczy, 2009).

1.1.2 Mechanisms of activation of Cdk-Cyclin complexes

Besides binding to a cyclin subunit, Cdk activation relies on phosphorylation by a Cdk-activating kinase, in order for the Cdk catalytic cleft to be fully opened (Kaldis, 1999). In addition, Cdk-Cyclin complexes are kept inactive by binding to a Cdk inhibitor (CKI) and/or through inhibitory phosphorylation by the Wee1 and Myt1 kinases (Malumbres...
and Barbacid, 2005), (Pines, 1999). There are two classes of Cdk inhibitors, the CIP/KIP family and the INK family and their members can associate with a broad variety of Cdks. Once CKI is released, the Cdk-cyclin activation is triggered and the inhibitory phosphate groups are removed through the action of Cdc25 phosphatase family members. The initial activation of Cdk-cyclin results in the phosphorylation and thereby further activation of positive regulators such as Cdc25, generating a robust positive feedback loop that activates the Cdk-cyclin complex (Kaldis, 1999).

Aside from these general mechanisms that regulate most Cdks, specific Cdks can be regulated by additional components. For example, Cdk1-Cyclin B is activated by the serine/threonine protein kinases Aurora A and by Polo-like kinase 1 (Hirota et al., 2003), (Hutterer et al., 2006).

\[ \text{Figure 1. The role of cyclins A, B, D and E in the eukaryotic cell cycle} \]
1.1.3 *Drosophila* cyclins and their role in development

In *Drosophila*, several of the Cdk-cyclin complexes have similar functions during the cell cycle as their mammalian counterparts, however with some exceptions. For example, unlike in mammals, the *Drosophila* Cdk4-Cyclin D complex is not required for cell cycle progression, but has distinct growth driving functions (Meyer et al., 2000). In the mitotic cycle, the association of S-phase cyclins with Cdk2 promotes entry into S phase and DNA replication, while the activation of Cdk1 by the mitotic cyclins promotes entry into mitosis (Knoblich and Lehner, 1993). In *Drosophila*, Cyclin E acts as the primary S phase cyclin, while Cyclin A, Cyclin B and Cyclin B3 function as mitotic cyclins (Lee and Orr-Weaver, 2003). Cyclin E and its partner kinase Cdk2 are required for DNA replication both during mitotic and endoreplicative cell cycles, however the mitotic cyclins are neither required nor expressed in most endocycling cells (Knoblich et al., 1994), (Lane et al., 2000), (Lilly and Spradling, 1996), (Lilly and Duronio, 2005). It should be noted that relatively little is known about the negative regulation of *Drosophila* cyclins and cdks since not many Cdk inhibitors have been found and characterized in the fly. The only gene that has been found is *Dacapo*, which belongs to the *Drosophila* Kip family and has been shown to inhibit proliferation during development (Lane et al., 1996). Conversely, no homologues of the CKI family members have been discovered to date.

Different cell cycle regulators very precisely regulate cell cycle transitions during every stage of *Drosophila* development. The particular cyclins involved are distinctly modulated during transition between S and M phase during early embryogenesis, transition from G2 to M phase at the mid-blastula transition and the G1 to S phase transition during organogenesis (Budirahardja and Gonczy, 2009). Additionally, aside from their roles in cell cycle progression, core cell cycle regulators have recently been appreciated to play a role in regulation of asymmetrical cell division and thereby fate determination (Chia et al., 2001).

1.1.4 Regulation of the *Drosophila* endocycle

Many cells during animal development become polyploid by going through endocycles or endoreplicating cell cycles. Endoreplication cycles occur by successive S phases
taking place without occurrence of mitoses, consequently increasing the cellular DNA content. The strategy of endoreplication is believed to be an efficient way of increasing cellular mass, i.e. cell growth, and is often found in differentiated cells that are large or highly metabolically active (Edgar and Orr-Weaver, 2001). Cell size for a given cell type is usually proportional to the nuclear DNA content, however it is still not completely clear if an increase in growth is preceded by increased endoreplication or vice versa. Experimental inhibition of endocycle progression by DNA replication inhibitors or mutations in genes essential for DNA replication shows that an increase in genomic DNA is required for both cell and organismal growth. For instance, *Drosophila* larval growth is accompanied by a high degree of endoreplication and experimental conditions that arrest or disturb growth of larvae always block endocycle progression, suggesting a tight regulatory connection between these two processes (Galloni and Edgar, 1999), (Zhang et al., 2000).

During *Drosophila* development, cells in many tissues initiate endoreplication cycles and as mentioned above, these cycles are regulated mainly by Cdk2-Cyclin E activity. Well characterised tissues that endoreplicate include the larval gut, fat body, Malpighian tubules, trachea and salivary glands (Knoblich et al., 1994). Endoreplication also occurs in several adult fly tissues, such as the ovarian follicle and nurse cells, sensory neurons in the wing and the gut enterocytes (Smith and Orr-Weaver, 1991), (Micchelli and Perrimon, 2006). The mechanism of endocycle regulation is dependent on oscillating activity of Cdk2-Cyclin E, demonstrated by blocked endoreplication when this kinase complex is over-expressed. This is thought to be due to required reduction in Cdk2-Cyclin E activity after S-phase, in order for the pre-replication complexes to assemble during G1 phase (Follette et al., 1998), (Weiss et al., 1998). In addition, some studies have implicated that Cdk2-Cyclin E oscillation frequency could be coupled to cell type specific characteristics such as metabolic rate. This is supported by several observations, one of them being the tight coupling of larval endocycles to an ongoing flux of dietary protein from feeding, thus implying that dietary amino acids provide a specific signal that controls the growth response in endocycling cells (Britton and Edgar, 1998). Furthermore, it was shown that these endocycles could be stopped, restarted, slowed down or accelerated by altering feeding regimes. With regard to the endocycle being responsive to dietary protein, it was shown that several genes required for protein synthesis and the *Drosophila*
Target of Rapamycin (\(dTOR\)) are also required for endocycling (Galloni and Edgar, 1999), (Zhang et al., 2000). \(dTOR\) is a protein kinase that links nutritional signalling to the protein synthetic machinery, via its downstream effectors 4E-BP and S6 kinase (s6K) (Zhang et al., 2000), (Miron et al., 2003).

The nutritional requirement for endocycling can be bypassed by forced expression of factors that autonomously promote cellular growth such as the \(dMyc\) transcription factor, the Cyclin D/Cdk4 complex, the catalytic subunit of phopshoinositide 3-kinase (PI3) and the \textit{Drosophila} insulin receptor (IR) (Johnston et al., 1999), (Datar et al., 2000), (Britton et al., 2002), (Edgar and Orr-Weaver, 2001). Forced expression of these growth promoters can also drive hyperpolyploidy of endoreplicating tissues in well-fed animals, one striking example being the over-expression of \(dMyc\) in the larval fat body (Johnston et al., 1999), (Pierce et al., 2004).

\section*{1.2 Cyclin D and Cdk4 in mammals and \textit{Drosophila}}

\subsection*{1.2.1 Functions of the \textit{Drosophila} Cyclin D and Cdk4}

Mammalian D-type Cyclins (CycD) and their accompanying cyclin dependent kinases 4 and 6 (Cdk4/Cdk6) are well characterised with respect to their role in growth factor responsive regulation of G1 cell cycle progression (Sherr and Roberts, 1999), (Sherr, 1996). This is achieved partly by phosphorylation and neutralization of Retinoblastoma (Rb) family proteins that include pRb, p107 and p130, which in their hypophosphorylated state bind transcription factors thereby repressing their activity. Amongst such transcription factors are the E2Fs, which are released by Rb pocket proteins following CycD/Cdk4 mediated phosphorylation, enabling them to promote transcription of a large number of cell cycle genes including enzymes for nucleotide synthesis and G1 cyclins such as Cyclin E (Dyson, 1998), (Harbour et al., 1999). One additional mechanism of cell cycle regulation is achieved by binding of D-type Cyclins and Cdk4/6 to CKI (p27) and sequestering them away from Cdk2/Cyclin E, allowing cell cycle progression to take place (Harbour et al., 1999).

In \textit{Drosophila} it has previously been shown that Cyclin D and its kinase Cdk4 are required for and promote cellular growth, but are surprisingly dispensable for cell cycle
progression (Datar et al., 2000), (Meyer et al., 2000), (Meyer et al., 2002). Flies carrying a null mutation of Cdk4 (cdk4\(^3\)) are viable with limited fertility, however they are noticeably smaller and lighter compared to control flies. In addition, they have fewer cells in the adult wing with the cells displaying a significant increase in cell size. The changes in cell number were attributed to the fact that cdk4\(^3\) mutant differentiating cells increase in mass slower and have a lengthened cell cycle through an increase in G1, S and G2 phases. Since the observed increase in cell size was contradictory to the notion that Cdk4 positively regulates growth, the authors hypothesised this was due to partial compensatory growth in the remaining cells, in order to keep an intact wing epithelium (Meyer et al., 2000).

In the same study described above, experiments were performed to address if the Cdk4 kinase domain is required for its growth driving function and if Cdk4 is the only Cyclin D-dependent Cdk. A transgenic construct carrying a kinase-inactive Cdk4 (UAS-Cdk4\(^{D175N}\)) was ubiquitously over-expressed in cdk4\(^3\) heterozygous and homozygous flies. The D175N mutation affects an aspartate residue, which is conserved in all protein kinases and is required for the phosphotransfer reaction (van den Heuvel and Harlow, 1993). Mutant Cdk4\(^{D175N}\) protein bound to Cyclin D with the same efficiency as kinase-active Cdk4 and was therefore expected to act in a dominant-negative fashion. Ectopic expression of Cdk4\(^{D175N}\) did not further reduce the weight of cdk4\(^3\) homozygous mutants, however it did result in a slight but significant weight decrease in heterozygous animals, supporting the idea that the D175N mutation acts in a dominant-negative manner. The interpretation of the results was slightly complicated by the fact that Cdk4\(^{D175N}\) expression caused a moderate weight increase when expressed in a homozygous mutant background, implying at least partially kinase independent mechanism of Cdk4 growth control. This alternative mode of action was explained by a possibility that Cdk4 could titrate members of the putative inhibitors of Cdk (CIP/KIP) family, as described for mammalian Cdk4/Cdk6 (Meyer et al., 2000), (Adams et al., 2000). Mammalian INK4 family members can specifically bind Cdk4 and Cdk6, which leads to loss of Cyclin D binding and catalytic inactivation, however so far, no INK homolog has been found in the fly (Sandhu et al., 2000).
1.2.2 Consequences on growth of Cyclin D mutations and ectopic Cyclin D/Cdk4 expression

Similarly to cdk4\textsuperscript{3} homozygous mutants, flies carrying Cyclin D null mutations (cycd\textsuperscript{1} or cycd\textsuperscript{2}) are smaller and lighter and display similar phenotypes with regard to adult wing cell number and cellular size (Emmerich et al., 2004). In the same study, cdk4\textsuperscript{3} and cycd\textsuperscript{1} single mutants were compared to double mutants of cycd\textsuperscript{1} and cdk4\textsuperscript{3} to uncover any Cyclin D functions independent of Cdk4. The comparison of the phenotypes did not show any significant differences between the single and double mutants, hence concluding that CycD does not have Cdk4 independent functions.

To further support the idea that the CycD/Cdk4 complex is required for growth in postmitotic Drosophila tissues, a study was carried out where Cdk4 and CycD were ectopically expressed in actively proliferating and post-mitotic tissues, either individually or cooperatively. Surprisingly, neither CycD, Cdk4 nor CycD/Cdk4 overexpression caused any detectable changes in cell cycle phasing or cell size in proliferating cells, supporting the observation that unlike in mammalian cells the Drosophila CycD/Cdk4 complex does not promote G1 to S transition \textit{in vivo}. However, CycD/Cdk4 over-expression had an immense impact on the growth of differentiated adult tissues, which was demonstrated by employment of adult eye- and larval salivary gland-specific Gal4 drivers. Eye-specific CycD/Cdk4 over-expression resulted in larger ommatidia as well as larger eyes that bulged out of the head. The overgrowth phenotype observed in the eye was the same as seen in the salivary gland, clearly indicating that Drosophila CycD/Cdk4 promotes cellular growth (Datar et al., 2000).

In summary, what is clear from the previous studies on the function of Drosophila CycD/Cdk4 complex is that it is required for growth induction in post-mitotic cells and not absolutely required for cell cycle progression. The main reason why this complex is dispensable for proliferation and cell cycle progression is because it acts in a redundant manner with Cyclin E/Cdk2. In addition, the growth promoting function might be nutrient responsive and act through targets other than RBF, however the catalytically active subunit of Cdk4 is still required (Datar et al., 2000), (Meyer et al., 2000).
In a more recent study, the growth promoting function of CycD/Cdk4 was shown to be dependent on mitochondrial activity, more precisely on the ribosomal protein mRpL12. mRpL12 is a mitochondrial ribosomal protein that was identified in a genetic screen for modifiers of a CycD/Cdk4 stimulated overgrowth phenotype. When CycD/Cdk4 was over-expressed in the adult eye in an mRpL12 heterozygous mutant background, no increase in growth was observed. mRpL12 mutant cells themselves show mitochondrial defects and have a cell-autonomous growth defect. In addition, experimental inhibition of mitochondria was performed, combined with clonal over-expression of CycD/Cdk4 and the result was evaluated in larval fat bodies and gut. The usual growth induction seen by CycD/Cdk4 over-expression was abolished in this experimental setting, further supporting the hypothesis that *Drosophila* CycD/Cdk4 complex requires mitochondria to drive growth (Frei et al., 2005) (Fig. 2a).

1.2.3 *Drosophila* Cyclin D/Cdk4 and links to other signalling pathways

All studies mentioned above provide quite convincing evidence that *Drosophila* Cyclin D/Cdk4 complex does indeed control growth, however the upstream signalling events

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**Figure 2.** Possible modes of regulation of *Drosophila* Cyclin D/Cdk4

a) Cyclin D/Cdk4 requires mitochondria to drive growth, as well as Fatiga, an enzyme involved in cellular sensing of hypoxia.

b) Cyclin D/Cdk4 regulates growth downstream of Hh signaling.
have remained elusive. Two separate investigations have implicated two distinct signalling pathways that act upstream and downstream of CycD/Cdk4 in context of cellular growth.

Duman-Scheel and colleagues identified a mutation in the Hedgehog (Hh) signaling inhibitor patched (Ptc) to be a regulator of the Rb pathway in *Drosophila*. Additionally, they showed that Hh signalling promotes transcription of Cyclin E and Cyclin D and that this transcriptional activation of Cyclin D mediates the ability of Hh to promote cellular growth. Ectopic expression of Ptc in wing disc clones inhibits cell growth, however co-overexpression of CycD/Cdk4 can suppress this effect. In agreement with these results, overexpression of Ptc in a Cdk4 mutant background did not cause a size reduction, implying that Hh signalling mediates growth induction through Cyclin D/Cdk4. Cubitus interruptus (Ci) is a transcription factor that mediates Hh signalling and induces growth when overexpressed. Ectopic expression of Ci in the wing disc increased Cyclin D transcript and protein, furthermore the effect on growth by Ci was abolished when induced in a Cdk4 mutant background, further supporting the idea that Cyclin D/Cdk4 is downstream of Hh signalling (Duman-Scheel et al., 2002) (Fig. 2b).

A study done by Chen *et al* showed that the Cyclin D/Cdk4 complex regulates the JAK/STAT signal transduction pathway in *Drosophila* embryos. Receptor-associated tyrosine kinases (JAKs) and their substrate transcription factors, signal transducers and activators of transcription (STATs) regulate many developmental processes, ranging from cell fate determination, cell polarity, cell proliferation and innate immune response (Jinks et al., 2000), (Zeidler et al., 1999), (Mukherjee et al., 2005), (Mukherjee et al., 2006), (Boutros et al., 2002). The *Drosophila* JAK is encoded by the Hopscotch (*Hop*) gene and the *Drosophila* STAT by the *Stat92E* gene (Binari and Perrimon, 1994), (Hou et al., 1996). The key findings in this investigation were that Cyclin D/Cdk4 binds to and regulates protein levels of STAT92E, while loss of Cdk4 leads to reduced STAT92E levels. These observations were not exclusive for Cyclin D/Cdk4 as over-expression of Cyclin E/Cdk2 also leads to increased levels of STAT92E, more protein stability and enhanced STAT92E activity. In addition, co-over-expression of *unpaired* (*upd*), a secreted extracellular ligand that activates the JAK/STAT pathway, together with CycD/Cdk4 leads to excessive HOP/STAT92E signalling and results in a tumour like eye phenotypes with extra ommatidia (Chen et
al., 2003). Despite the above-mentioned observations, the actual physiological role and relevance of the Cyclin D/Cdk4 – STAT interaction still has to be further investigated and determined.

1.2.4 Role of mammalian Cyclin D/Cdk4 beyond cell cycle control

Mammalian D-type cyclins are unusual components of the cell cycle machinery in that they lack the periodical induction during cell cycle progression that is observed for other cyclins. The levels of D-type cyclins are controlled by the extracellular mitogenic environment and are therefore believed to serve as a link between the extracellular environment and the core cell cycle machinery (Sherr and Roberts, 1999, 2004). Recently, several studies have emerged suggesting additional roles for the mammalian Cyclin D/Cdk4 complex, these roles being partially separate from the requirement of CycD/Cdk4 in cell cycle progression. In addition, Cyclin D/Cdk4/6 complexes perform an important kinase-independent function by titrating away cell cycle inhibitors p27<sup>Kip1</sup> and p21<sup>Cip1</sup> from the Cdk2-containing complexes (Polyak et al., 1994; Sherr and Roberts, 1999).

A study by Abella et al suggests a requirement for Cdk4 for proper differentiation of murine adipocytes through binding to PPAR-γ and subsequent activation of adipocyte specific and gluconeogenic genes (Abella et al., 2005). This supports the emerging role of D-cyclins and Cdk4/6 in transcriptional control, through binding to various transcription factors. In addition to PPAR-γ, mammalian D-cyclins were shown to activate and repress transcription factors independently of Cdk4, such as estrogen, androgen and thyroid hormone receptors, STAT3 and others (Bienvenu et al., 2001; Coqueret, 2002; Lamb et al., 2000; Lin et al., 2002; Reutens et al., 2001). A very recent study also showed that Cyclin D1 has in vivo transcriptional functions through occupation of promoters of abundantly expressed genes during mouse development, demonstrated through genome-wide location analyses (Bienvenu et al., 2010).

Loss-of-function studies with mice lacking individual D-cyclins, Cdk4 or Cdk6 have been conducted and demonstrate that each strain is viable and shows very narrow, tissue-specific defects. Cdk4 mutant mice display smaller body and organ size,
including a lean phenotype. These mice are in addition diabetic due to pancreatic beta cell failure which is a consequence of β-islet cell hyperplasia, concluding that Cdk4 controls proliferation of these cells (Rane et al., 1999), (Martin et al., 2003). The phenotypes of mutations in the individual D-cyclin genes in mice range from developmental neurological abnormalities, hypoplastic retinas to female sterility, cerebellar abnormalities and impaired development of immature T-lymphocytes (Kozar and Sicinski, 2005). Mice lacking all three D-cyclins are embryonic lethal before E17.5 due to severe anemia and cardiac abnormalities, however they develop normally until E13.5. The hematopoietic cells are severely impaired in their proliferation but most other cell types proliferated normally (Kozar et al., 2004). One major complication in studying the exact developmental role of the mammalian D-type Cyclins and Cdk4/6 is the high level of redundancy. In contrast, Drosophila contains only a single gene encoding for Cyclin D and a single gene for Cdk4. This makes Drosophila an ideal system to study the developmental role of this complex.

In a very recent study, a new link was established between control of cell proliferation and metabolism, based on the well-studied Cdk4-pRb-E2F1 connection. The authors made the observation that E2F1, Cdk4 and pRb are expressed in almost all insulin producing pancreatic β-cells, despite most of these cells being non-proliferating. E2F1 was shown to directly control the expression of Kir6.2, a key component of the K-ATP channel involved in the regulation of glucose induced insulin secretion. Cdk4 inhibition or genetic inactivation of E2F1 resulted in decreased Kir6.2 expression, impaired insulin secretion and glucose intolerance in mice. It was also shown that Cdk4 can be activated by glucose through the PI3 kinase pathway and thus lead to increased Kir6.2 expression (Annicotte et al., 2009).

In the light of the emerging studies pointing towards more versatile roles of the mammalian Cyclin D/Cdk4 complex, we sought to investigate if the metabolic aspect of its newly-discovered functions has any parallels in Drosophila. Moreover, the implication that the Drosophila CycD/Cdk4 might be driving growth in response to nutrients makes the possible functional connection to metabolism more likely.
1.3 Regulation of metabolic homeostasis in adult *Drosophila*

Maintenance of sugar and lipid homeostasis involves important regulatory systems, ensuring functionality and survival of an organism even at times when the food supply is scarce. Mechanisms of fat storage and glucose regulation are conserved in organisms as different as mammals and *Drosophila*. As previously mentioned, mammalian studies have shown that D-type Cyclins have important roles in adipocyte differentiation, adipose mass regulation as well as beta-oxidation and fatty acid synthesis (Nerlov, 2007), (Sarruf et al., 2005), (Abella et al., 2005). These data suggest that D-type cyclins bound to Cdk4 or Cdk6 have metabolic functions that are different from the well-characterized functions in cell cycle progression. Our own unpublished observations and the studies on mammalian CycD/Cdk4 led us to become interested in how the *Drosophila* CycD/Cdk4 complex might be involved in regulating whole animal metabolism, both during the larval stages and in adult flies. Our initial organ of interest was the larval and adult fat body, which is the major lipid storage organ in flies and is functionally equivalent to vertebrate adipose tissue and liver (Baker and Thummel, 2007). Under feeding conditions dietary lipids absorbed in the midgut are transported to the fat body, where the greatest majority of the lipids is stored as triacylglycerol (TAG) inside fat body cells (Teixeira et al., 2003), (Beenakkers et al., 1985), (Canavoso et al., 1998). When the need arises, TAG can be broken down into diacylglycerol (DAG), which is exported from the fat body and transported to the peripheral tissues where the energy demand is high. Here, DAG is further broken down by tissue specific lipases into monoacylglycerol and eventually free fatty acids that are used for energy production through the mechanism of β-oxidation (Athenstaedt and Daum, 2006) (Fig. 3). The fat body is also the site of hemolymph protein synthesis and the major storage site of glycogen (Candy et al., 1997).
In the recent years an increasing number of studies involving *Drosophila* metabolic homeostasis have emerged, consequently elucidating the molecular and functional mechanisms involved. Dysfunctions in lipid homeostasis in *Drosophila* are connected to diverse regulatory pathways and molecules, such as Insulin and Hedgehog signaling, de novo TAG synthesis, mitochondrial functions, lipolysis and lipid surface associated proteins (Teleman et al., 2005), (Britton et al., 2002), (Suh et al., 2006), Suh JM, (Okamura et al., 2007), (Gronke et al., 2007), (Mourikis et al., 2006), (Gronke et al., 2003).

As previously mentioned, the fat body does not only store nutrients as lipids, but also stores carbohydrates in form of glycogen. Glycogen is a polymer of many glucose residues and thus the main storage form of glucose, which is necessary for fueling glycolysis (Steele, 1982). The amount of stored glycogen in insects is usually lower compared to stored lipids and fluctuates considerably based on physical or short time feeding activities or environmental conditions such as starvation (Anand and Lorenz, 2008), (Steele, 1982), (van Marrewijk et al., 1986), (Satake et al., 2000). Glycogen is synthesized from uridine diphosphate (UDP) glucose mainly derived from dietary carbohydrates and amino acids. UDP-glucose is also used for synthesis of trehalose, the main circulating sugar in the hemolymph, which is the equivalent of mammalian blood (Thompson et al., 2003). When trehalose concentration in the fat body reaches a certain level, its synthesis is inhibited and UDP-glucose is stored as glycogen instead, which when the need arises can be used as a source of material for trehalose and lipid production (Friedman, 1978), (Fig. 4).

**Figure 3.** Schematic overview of triacylglycerol breakdown in the insect fat body and its transport to and utilization by peripheral tissues. DAG: diacylglycerol, LDL: low-density lipoprotein, HDL: high density lipoprotein.

![Diagram of triacylglycerol breakdown](image-url)
Trehalose is a disaccharide and the major transport form of carbohydrate in insect hemolymph. Its concentration in the hemolymph is as high as 2% (w/v), indicative of the importance this sugar has for fueling flight muscles during flight (van Marrewijk et al., 1986), (Downer and Matthews, 1976), (Candy et al., 1997). Maintaining constant high levels of trehalose is thus of utmost importance and as mentioned above, can be guaranteed through a mechanism of glycogen breakdown.

1.3.1 The unappreciated role of the gut in nutrient homeostasis

The numerous metabolic studies in the fruit fly, both in larvae and adults, have been informative in beginning to unravel the complex mechanisms underlying control of metabolic homeostasis. However, control of whole body metabolism does not only depend on what occurs in the fat body or the functionality of the link between the brain and the fat body, but also on another key organ, namely the fly intestine. The intestine is the site at which nutrients and energy sources are absorbed by the organism. In insects, TAG breakdown, DAG re-assembly (Fig. 5, lower panel) and glucose uptake (Fig. 5 upper panel) occur in the intestinal lumen and intestinal epithelial cells. Studies presented in this thesis have uncovered a novel role for Drosophila Cyclin D/Cdk4 in gut epithelial homeostasis and illustrate a previously

![Figure 4. Interconversions of glycogen, glucose and trehalose in insects.](image)

underappreciated role of the gut in controlling whole organism metabolism. In the next part of the introduction the insect intestinal physiology will be reviewed, together with its functional importance.

Figure 5. Upper panel: Schematic overview of possible pathways involved in glucose absorption and metabolism in insects. Some mechanisms have been described for single species only. Lower panel: Schematic overview of insect dietary lipid breakdown in the intestinal lumen and re-assembly in the enterocytes. Pictures adapted from "Biology of the insect Midgut", Lehane 1996
1.4 Structure and function of the insect and *Drosophila* gut

As mentioned, the *Drosophila* larval and adult fat body, their functions and means of energy homeostasis have received great deal of experimental attention in the past years, while another important organ remained neglected in comparison, namely the fruit fly intestine. The intestine of larger insects, especially the midgut, has been characterized to a much higher degree than in *Drosophila*, with respect to its morphology and its digestive and absorptive functions.

The insect gut can be divided into separate morphological and functional entities (segments), the foregut, midgut and hindgut (Fig. 6 and Fig. 7A) and each of these parts will be reviewed separately.

1.4.1 The insect foregut

The insect foregut originates from the invaginating ectodermal tissue, which occurs during embryonic development, and can in many insect species be divided into a buccal cavity (mouth), pharynx, oesophagus, crop and proventriculus. Epithelial cells in the foregut are usually flattened squamous cells that do not secrete digestive enzymes into the lumen of the foregut. The insect salivary glands are blind tubes attached to the anterior part of the foregut that secrete fluid which contains amylases (carbohydrate digestion), lubricate the food and contribute to digestion in the crop. In Drosophila, the crop is connected to the uppermost part of the proventriculus and is the storage site of ingested food before it is passed on further for digestion in the midgut (Fig. 6). In other insects, the crop releases food to the midgut at a rate that allows the nutrients to be digested and absorbed more efficiently (Nation, 2002).

1.4.2 The insect midgut

The largest part of the insect intestine is the midgut, which has endodermal origin, and can be divided into additional segments known as the anterior-, mid- and posterior midgut (Fig. 6). The different sections of the gut have very distinct intraluminal pH levels, ranging from pH 7-9 in the anterior and posterior midgut to below pH 4 in the middle midgut region, implying very different functions (Shanbhag and Tripathi, 2009), (Fig. 7A). The anterior midgut segment is believed to be absorptive, based on the observation that the enterocytes in this region are taller with a central cytolasmic dome covered with microvilli, which protrudes into the lumen (Fig. 7B). The much shorter acidic middle region has an interesting cellular morphology and based on ultrastructural examinations these cells are denoted as being both absorptive and secretory (Fig. 7D). Despite many studies, the exact secretory function of these cells is still unclear, as is the composition of the secreted content and regulation of secretion (Lehane and Billingsley, 1996), (Yao and Forte, 2003). One type of cell in the middle midgut that has been studied in more detail in Drosophila larvae are the copper cells, named for their ability to accumulate dietary copper. Recent studies showed that copper cells share several similarities with the acid-producing gastric parietal cells of the mammalian stomach and they are indeed at least partially responsible for the acidification of the middle midgut, implying an important role in food digestion.
(Dubreuil et al., 1998), (Dubreuil, 2004). Cells in the posterior midgut epithelium are large, cuboidal and have an extensively dilated basal extracellular labyrinth compared to the anterior segment cells, however not much is known about the exact function of these cells (Shanbhag and Tripathi, 2009).

Another important constituent of the insect midgut is the peritrophic membrane (PM), which are single or double membranes forming a tube around the ingested food and physically separating it from the intestinal epithelium. The exact type and structural components of the PM vary greatly across different species and their expression and structure can differ between various developmental stages of the same insect. The main roles of the PM are described as digestion and protection of the midgut epithelium (Lehane and Billingsley, 1996).

One of the major functions of the midgut is to digest dietary lipids in the midgut lumen and to absorb and process the digestion products for export into the hemolymph (Beenakkers et al., 1985), (Turunen S, 1993). The digestive process of ingested lipids exhibits two different modes of breakdown in the midgut; the complete hydrolysis of TAG to fatty acids and glycerol (Weintraub and Tietz, 1978), (Tsuchida and Wells, 1988) and the formation of fatty acids and monoacylglycerol in the enterocytes (Hoffman and Downer, 1979), (Male and Storey, 1981). Fatty acids are efficiently absorbed into midgut enterocytes and since fatty acid uptake is the rate-limiting step for diacylglycerol production, this suggest that fatty acid transporters might reside in the luminal membrane of enterocytes (Tsuchida and Wells, 1988), (Hui and Bernlohr, 1997).

Carbohydrates are necessary dietary components of most insect species and most ingested carbohydrates are polymerized and must be broken down to monomers. The best-utilized monomers are glucose and fructose, constituting starch, glycogen and sucrose and galactose and mannose (Dadd, 1985). The enzymes that digest carbohydrates vary greatly across the insect species, however many of them belong to the class of glycosidases (amylases, cellulases, glucosidases, etc). Despite carbohydrates being used by all insects, very few publications describe how they are absorbed in the midgut and most indicate that it occurs by diffusion (Lehane and Billingsley, 1996).
The insect hindgut

The hindgut, like the foregut, develops in the *Drosophila* embryo from ectodermal imaginal discs and starts immediately after the Malpighian tubules (Takashima et al., 2008) (Fig. 6). The terminal part of the hindgut is the rectum or the anal pads. Both
circular and longitudinal muscles lie on the outer or hemolymph side of the hindgut, however the arrangement of these muscles varies greatly along different insect species (Gupta and Berridge, 1966). The cells of the hindgut are usually arranged in a single layer of irregularly shaped epithelial cells that are held together by numerous septate desmosomes that connect the later borders of these cells. The rectum plays an important role in the reabsorption of water, ions and dissolved substances from the primary urine flushed into the hindgut by the Malpighian tubules. The rectal pad cell and rectal papillae are specialised cells found in the rectum of many insects, and have characteristic ultrastructure and physiological mechanisms that promote reabsorption (Nation, 2002).

**1.4.4 Cell types in the Drosophila midgut and the regulation of gut homeostasis**

In the very recent years, several new discoveries have been made in the field of Drosophila intestinal physiology and especially gut homestasis. Most previous research focusing on the function of the fruit fly gut was focused mainly on the involvement of this organ in the immune response and defence against ingested pathogens (MacDonald and Pettersson, 2000), (Boulanger et al., 2001), (Ha et al., 2005). It was long known that the main cell type in the gut is the enterocyte and a few studies have been conducted to characterise its structure and function, with the functional focus being on the brush border as part of the first line of defense (Hegan et al., 2007), (Cario et al., 2002).

As there are several similarities between the mammalian and the Drosophila gut, it appeared quite likely that the fly gut also accommodates intestinal stem cells, comparable to stem cells of its mammalian counterpart. Two important studies shed new light on the matter and provided evidence that stem cells do in fact reside in the Drosophila midgut epithelium ( Micchelli and Perrimon, 2006), (Ohlstein and Spradling, 2006). In the same studies, it was concluded that the Drosophila intestinal epithelium is a monolayer of large, polyploid enterocytes (ECs), which is interspersed with small, diploid and basally embedded intestinal stem cells (ISCs), enteroendocrine cells (EEs) and more apically located enteroblasts (EBs) (Fig. 8).
Adult stem cells are of utmost importance in maintaining tissue homeostasis throughout the course of a lifetime and in promoting tissue repair after injury or disease (Radtke and Clevers, 2005). A key feature of stem cells and their progenitors is the capacity to balance self-renewal/proliferation and differentiation (Yamashita et al., 2010). It is now relatively clear that midgut stem cells divide at a low and steady rate throughout the entire lifetime of a fly, producing a daughter cell while one cell retains stem cell properties. The daughter cell (enteroblast) can subsequently give rise to either enterocytes or enteroendocrine cells, which are the absorptive and secreting cell types respectively (Fig. 9).

**Figure 8.** Schematic drawing of the *Drosophila* intestinal epithelial layer.

**Figure 9.** Schematic representation of *Drosophila* intestinal stem cell proliferation and differentiation. Percentages represent the ratio of enteroblasts that become either enterocytes or enteroendocrine cells. 2n: diploid cell, 8n: endoreplicative cell.
1.4.5 Regulation of *Drosophila* ISC proliferation, self-renewal and differentiation by Notch and Wnt signalling pathways

Intestinal stem cells express the Notch ligand Delta, which is expressed in cytoplasmic vesicles and equally passed on to the enteroblast after cell division. Delta expression is presumably rapidly and actively down-regulated in the daughter cell, followed by differential activation of Notch signalling and the final commitment towards an enterocyte or enteroendocrine cell fate. Delta signal from the ISC to the EB, and subsequent Notch activation, is functionally required for the EB to exit the mitotic cell cycle and differentiate into an EC. In accordance with these findings, loss of Delta in ISCs or low Notch activation in the EBs leads towards an enteroendocrine cell fate, as demonstrated by Notch pathway mutant clones. Stem cells mutant for Delta developed into tumours exhibiting mosaic cell populations with some cells expressing the enteroendocrine cell marker Prospero and some ISC-like cells lacking Prospero expression (Ohlstein and Spradling, 2007). On the contrary, excessive activation of Notch signalling leads to decreased proliferation of ISCs and a reduction in overall abundance of ISC and EB cells (Micchelli and Perrimon, 2006).

In addition to the requirement for Notch signalling in regulating the fate of stem cells, there is clear evidence that this is done in concert with Wnt signalling. The Wnt ligand wingless (Wg) is expressed specifically in the circular muscles surrounding the gut epithelium close to the intestinal stem cells. Reduced function of wg causes ISC quiescence and differentiation, while ectopic expression of wg leads to accumulation of ISC like cells that express high levels of Delta. Moreover, clonal analysis showed that the main downstream components of the Wg pathway, including Frizzled, Dishevelled and Armadillo, are autonomously required for ISC self-renewal (Lin et al., 2008).

1.4.6 Regulation of *Drosophila* midgut homeostasis and regeneration by Cytokine/JAK/STAT signalling

As previously mentioned, JAK/STAT signalling pathway provides a variety of important developmental functions, two crucial roles being establishment of cell fate determination and promotion of proliferation (Jinks et al., 2000; Mukherjee et al., 2005;
Zeidler et al., 1999). Furthermore, the JAK/STAT pathway has a prominent role in cytokine mediated signalling induced by enteric infection or stress. According to one study, damaged or stressed enterocytes produce a range of unpaired (upd, upd2, upd3) cytokines, activating the JAK/STAT pathway in ISCs and thereby promoting their cell division and differentiation. Experimental ablation of enterocytes, enteric infection and JNK-mediated stress signalling can also induce cytokine production and rapid regeneration of the midgut epithelium (Jiang et al., 2009).

1.4.7 Aging related loss of Drosophila intestinal homeostasis

In older flies the overall intestinal homeostasis is highly aberrant, demonstrated through a variety of age-related defects including functional degeneration. One important cause for this age-related decline in homeostasis is damage by oxidative stress and other environmental challenges (Finkel and Holbrook, 2000), (Stadtman, 2001). Signalling pathways that promote stress tolerance have therefore emerged as important regulators of life span, one such pathway being the Jun N-terminal kinase (JNK) pathway. JNK is activated by a range of environmental challenges and increases stress tolerance and longevity in flies and worms (Oh et al., 2005), Oh 2005, (Wang et al., 2003), (Wang et al., 2005). In a variety of animal tissues, replacement of damaged cells through newly formed progeny deriving from pluripotent stem cells is an efficient way to delay tissue degeneration. The age related decline in regenerative potential of highly proliferative tissues implies that aging is caused by a loss of stem cell function (Rando, 2006), (Rossi et al., 2008).

It has been shown that JNK signalling is noticeably activated in aging flies following an induction of ISC overproliferation, possibly as a response to loss of differentiated cells. As a consequence of increased JNK activity, the Delta/Notch expression is disturbed, resulting in cells that retain Delta and simultaneously show activity of the Notch reporter “suppressor of hairless” (Su(H)Gbe-lacZ). The retention of Delta expression in other cell types other than the stem cell is believed to be a hallmark of aging-related loss of differentiation. Enteroblasts expressing Delta do not terminally differentiate into either enterocytes or enteroendocrine cells, hence they accumulate and disrupt overall homeostasis. In addition, these misdifferentiated cells are often polyploid EC-like cells
that do not express EC markers (Biteau et al., 2008). Aging guts also display an overall increase in cell number through increased proliferation and misdifferentiation of ISCs, as the density of normal ECs is decreased and the EE numbers are unchanged (Choi et al., 2008), (Biteau et al., 2008). As mentioned, this deterioration was shown to be due to activated JNK signalling in ISCs and/or EBs of old and stressed intestines. In addition, an interaction between JNK and Notch is required in the ISC lineage to coordinate stress-induced cell proliferation and differentiation.

1.4.8 Models for damage repair in the fly intestine

Several genetic pathways have been shown to regulate intestinal homeostasis in healthy, diseased, stressed and aged flies through control of ISC proliferation. However until recently it was not known if epithelial injury, aside from that caused by pathogens can change ISC division rates as well.

One study addressed this question by causing damage in the fly intestines and examining the ensuing increase in ISC proliferation. Adult flies were fed dextran sulfate sodium (DSS), which has been shown to cause injury in the intestines of experimental animals (Mizoguchi et al., 2008). DSS feeding in flies caused an increase in ISC division and also a marked increase in number of enteroblasts. However, this was not concurrent with an increase in mature enterocyte number suggesting that DSS feeding induces enteroblast accumulation and not differentiation into enterocytes. Based on these data and stainings for basement membrane structure, the idea was postulated that DSS disrupts basement membrane organisation, which is sensed by the ISCs and thereby inducing their cell division. The lack of cell loss amongst the EC cell population was accredited to the absence of the specific signal required to stimulate enteroblast differentiation. In contrast, damage induction specifically in the enterocytes did induce ISC division and enteroblast differentiation (Amcheslavsky et al., 2009). Additionally, one other study reported that forced expression of cell death specifically in the enterocytes also induces substantial proliferation of stem cells and gut renewal (Jiang et al., 2009).
Introduction

In summary, the *Drosophila* mid-gut epithelium is composed of several different types of specialized cells that derive from intestinal stem cells. ISCs are important for maintenance of normal homeostasis and repair of damage to the epithelium.

1.5 Aim of thesis

The initial aim of this thesis was to investigate whether the *Drosophila* Cyclin D/Cdk4 complex plays a role in regulating metabolism in the whole organism and energy storage. Indeed, flies mutant for either of these genes demonstrated defects in lipid and glycogen storage. These defects were traced to an unexpected role of Cyclin D/Cdk4 in maintenance of gut epithelial homeostasis and enterocyte growth. Therefore an additional aim of this thesis was to investigate the nature of the homeostatic defects and link these more generally to control of energy metabolism in the whole fly.
Results

2. RESULTS

2.1 Role of Cyclin D/Cdk4 in the larval and adult fat body

2.1.1 No difference in lipid levels between wt and cdk4\(^3\) mutant larvae

In the light of the existing studies pointing towards roles for the mammalian Cyclin D/Cdk4 that extend beyond the partial requirement of this complex in cell cycle control, we wanted to investigate if the *Drosophila* CycD/Cdk4 complex has functions other than controlling growth. Since it was shown that the existence of mammalian CycD/Cdk4 seems to be of particular importance in metabolically active tissues, our investigation initially focused on an exceptionally metabolically active stage of *Drosophila* development, namely the larval stage. The fruit fly larvae go through three distinct larval stages (L1, L2 and L3) and during this time-period the total weight gain is between 200-300 fold. The increase in larval size and weight is concurrent with extended feeding periods and tremendous accumulation of dietary fat into the larval fat body, the stored lipids later being required for metamorphosis (Mirth and Riddiford, 2007).

One of several questions we wanted to address was whether harbouring a Cdk4 mutation causes any metabolic phenotypes during the intermediate larval stages (late L2 and early L3) under both fed and starved conditions. Total levels of larval lipids were quantified as total triacylglycerol and glycerol. In addition, lipids were visualised using a red fluorescent dye that stains neutral lipids (Nile Red). The experimental animals used were homozygous for the mutant allele *cdk4*\(^3\), which is a null mutation created through mobilization of a 1.8kb intragenic deletion eliminating essential kinase domains (Meyer et al., 2000). As controls, heterozygous *cdk4*\(^3\) mutant animals were used, if not otherwise indicated. To circumvent any sex-specific bias that might exist, early L3 larvae were separated by gender and lipid quantification was done under fed conditions. The *cdk4*\(^3\) mutant larvae of both genders had more lipids than wild-type larvae when normalised against body weight (Fig. 10). Despite these data, when the fat bodies of mutant male larvae were examined by Nile Red staining and compared to heterozygous mutant controls they did not exhibit any obvious increase in lipid
Results

**Figure 10.** No differences in total lipid levels in male compared to female larvae, however cdk4<sup>3</sup> mutant males and females have higher lipid levels compared to respective control. Age of larvae: early L3 (69-72 hours after egg-deposition, AED). Measurement done in duplicates.

**Figure 11.** No difference in lipid accumulation between male cdk4<sup>3</sup> mutant and control larval fat bodies. Lipids are stained with Nile Red. Age of larvae: late L2 (68-70 hours AED) Scale bar: 20 µm

abundance or changes in lipid droplet morphology (Fig. 11). Therefore we hypothesized that the observed increase in lipid levels in mutant males and females most probably is due to the larvae being lighter and smaller than the control, thus meaning they have relatively abundant lipid levels for their body size.

Additionally, to metabolically challenge the larvae, animals were starved on PBS for 20 hours and their fat bodies were stained with Nile Red to examine the extent of reduction in lipid droplet size and abundance. Fat bodies of male cdk4<sup>3</sup> mutant and
control larvae showed similar levels of depletion after PBS starvation, the same was true for male larvae homozygous for a hypomorphic allele of Cdk4 ($cdk4^{SH0671}$, Chen et al., 2003) and $cdk4^{SH0671}$/$cdk4^{SH0671}$ transheterozygous animals, compared to their respective controls (Fig. 12 and data not shown). On the other hand, fat bodies from female larvae showed in general a higher degree of lipid depletion compared to male larvae, with $cdk4^3$ mutant females showing the highest level of depletion (Fig. 12). A possible explanation behind the increased lipid depletion of mutant females could be higher metabolic activity in female larvae, as control females deplete lipids faster compared to control males (data not shown).

### 2.1.2 Cdk4 does not regulate growth of larval fat body cells.

In addition to addressing the question if Cdk4 is required for normal lipid accumulation in larvae, we wanted to know if Cdk4 might have any role in regulating larval fat body nuclear and cellular size? Homozygous $cdk4^3$ mutant clones were induced in the larval fat body using the FRT-FLP technique and fat bodies were stained with Phalloidin to visualize actin filaments defining cell-boundaries. No obvious difference in cell size or nuclear size was observed when comparing mutant clones to the control cells (Fig. 13).

Taken together, we conclude that the Cyclin D/Cdk4 complex most probably does not play any significant role in specifying or maintaining the larval fat body, neither from a cellular nor metabolic point of view. Next in our investigation, we focused on the importance of this complex in the metabolism of the adult fly.

**Figure 13.** $cdk4^3$ mutation does not affect the cell and nuclear size of *Drosophila* larval fat body cells. *Flp/Flp;FRT42- cdk4^3/FRT42-GFP* clones were induced with heat-shock, mutant clones are marked by absence of GFP. Phalloidin marks cell boundaries. Age of female larvae: early L3.
Results

Figure 12. Weak lipid phenotype in cdk4^{SH0671} and cdk4^{3} homozygous mutant females (Early L3 larvae (69-72 h AED), duration of PBS starvation: 20h, tissue: fat body, cdk4^{3}: null mutation, cdk4^{SH0671}: hypomorphic allele. Scale bar: 20 µm.
Results

2.2 Involvement of *Drosophila* Cyclin D/Cdk4 in adult metabolism

In order to investigate potential metabolic roles of the Cyclin D/Cdk4 complex in adults, multiple metabolic analyses were performed on homozygous *cdk4*3 and *cycd*1 male flies; this comprised the previously mentioned lipid quantification, as well as investigation of glycogen and trehalose levels. Quantification of whole animal lipids, glycogen and trehalose is commonly used as a way to assess the existence of metabolic dysregulation, either in *Drosophila* larvae or adults. Furthermore, for reasons of simplicity only adult male flies were used in our investigation, since metabolic studies in female flies are complicated by factors such as egg-production, egg-laying frequency and if a female is mated or not (Ballard et al., 2007; Giesel et al., 1989).

2.2.1 *cdk4*3 and *cycd*1 mutant males have reduced body lipids and are starvation sensitive.

Lipid quantification of whole adult males at different ages revealed a significant decrease in total body lipids in *cdk4*3 homozygous mutants when compared to heterozygous animals starting at 5 days of age, with the mutants exhibiting increasingly less lipids with age, compared to control (Fig. 14a,b and 15a). Similar results were obtained for *cycd*1 mutants (Fig. 15c), as well as animals transheterozygous for the *cdk4*3 and *cdk4*SH0671 alleles (Fig. 15b), indicating that the observed phenotype is Cyclin D dependent and most likely Cdk4 specific. Additionally, both mutant genotypes proved to be highly starvation sensitive under complete starvation, which is a likely consequence of reduced lipid storage (Fig. 15d and e). As a complement we investigated whether *cdk4*3 mutants show a reduction in lipids in their fat bodies. Most of the adult fat tissue can be found in the abdomen, but some also resides in the head and parts of the thorax. Microscopical examination of frozen, cryosectioned fed adult flies that were stained with a lipid stain (Oil Red O, ORO) revealed that *cdk4*3 mutants do indeed have smaller lipid droplets in their fat tissues compared to wild-type (Fig. 16).
Results

Figure 14. *cdk4* ∆ adult males exhibit a significant decrease in lipid levels during their lifetime compared to control flies (a and b). Analysis of total body lipids normalized against body weight of freshly hatched flies (FH, 1 day old) or flies at varying ages between 3 and 32 days. All measurements were done in triplicates if not otherwise indicated and results are shown as mean and SD. Note, at 5 days of age the lipid decrease in mutants is 29 percent, however the measurement of mutant samples was done in duplicates, hence it is not significant.

Figure 16. *cdk4* ∆ mutants have reduced lipid droplet size in the fat body of head and abdomen. Oil red O staining of frozen cryosections of 7 days old adult males. Upper and lower right are close ups of adult fat bodies just under the cuticula. As controls, yw flies were used.
In order to ensure that the observed lipid and starvation phenotypes are truly due to absence of Cdk4 and not secondary mutations, rescue experiments were performed where a Cdk4 transgenic construct was expressed ubiquitously using the Gal4 driver *daugtherless* (*da-gal4*) in a *cdk4* homozygous background. This was sufficient to rescue both the lipid phenotype and the starvation sensitivity (Fig. 17a and 17c), thus confirming that the reduction in total lipid levels and starvation sensitivity is specifically due to absence of Cdk4. Furthermore, ectopic expression of a kinase-dead Cdk4

Figure 15. *cdk4* and *cycd1* mutant males have decreased body lipid levels and are highly starvation sensitive. Total body lipids were quantified from a) 7-8 days old male flies, b) 18 days old flies and c) 7 days old flies. Shown is mean and SD from 3 biological replicates. d) and e) Starvation experiments were performed by placing adult flies on vials with PBS and numbers of dead/alive animals at a certain time-point were scored. Shown is mean of three groups of animals. Age of flies at the start of starvation: 7 days old. Numbers of flies used in d) control n=42, mutant n=45 and e) control n=44, mutant n=47. Statistical significance between survival curves was calculated using Log-rank survival test.
transgene (UAS-Cdk4\textsuperscript{D175N}) unexpectedly also gave a complete lipid rescue, this being contradictory to the commonly-accepted mode of action of Cdk4 (Fig 17a). It was previously described that ubiquitous expression of UAS-Cdk4\textsuperscript{D175N} in flies heterozygous for cdk4\textsuperscript{3} mimics the phenotype of homozygous mutants, resulting in smaller and lighter animals, indicating that growth-promoting functions of Cdk4 require a functional kinase domain (Meyer et al., 2000). Bearing this in mind, the above-described results are quite puzzling, implying that the lipid phenotype represents a Cyclin D-dependent, yet kinase-independent function of Cdk4. The meaning and consequence of these results will be discussed subsequently throughout the thesis.

Since lipid phenotypes often underlie very complex mechanisms and dysfunctions and can also be due to metabolically unrelated secondary effects, it is important to consider multiple possibilities behind a specific lipid phenotype. We considered and examined several plausible causes, such as:

1. Is de novo lipogenesis affected in mutant animals?
2. Do cdk4\textsuperscript{3} mutants have dysregulated lipid breakdown when starved?
3. Is carbohydrate metabolism dysregulated (trehalose/glycogen)?
4. Can free fatty acid feeding rescue the lipid phenotype?
**Results**

**Figure 17.**

a) Reduction in stored lipids in \( cdk4^3 \) mutant adult males is specifically due to absence of Cdk4, as the lipid phenotype can be rescued with ubiquitous re-expression of Cdk4 (Cdk4). Additionally, kinase inactive Cdk4 (Cdk4\(^ {D175N} \)) can completely rescue the lipid phenotype. Percentages are calculated based on two separate experiments.

b) Ubiquitous expression of kinase inactive Cdk4 causes increased lipids in a \( cdk4^3 \) heterozygous background. Age of flies: 8-9 days. Shown is mean and SD from 4-6 biological replicates.

c) Starvation sensitivity of \( cdk4^3 \) mutants can be rescued with Cdk4 re-expression. Starvation experiments were done on PBS only and numbers of dead/alive animals at a set time-point were scored. Numbers of flies used: \( +/^-da-g4 \) n=88, \( -/-da-g4 \) n=81, \( +/-da-g4 \) UAS-cdk4 n=27, \( -/-da-g4 \) UAS-cdk4 n=51. Shown is mean. All experiments are done with mature 7-8 days old adult male flies. Lipid levels are shown as mean and SD, if not otherwise stated.
Results

2.2.2 cdk4^3 mutant males respond to diet-induced obesity

Lipogenesis (de novo lipid synthesis) is a process where fatty acids are synthesised from acetyl CoA, following incorporation mainly into triglycerides (Bailey et al., 1975; Soulages and Wells, 1994). Increased lipid storage can also result from lipogenesis in the fat body from stored carbohydrates such as glycogen (Beenakkers et al., 1985). The insect fat body generally has a higher ratio of lipid compared to glycogen storage, which in some species is due to the higher capacity of the fat body to perform lipogenesis from glucose (Zhou et al., 2004). Since the underlying cause of observed lipid phenotypes in insects can be dysregulated lipogenesis, we investigated whether the reduced lipid levels in cdk4^3 mutant flies could be due to less lipid synthesis (Kunte et al., 2006; Okamura et al., 2007).

Fatty Acid Synthase (FAS) and acetyl-Co-A carboxylase (ACC) are two well characterized rate-limiting enzymes that regulate the production of fatty acids for TAG storage in mammals. Changes in their mRNA expression can be indicative of disrupted fatty acid production (Foufelle et al., 1996; Kunte et al., 2006; Okamura et al., 2007). Quantitative Real-Time PCR (qRT-PCR) performed on whole male flies

Figure 18. Mutant adults respond to diet induced obesity.

a) FAS transcript shows a significant decrease in mutant animals compared to control. Quantitative RT-PCR was performed on RNA from whole adult males (7 days old). b) Lipids were measured at the start of the experiment in 14-15 day old males (White bar, Fed) and compared to control males fed on normal food for an additional 7 days (Black bar, Fed + 7d) or to males fed with only 20% sucrose for 7 days (Grey bar, Fed +7d sucrose). All lipid levels were quantified simultaneously. Shown is mean and SD. Experiments were performed in duplicates.
Results

showed a 36.6 percent decrease in gene expression of FAS, however mRNA levels of ACC were unchanged (Fig. 18a). The reduction in FAS gene-expression could be a sign of a primary defect of mutant animals in performing lipogenesis or simply reflect decreased lipid synthesis due to a change in upstream metabolic flux.

In order to challenge the lipogenic capabilities of cdk43 mature adults, flies were kept on a sucrose/PBS only diet for one week followed by lipid level quantification. Sucrose is a disaccharide derived from glucose and fructose and as mentioned, can be readily used by insects for lipid synthesis. In mammals, glucose can be used for de novo lipid synthesis through conversion to pyruvate, followed by further conversion to Acetyl CoA via the TCA cycle and via conversion of glucose to glycerol. Acetyl CoA is used for making free fatty acids that combine with glycerol to form TAG (Ducharme and Bickel, 2008). Lipid quantification revealed that cdk43 mutant flies could in fact use glucose derived from sucrose for de novo lipid synthesis to the same extent as control flies (Fig. 18b). This also supports the notion that down-regulated FAS gene-expression is not the cause of the lipid phenotype, but most probably reflects lower overall metabolic activity/flux in the mutants.

2.2.3 cdk43 mutant males do not have increased starvation-induced lipolysis.

To address if the starvation sensitivity of mutant males is due to increased or inhibited lipid breakdown (lipolysis) during starvation, time-course experiments of PBS starvation were performed. Increased depletion of lipid storage or inhibition of lipolysis have been shown to be causes of increased sensitivity to starvation. Lipid breakdown in the insect fat body is controlled by hormones and lipases, the latter being enzymes that can hydrolyze both dietary and stored TAG (Asher et al., 1984; Gronke et al., 2005; Gronke et al., 2007). Over-expression of the starvation-induced TAG lipase Brummer (Bmm) leads to excessive lipase activation, which results in abnormally rapid TAG breakdown, rendering flies starvation sensitive (Gronke et al., 2005; Gronke et al., 2007). Conversely, flies harbouring a bmm loss-of-function mutation display highly increased TAG storage and as a consequence are extremely starvation resistant. However, despite being considerably more starvation resistant compared to control
flies the \textit{bmm} mutants still have a lipolysis defect, demonstrated by remaining excessive TAG levels at their time of death (Gronke et al., 2005).

cdk4\textsuperscript{3} mutant and control males were starved for a defined time periods and lipids were quantified (Fig. 19). Both genotypes showed a similar depletion of lipids during starvation, demonstrating that cdk4\textsuperscript{3} mutant males do not exhibit decreased or increased lipolysis when starved. However, this does not exclude that basal lipolysis is increased prior to starvation, as this matter was not addressed.

![Figure 19](image_url)

\textit{Figure 19.} cdk4\textsuperscript{3} mutant males respond normally to starvation by inducing lipolysis. Experiment was done with mature, 7 days old adult flies. Starvation was done with PBS only. Decrease in lipid levels is indicated as percentage and shown is mean and SD from two biological replicates.

\subsection*{2.2.4 cdk4\textsuperscript{3} mutant males have decreased glycogen levels}

In order to fully characterise the metabolic status of cdk4\textsuperscript{3} mutant flies, two other metabolic read-outs were used, namely levels of stored glycogen and stored/circulating trehalose. Quantification of glycogen levels in mature, fed adult males showed that cdk4\textsuperscript{3} mutants have significantly decreased glycogen storage compared to control animals (Fig. 20a), however trehalose levels were relatively unchanged (Fig. 20b). A rescue experiment was performed to confirm that the decrease in glycogen is specifically due to absence of Cdk4, which proved to be the case as glycogen levels can be completely restored with ectopic expression of Cdk4.
Results

Additionally, over-expression of a kinase inactive Cdk4 transgene lead to significantly elevated glycogen levels compared to heterozygous control flies with and without expressing a kinase active Cdk4. The same transgene caused a significant increase in glycogen when expressed in a cdk4<sup>3</sup> homozygous mutant background compared to mutant controls, however not as dramatically when compared to cdk4<sup>3</sup> heterozygous animals.

Taken together, our data show that lack of Cdk4 results in nutrient storage defects, demonstrated through a dramatic decrease in lipid and glycogen levels in cdk4<sup>3</sup> mutant adult flies.
Results

2.2.5 Oleic acid feeding does not rescue the mutant phenotype.

Since breakdown of dietary fat by lipases is a crucial step in absorption and subsequent storage of lipids, as the next step we wanted to investigate if lipase expression was altered in the mutants. As mentioned earlier, lipases are enzymes that catalyse the hydrolysis of ester chemical bonds in lipid substrates, thereby releasing free fatty acids from their glycerol backbone (Ducharme and Bickel, 2008). Gene-expression levels were examined in whole flies of a number of Drosophila lipases, shown grouped as either acidic or neutral/phospholipases. This classification derives from a study that used sequence and functional information across five holometabolous insect species to group lipases into distinct families. Motif search of

![Graph](image)

**Figure 21.** Gene expression profiling of a number of Drosophila lipases shows that some are significantly down-regulated in mutant animals. a) Neutral lipases, except CG8552 which is a phospho-lipase and Bmm, which is a ATGL lipase. b) Acidic lipases qRT-PCR was performed on RNA extracted from whole male flies and performed in biological triplicates. Shown is mean and SD.
Results

catalytic residues of neutral and acid lipase protein sequences were analysed using BLASTN and annotated (Horne et al., 2009). The results show that four out of eleven examined lipases were highly down-regulated in cdk4\(^3\) mutant animals compared to control (Fig. 21). These data could potentially indicate that defects in dietary lipid breakdown could be the cause of the lipid phenotype. Unfortunately very few lipases have been functionally characterised in Drosophila, making it difficult to draw conclusions regarding what the actual function of the down-regulated lipases might be. Additionally, there are over 50 genes believed to encode Drosophila lipases, therefore even more lipases could potentially be down-regulated in the mutants aside from the ones detected here.

Nevertheless, we sought to examine the possible connection between reduced lipase expressions in the mutant flies with reduced lipid levels. A feeding experiment was performed where the requirement for functional lipases was partially circumvented by supplementing normal fly food with oleic acid (OA). Oleic acid is a mono-unsaturated free fatty acid that is readily taken up by Drosophila S2 cells and the fly intestine and is consequently used for de novo lipid synthesis (Guo et al., 2008; Kunte et al., 2006;

![Graph](image)

**Figure 22.** Lipid quantification of adult flies fed with either Oleic Acid (OA) supplemented or normal (N) fly food for 8 or 15 days (OA concentration 400 \(\mu\)M, concentration starting age 8-9 days). OA feeding for 15 days caused a slight increase (8.4%) in total lipids in control flies when compared to flies of the same genotype fed on normal diet for 15 days. OA feeding had no effect on mutant flies. Shown is mean and SD of three biological replicates.
**Results**

Sieber and Thummel, 2009). Sixteen days of OA feeding can lead to an increase in lipids in wild-type flies of up to 25.6 percent compared to flies at the start of OA feeding (data not shown). In another experiment OA feeding was performed for fifteen days and led to an 8.4 percent lipid increase in cdk4\(^3\) heterozygotes compared to flies kept on normal food for the same length of time (Fig. 22). However, oleic acid feeding of cdk4\(^3\) mutant males had no effect on their total lipid storage after fifteen days, compared to control mutants kept on normal food (Fig. 22). From these results we could not conclude with certainty that the observed decrease in lipase gene-expression indeed has any direct effect on dietary lipid breakdown, nonetheless we concluded that cdk4\(^3\) mutants might have impaired intestinal absorption. Unfortunately, attempts to directly study intestinal absorption in mutant animals were unsuccessful, however the failure to accumulate lipids following OA feeding together with reduction in both lipids and glycogen hinted towards intestinal defects. Moreover, the observed down-regulation in lipase expression would only be able to possibly explain the decrease in lipid and not glycogen levels, as other enzymes are required for cleavage of dietary carbohydrates. Because the adult intestine is responsible and required for absorption of all nutrients, defects in this organ could explain the dual nutrient storage defects in cdk4\(^3\) mutant animals. Therefore, we turned our attention to examining any potential dysfunctions in the intestine of mutant animals and if the *Drosophila* Cyclin D/Cdk4 complex has any role in intestinal physiology and function.

**2.3 Role of CycD/Cdk4 in adult intestinal physiology**

**2.3.1 cdk4\(^3\) mutant males have aberrant intestinal clearing rates.**

At the present, no straightforward or reliable quantitative nutrient absorption assay for *Drosophila* has been developed, one of the obstacles being the small size of the fruit fly or the limitations of feeding dyes that can be used for such experiments. Nonetheless, a few assays exist that address intestinal absorption defects in an easy but more indirect way, one of them being a quantitative gut clearance assay. The principle of the assay is feeding of flies with dye coloured fly food for a set amount of time, followed by a time-course where flies are allowed to clear their intestines on normal food. One previous study has shown that de-regulated rates of gut clearance
Results

can be correlated with intestinal absorption defects and nutrient storage related phenotypes (Bland et al., 2010).

cdk4^3 mutant males and controls were allowed to feed on normal fly food containing the non absorbed food dye Allura Red (AR) for around twelve hours (over-night), subsequently followed by gut clearance on normal food for 20 or 60 minutes. Mutant males contained higher levels of dye already at time-point zero and exhibited very low

![Image of cdk4^3 mutants with AR dye](image)

**Figure 23.** cdk4^3 mutants have aberrant intestinal clearing rates, indicated by Allura Red quantification (AR).

a) Upper panel shows dissected adult guts after feeding with AR over-night. Lower panel is a close-up of one gut per genotype from upper panel.

b) Quantification of absorbance of homogenized adult flies fed AR over-night to measure the amount of AR present in the flies at indicated time-points. Red line indicates the absorbance of control flies not fed AR. Experiment was done in triplicates and bars show mean ± SD.
Results

level of gut clearance after 60 minutes compared to controls (Fig. 23b), which hinted towards reduced food absorption in mutant animals. In addition, microscopical investigation of dissected intestines pre-fed with AR revealed that mutant guts not only have higher levels of AR in the gut, but their crops were clearly extensively dilated and filled with red coloured food (Fig. 23a). In higher insect species the meal size kept in the crop prior to being passed on into the intestine is controlled by the digestive rate in the anterior part of the midgut (Nation, 2002). Hence, the excessive food accumulation in the crops of cdk43 mutant flies could imply possibly reduced midgut digestion or muscle related intestinal clearance issues. Either way these results provided further incentive to more closely investigate the mutant intestine.

2.3.2 cdk43 mutant males have multiple intestinal phenotypes

As previously discussed, the Drosophila adult intestine comprises multiple cell types, the main cell type being the absorptive enterocyte (EC) interspersed with intestinal stem cells and enteroendocrine cells (EEs) (Fig. 8). Intestinal stem cells give rise to both enterocytes and enteroendocrine cells through the mechanism of asymmetrical cell division. Differential levels of Notch signalling in the daughter cell (the enteroblast) determine the cell fate. Enterocytes are easily recognised as they are endoreplicating cells and thus have large nuclei (8N). Enteroendocrine cells are small, diploid cells that stain positively for the transcription factor Prospero and intestinal stem cells are diploid with small nuclei and stain cytoplasmically for the Notch ligand Delta (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). Microscopical examination of the cdk43 mutant intestines was performed using these morphological and marker criteria to distinguish the different cellular populations in the intestine.

2.3.3 cdk43 mutant males have reduced EC and EE numbers

The initial analysis of mutant intestines from mature adult males revealed greatly reduced numbers of enterocytes and enteroendocrine cells (Fig. 24a,b and 24d,e respectively). The EE numbers were counted across the length of the whole gut and the EC numbers were quantified separately in the anterior and posterior midgut, due to their different morphology and cell composition. Throughout this thesis all immunofluorescent pictures show the anterior midgut if not otherwise stated.
Results

Additionally, the enterocytes in cdk4^3 mutant intestines displayed smaller nuclei (Fig 24f,g) that stained weaker with 4',6-diamidino-2-phenylindole (DAPI), yet these enterocytes had larger cellular area (Fig. 24c). In order to examine if the decrease in enterocyte number and increase in cellular area were acquired phenotypes that appear with age, mutant intestines of newly eclosed male flies were examined. In intestines of immature and very young flies the enterocytes have a smaller cellular area than in older flies, demonstrated by the nuclei lying very closely together with a smaller cytoplasmic area. In immature mutant intestines the enterocyte number was already greatly reduced and the difference in cellular area compared to control intestines was even more dramatic than in older flies (Fig. 25).

The reduced nuclear size in mutant enterocytes supports a hypothesis that Cyclin D/Cdk4 complex is most likely involved in regulating their growth. Since, as mentioned before, growth and endoreplication are two tightly linked cellular processes. Based on the smaller EC nuclear size and weaker DAPI staining in mutant intestines, we presumed that the mutant ECs might have endoreplication defects. An assay was performed to address the extent of endoreplication in mutant and control intestines, however these results will be described in more detail later.

Although the observation that mutant EC cells simultaneously have a decrease in nuclear but an increase in cellular area is somewhat contradictory, this could be a very likely outcome of growth impairment in a monolayered tissue. Reduction in cellular number, but increase in cellular size has been described previously for other cdk4^3 mutant epithelia such as the wing (Meyer et al., 2000). A plausible explanation behind increased enterocyte area could be that the cells are compensating for the vast decrease in cell number by simply stretching in order to maintain epithelial integrity. Reasonably, this should lead to decreased total thickness of the epithelium and not increased compensatory growth.
Results

Figure 24. Mutant intestines have decreased numbers of enterocytes and enteroendocrine cells. a) DAPI and armadillo stainings. b) Quantification of EC number. c) Quantification of EC cellular area. d) Overlay of Prospero and DAPI staining. e) Quantification of the number of Prospero positive EE cells per gut. f) Overlay of DAPI and lamin staining. g) Quantification of EC nuclear area.

Numbers of quantified cells in c) control: n=47, mutant n=74 and g) control n=52, mutant n=51. In b) 5 pictures were used for quantification. In e) prospero positive cells in 4 guts/genotype were counted. Age of all flies: 7-8 days old. EC number graph and prospero graphs shown as mean and SD. EC cellular and nuclear size is shown as mean and SEM. Scale bars in: a) 25 µm, and f) 10 µm. All pictures are taken in the anterior midgut, except d) that was taken in posterior midgut.
2.3.4 cdk4<sup>3</sup> mutants have reduced gut gene expression of β-trypsin and tachykinin.

In association with the results showing that cdk4<sup>3</sup> mutant intestines have significantly reduced enteroendocrine numbers, we wanted to investigate if this reduction in cell number could potentially have physiological consequences. As mentioned, enteroendocrine cells are considered the main secreting cell type in the insect gut, their secretory products ranging from enzymes breaking down nutrients, to hormones and neuropeptides that control the muscles innervating the intestine and thus food
Results

passage. Additionally, in light of the observation that mutant flies have reduced rates of gut clearance, this could be a phenotype plausibly connected to having fewer EE cells. Therefore we examined the mRNA expression levels of genes encoding for trypsin and various neuropeptides such as allatostatins and neuropeptide F found in the Drosophila gut.

Trypsins are serine proteases that belong to the group of endopeptidases and are enzymes specialized in breakdown of proteins, with specificity for the peptide bond formed by the carboxylic side of Arg or Lys residues (Terra and Ferreira, 1994). Invertebrate trypsins have been widely studied, however they show very species-specific characteristics and more knowledge is available for insects other than Drosophila (Muhlia-Almazan et al., 2008). Four genes belonging to a clustered gene family coding for trypsin genes in Drosophila have been isolated, however their exact function is not known so far (Davis et al., 1985), (Wang et al., 1999). In vertebrates, trypsins are synthesized in specific cells in the pancreas and secreted into the gut in their inactive form, to be subsequently activated by another serine protease. Some of the mechanisms of storage, secretion and activation have also been shown to function in a similar manner in some invertebrates such as mosquitoes and other hematophagous insects (Lehane et al., 1998). In a recent publication, expression of three trypsin genes was used as a read-out for general functional deterioration of the Drosophila intestinal mucosa. The expression of the most abundant trypsin gene, β-trypsin, was reduced by around 60 percent in 30 days old fly intestines compared to 3 day old flies (Biteau et al., 2008).

Allatostatins are neuropeptides known to inhibit juvenile hormone production in the ring gland, the major endocrine organ, and can in addition act as antagonists and slow down gut peristalsis. They are believed to be secreted by endocrine cells present throughout the entire intestine and to date, four different Allatostatin peptide families have been found in Drosophila (Veenstra et al., 2008), (Lehane and Billingsley, 1996).

Tachykinins belong to the myotropic neuropeptide family and their main function is to control muscle contraction, although other unknown functions may exist (Champagne and Ribeiro, 1994), (Nassel et al., 1995). The same type of enteroendocrine cells that secrete tachykinins often also secrete Neuropeptide F, which is found in both the
midgut and the central nervous system. Its main function is though to be control of feeding behaviour (Veenstra et al., 2008).

Quantitative RT-PCR was performed to examine gene expression of three different trypsin genes (β-, ε- and γ–trypsin), three allatostatin isoforms (A, B and C) and two types of neuropeptides (Tachykinin and Neuropeptide F). The analysis revealed that mutant intestines show a significant down-regulation in expression of β-trypsin and tachykinin (Fig. 26a and 26b respectively). Since tachykinin is involved in controlling gut muscle contraction it is possible that its reduced gene expression could have an adverse affect on muscle contraction in mutant animals. Why this is most likely not the case will be discussed later. It is difficult to speculate about the physiological significance of reduced β-trypsin mRNA levels, as a clear role for Drosophila trypsins has not been defined. One possibility is that mRNA levels correlate with a decrease in EE numbers, however older flies exhibit a decrease in trypsin mRNA but not a corresponding reduction in cell number (Biteau et al., 2008). If reduced β-trypsin levels truly reflect mucosal integrity and function, the reduced gene expression could be connected to decreased absorptive functions.

Figure 26. a) and b) Mutant adults have reduced mRNA levels of midgut β-trypsin and tachykinin. Ast: Allatostatin, Tk: Tachykinin, Npf: Neuropeptide F. (γ–trypsin transcript could not be detected). qRT-PCR was performed on dissected midguts from 7-8 days old male flies. Shown is mean and SD of three biological replicates.
Results

2.3.5 cdk4<sup>3</sup> mutants have reduced lipase expression in the midgut

In connection with attempting to find a link between decreased EE numbers and affected gene expression of EE secretive products, we examined in addition if there is a similar consequence of having fewer EC. Since we could show that cdk4<sup>3</sup> mutant adults have reduced whole body lipase gene-expression, we examined if lipase mRNA was reduced specifically in adult midguts as well. Lipases were again grouped in the same way as in the previous experiment and the down-regulation of several lipases could be confirmed (Fig 27a and 27b). mRNA levels of lipase<sub>3</sub> and CG8093 were not statistically significantly different due to the large standard deviation, however the decrease in gene expression based on the means was 78.4 and 60.64 percent respectively. Since most of the examined lipases have not been functionally characterized, we wanted to know if RNAi based knock-down of these lipases specifically in the enterocytes could have any influence on stored lipid levels. NP1-gal4 driven RNAi of lipase<sub>3</sub>, CG8093 and CG6529 resulted in 5.36 and 5.68 percent decreases in lipids and a 12.24 percent increase, respectively (data not shown). The lack of dramatic lipid phenotypes may potentially be due to the very large family of lipases exhibiting a certain degree of functional redundancy. It may require simultaneous knockdown of multiple lipases to have an effect on dietary lipid breakdown and storage. Therefore we cannot conclude with certainty that the

![Figure 27](image-url)  
**Figure 27.** Mutant flies have decreased levels of several lipases in their midgut. qRT-PCR was performed on dissected midguts from 7-8 days old male flies. Mean and SD from three biological replicates is shown.
decrease in lipase expression has a definite effect on the observed mutant lipid phenotype, or if it simply reflects functional defects in mutant ECs.

**2.3.6 cdk4^3 mutant intestines undergo loss of intestinal stem cells and enteroblasts with age**

As previously mentioned, the requirement for intestinal renewal is fulfilled by increased cell division of intestinal stem cells and the direction of cell fate is decided accordingly to the homeostatic needs. Since mutant intestines display a reduction in both enterocytes and enteroendocrine cell number, it is reasonable to assume that the cause behind this dual phenotype must partially or entirely lie within the intestinal stem cells. The only way to reliably detect *Drosophila* ISCs is by expression of Delta and/or GFP expression driven by the Gal4 driver *escargot* (*esg*), which is strongly expressed in ISCs and enteroblasts and very weakly in immature enterocytes (Micchelli and Perrimon, 2006). Delta is confined in vesicles in the cytoplasm of ISCs and can also be detected in the newly formed daughter cells, however only as single Delta positive vesicles (Ohlstein and Spradling, 2007). Therefore, intestinal stem cells and enteroblasts are distinguished by presence of *esg*-GFP and presence or absence of Delta respectively. Mis-expression of Delta in cells that do not appear to be stem cells, i.e. that are enteroblasts, points towards differentiation defects and loss of “stem-cellness” (Biteau et al., 2008; Ohlstein and Spradling, 2007).

The abundance of intestinal stem cells and enteroblasts was examined in mutant intestines by expressing GFP driven by *esg-gal4* (*esgGFP*) in a *cdk4^3* mutant background, combined with prospero antibody staining to specifically mark EE cells. Already the intestines of newly eclosed mutant flies showed a marked decrease in the abundance of GFP positive cells compared to control intestines and this decrease became even more dramatic in slightly older flies (6-7 days old) (Figure 28a and 28b respectively). In addition, the GFP signal of numerous cells in the mutant intestines is extremely weakened and appears to be disappearing, which is something not observed in control intestines. The quantification of GFP positive cells could not in itself reliably reveal which fraction of the cell population was in fact reduced. ISCs and EBs express GFP in slightly different patterns, ISCs expressing a weaker, more nuclear GFP and EB expressing a stronger, both nuclear and cytoplasmic GFP signal.
Results

Despite co-labelling with Delta in order to reliably detect stem cells, no counting of Delta positive cells could be performed, as the staining was most often too weak to be detected and counted reliably.

Since these results were highly important for understanding the disrupted intestinal homeostasis in mutant animals, it was of utmost importance to unravel the exact defect in the stem cells. Loss of stem cells usually implies loss of stem cell identity and the underlying mechanisms are rather diverse and complex. These mechanisms range from cell autonomous defects through disruption or loss of asymmetrical stem cell division, to cell non-autonomous defects such as cell adhesion, spatial cues and in a few cases even physical or mechanical disruption of the stem cell niche (Jones and Wagers, 2008; Lin, 2002; Wu et al., 2008).
Results

At present, it is not fully understood how *Drosophila* intestinal stem cell maintenance is controlled through regulation of differentiation and proliferation. A few key pathways have been characterised for their involvement in controlling ISC maintenance and thus intestinal homeostasis, such as the Wnt/Wingless, Notch, JAK/STAT and JNK pathways (Biteau et al., 2008; Jiang et al., 2009; Lin et al., 2008; Ohlstein and Spradling, 2007). The Notch signalling pathway has been established as a major regulator of intestinal stem cell fate and differentiation, as differential activation of Notch decides the cell lineage direction. These stem cell fate decisions are at least partially decided upon together with the Wnt/Wingless (Wg) pathway. In turn, loss of Wnt/Wingless signalling through loss of its downstream pathway components leads to less stem cell proliferation, however without the overall differentiation being affected, demonstrated through normal enterocyte and enteroendocrine numbers. A similar reduction of ISC proliferation can be observed in loss of function alleles of Armadillo or Dishevelled, two Wg components, that similarly to loss of Cdk4 lead to loss of intestinal stem cells over time (Lin et al., 2008).

To examine if the described loss of stem cells and enteroblasts in *cdk4<sup>3</sup>* mutant intestines could be due to differentiation defects in these two cell types, *esgGFP* expression and co-labeling with Delta was performed to address this question. Shown in Fig. 29 is a mutant intestine demonstrating several different combinations of *esgGFP* and Delta expression. While many cells display the normal expression pattern, one cell expressing GFP and with a nucleus larger than observed in stem cells stains positive for Delta. As mentioned above, mis-expression of Delta in cells that do not appear to be stem cells points toward differentiation defects and/or terminal differentiation of a stem cell. Thus, the presence of mis-differentiated ISCs/EBs could explain the decrease in intestinal stem cell numbers in *cdk4<sup>3</sup>* mutants. The nature of possible differentiation defects needs to be further investigated experimentally (see Discussion).
Results

2.3.7 Proliferation rates of intestinal stem cells are reduced in \textit{cdk4}^3\ mutant guts

An important question was if the mutant stem cells that are still present have changed proliferation rates. To investigate the proliferation rate of \textit{cdk4}^3\ mutant intestinal stem cells a BrdU (5-bromo-2-deoxyuridine) assay was performed, where BrdU is incorporated into newly synthesized DNA during replication and in endoreplicating cells (Micchelli and Perrimon, 2006). BrdU feeding was performed with \textit{cdk4}^3\ mutant and control animals for 48 hours and started at 2 days of age. When scoring for BrdU incorporation, distinction was made between BrdU positive enterocytes (large nuclei) and stem cells/enteroblasts (small nuclei). Young, control flies show a relatively high level of BrdU incorporation in small cells, reflecting self-renewing proliferation of stem cells in healthy intestines. On the other hand, BrdU incorporation is very low in large

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{merge.png}
\caption{Enteroblasts in \textit{cdk4}^3\ mutant intestines show differentiation defects. Arrows are pointing out:
a) Solitary ISC expressing Delta. b) ISC expressing Delta and adjacent to it an EB without Delta, expressing GFP. c) ISC and EB both expressing Delta, possibly very recent division. d) Larger cell, most likely an EB abnormally expressing Delta. Age of flies: 7 days old. Scale bar: 15 \(\mu\text{m}\).}
\end{figure}
Results

nuclei, indicating that few new enterocytes were born, as would be expected in young intestines with little environmental damage (Fig. 30). Strikingly, this ratio of BrdU positive nuclei is reversed in cdk4<sup>3</sup> mutant intestines, where incorporation into small nuclei is significantly lower compared to the control and incorporation into large nuclei is abnormally high (Fig. 30). This would suggest low proliferation of intestinal stem cells and increased turnover of endoreplicating enterocytes, which is quite contradictory, as new enterocytes require more stem cell proliferation. One way to

![Graph showing BrdU incorporation in small and large nuclei for cdk4<sup>3</sup>/+ and cdk4<sup>3</sup>/cdk4<sup>3</sup> genotypes.]

**Figure 30.** Stem cells in cdk4<sup>3</sup> mutant intestines proliferate less compared to control intestines shown by decreased numbers of cells with incorporated BrdU. However, mutant enterocytes show significantly increased BrdU incorporation.

Duration of BrdU feeding: 2 days, started 2 days after eclosion. Shown is mean and SD. Counting of BrdU positive nuclei was done in 10 intestines for control and 8 for the mutant genotype.

directly detect proliferating cells is to stain for the mitotic marker phopho-histone-3 (pH3). If there were increased enterocyte turnover in mutant intestines one would expect more pH3 positive cells, which was not the case (data not shown).

Importantly, incorporation of BrdU into newly synthesised DNA does not only occur during replication, as DNA damage can also trigger DNA synthesis and consequently lead to BrdU incorporation. This means that BrdU positive large nuclei could suggest increased DNA damage in ECs. Another possibility is that mutant enterocytes endoreplicate at a slower rate and thus more cells at any timepoint in mutant guts are “trapped” in the process endoreplication, and hence incorporate BrdU, than in control
guts. In conclusion, since $cdk4^3$ mutant enterocytes can incorporate BrdU, indicative of endoreplication, but exhibit smaller nuclei and weaker DAPI staining, it appears that Cdk4 is necessary for fully efficient endoreplication in ECs.

### 2.3.8 $cdk4^3$ mutant intestinal epithelium displays changed abundance of junction proteins

To date, there is no clear evidence that a specified physical gut stem cell niche exists and the only known niches are either transient or partially specified by muscles surrounding the gut epithelium (Lin et al., 2008; Mathur et al., 2010). The results reviewed above, with regard to significant intestinal stem cell loss in $cdk4^3$ flies, suggests that the mutant epithelium cannot support either normal proliferation and/or differentiation of stem cells. The requirement for spatial orientation and/or polarity cues offered by a physical stem cell niche and its signalling environment, in order to support stem cell division, has been well established for example in the *Drosophila* male and female germline and the dividing stem cells of the peripheral and central nervous system (Egger et al., 2008; Kiger et al., 2001; Kiger et al., 2000; Tulina and Matunis, 2001; Xie and Spradling, 2000). Therefore, it is plausible to assume that intestinal stem cells also reside in a permanent physical stem cell niche. Since the intestinal epithelium has quite a distinct cellular architecture and disruptions of this organisation can induce stem cell proliferation, we wanted to investigate if there are any issues in the structural arrangement of mutant epithelial cells. Dissected mutant and control intestines were stained for different proteins located in specific epithelial cellular junctions that reside between the intestinal cells (for schematic overview, see Fig. 31). The adherens junctions were stained with the *Drosophila* β-catenin homolog Armadillo (Arm) and the septate junctions with Discs Large (Dlg) and α-spectrin.

Armadillo is a cytoplasmic protein that binds directly to its transmembrane protein DE-cadherin and thus is an essential part of the epithelial cadherin-catenin complex (CCC) that resides in adherens junctions. Adherens junctions play a key role in cell-cell adhesion and in organizing cells into epithelia and disruptions of the CCC leads to loss of factors from the apical membrane (Oda et al., 1994; Oda et al., 1997). Several studies have emphasized the importance of Arm for adherens junction assembly, cell polarity and morphogenesis during *Drosophila* embryogenesis and oogenesis. For
example one such study showed that partial reduction in *arm* function in embryos was sufficient to cause loss of epithelial integrity (Cox et al., 1996; Peifer, 1993; Peifer et al., 1993). Aside from being a part of the CCC complex, Arm is an important effector of the Wingless (Wg) signalling, however the relationship between the cytosolic and junctional Arm remains unclear.

Residing underneath the adherens junctions are the septate junctions (SJs), which have been proposed to play a role in formation of trans-epithelial diffusion barrier, establishing and/or maintaining cell polarity, cell adhesion and mediating interactions between cells (Baumgartner et al., 1996; Lamb et al., 1998). A specific component of SJs of imaginal discs and in late embryos is the MAGUK (membrane-associated guanylate kinase) protein Dlg. Originally, *dlg* was identified as a tumour suppressor gene that controls proliferation and integrity of imaginal discs, however it plays an important role in maintaining epithelial integrity and cell polarity as well (Bilder et al., 2000; Woods and Bryant, 1991; Woods et al., 1996).

One important aspect of sustaining epithelial organisation is through epithelial polarity and thus the polarisation of the underlying actin-based cytoskeleton. The polarisation
of this cytoskeleton occurs through interactions between polarised transmembrane proteins, membrane-associated cytoplasmic proteins and cytoskeletal proteins (Yeaman et al., 1999). Epithelial cells contain a polarised spectrin cytoskeleton that associates with the apical or basolateral membrane and consequently contributes to polarized membrane organisation by binding membrane proteins at the basolateral surface (McNeill et al., 1990), (Tepass et al., 2001). The spectrin protein is a tetrameric actin crosslinking protein and Drosophila has three different spectrin subunits α,β and βH-Spectrin, which assemble into two different isoforms α₂β₂ and α₂β₁H₂-Spectrin (Dubreuil et al., 1998; Thomas, 2001). These isoforms can be found at the basolateral membrane in a complex with Ankyrin and enriched in the marginal zone, respectively (Dubreuil et al., 1997), (Lee et al., 1993), (Thomas, 2001).

The intestinal stainings against the above-reviewed epithelial junction proteins and the following microscopical examination revealed that the abundance of these proteins was affected in cdk4<sup>3</sup> mutants. Mutant guts stained weaker for Arm protein compared to control guts (Fig. 32a), but significantly stronger for Dlg (Fig. 32b). Staining against α-spectrin protein stained all intestines weakly in a similar pattern as Arm protein, implying that it stains adherens junctions as suggested previously (data not shown, (Thomas and Kiehart, 1994; Thomas et al., 1998). Most noticeably, α-spectrin stained underneath small cells, most likely stem cells, in a region that appeared to be between the basal extracellular matrix and the basement membrane. In mutant intestines the protein seemed to either be more abundant or deposited in a denser manner (Fig. 32c).

Taken together, these data demonstrate that mutant intestines display changes in protein abundance of junction proteins, consistent with the epithelial disorganisation observed in the previous cellular analysis. This is demonstrated by less Arm protein and strong accumulation of α-spectrin and Dlg proteins, which is not observed in control intestines. The possible explanation behind these results and their consequence will be discussed later.
Mutant intestines show changed abundance of epithelial junction proteins. 
a) Staining for Armadillo is weaker in $cdk^4$ intestines compared to control, however Dlg and spectrin stainings are stronger in the mutant compared to control (b and c respectively). 
Age of flies: 7-8 days. Scale bars: 20 µm.
Results

2.3.9 Re-expression of Cdk4 in enterocytes rescues lipid phenotype in cdk4<sup>3</sup> mutants

To uncover the tissue-specificity of the lipid phenotype in cdk4<sup>3</sup> mutants we performed rescue experiments using Lsp3.1-gal4 (fat body specific driver) however it did not affect the low lipid levels, suggesting that the adult fat body is not the origin of the phenotype (data not shown). Based on the understanding that the mutants have a reduction in overall nutrient storage and dramatically decreased numbers of absorptive cells in the gut, we performed rescue experiments specifically in enterocytes. NP1-gal4 is an enhancer trap insertion into the gut-specific brush border myosin 1A gene and is also known as Myo1AGal4 (Morgan et al., 1994). Expression of GFP driven by NP1-gal4 shows strong GFP expression specifically in immature and mature enterocytes in the anterior and posterior midgut, with no expression in ISCs, EBs and EEs (Jiang et al., 2009). myosin 1A (Myo1A, CG7438) belongs to class I myosins together with myosin 1B (Myo1B, CG9155), however these two unconventional myosins are very divergent in their sequences and also display distinct patterns of temporal expression (Morgan et al., 1994). Both of these myosins are expressed in the apical brush border (BB) domain of the midgut epithelium in late-stage embryos, larvae and adults, with the better characterised Myo1B being present specifically in the microvillus of the apical BB (Hegan et al., 2007; Morgan et al., 1995). It is hypothesized that since Myo1A and Myo1B proteins can be found and co-localised to the BB of every part of the larval and adult gut, their function is probably not exclusively secreting or absorptive but might in addition be involved in regulating membrane dynamics, transport vesicles or structural elements in the gut BB (Morgan et al., 1995).

NP1-gal4 was used to over-express Cdk4 in a cdk4<sup>3</sup> mutant background, which completely rescued the mutant lipid phenotype (Fig 33a). Furthermore, ectopic expression of the kinase-inactive transgene UAS-Cdk4<sup>D175N</sup> also gives a full lipid rescue (Fig. 33b), comparable to the results of rescue experiments where this transgene was over-expressed using da-gal4 (Fig. 17a).
Results

Figure 33. Ectopic expression of Cdk4 using NP1-gal4 rescues the mutant lipid phenotype (a) and the decrease in enterocyte cellular number (c).

b) Expression of kinase inactive (D175N) Cdk4 also rescues the lipid phenotype. Lipid quantification in a) and b) was done in the same experiment and the numbers are therefore directly comparable across graphs.

c) Re-expression of Cdk4 in mutant ECs restores the increase in cellular area observed in the mutant ECs (5 pictures/genotype were used for quantification).

d) EC specific Cdk4 over-expression does not rescue the reduction in enteroendocrine cell numbers, shown as number of prospero positive cells per whole gut (cells were counted in 4 guts/genotype).

e) Abdomens of NP1-gal4 rescued mutant males appear larger compared to control mutant males. 7-8 days old male flies were used in all experiments. Lipid levels are shown as mean and SD.
Results

2.3.10 *NP1-gal* driven Cdk4 over-expression rescues multiple enterocyte phenotypes

Loss of Cdk4 leads to fewer enterocytes, larger EC cellular area and smaller nuclear area, suggesting growth and endoreplication defects specifically in this cell type. Ectopic expression of Cdk4 with *NP1-gal*4 reverses these phenotypes and results in increased enterocytes numbers (Fig. 33c), smaller cellular area (Fig. 34) and larger nuclear area (Fig. 35), arguing that the reduction in lipid and glycogen stores in mutant flies is intimately linked with cellular changes in the enterocytes. However, these experiments also show that enteroendocrine cell number is not completely rescued (Fig. 33d).

The observation that enterocyte numbers have been restored in the rescued flies is quite remarkable when it is taken into consideration that *NP1-gal*4 is an enterocyte specific Gal4 driver and is not expressed in any other intestinal cell type. As intestinal stem cells are the only cell type that can produce increased numbers of enterocytes, this suggests that mutant ECs re-expressing Cdk4 support increased proliferation of ISCs and encourage differentiation along an EC and not EE lineage. This idea was tested in the experiment described in the following section.
Results

Figure 34. Re-expression of Cdk4 in mutant enterocytes reverses the increase in EC size. Ectopic expression of Cdk4 leads to an increase in EC cell area in a heterozygous background compared to controls not expressing Cdk4, however this is not significantly different when compared to homozygotes expressing Cdk4. Age of flies: 7-8 days adult males. Scale bar: 20 µm. Shown is mean and SEM. Number of cells quantified: +/- n= 80, +/- n= 65, +/-;↑Cdk4 n= 61, +/-;↑Cdk4 n=113
Results

Figure 35. Re-expression of Cdk4 in enterocytes of mutant flies rescues the decrease in nuclear size observed in mutant flies. Over-expression of kinase dead Cdk4 (D175N) in ECs in a cdk4<sup>3</sup> heterozygous background can repress nuclear growth, however the expression of the same transgene rescues cdk4<sup>3</sup> mutant nuclear size to same size as in control. Immunofluorescence pictures show DAPI and lamin staining. Age of flies used: 8-9 days. Nuclear area is shown as mean + SEM. Scale bar: 20 μm. Numbers of cells used for quantification, in the order: upper row, left to right and lower row, left to right: n=175, n=210, n= 205, n= 227, n= 230, n= 246.
2.3.11 Enterocyte specific Cdk4 over-expression rescues ISC proliferation defects in mutant intestines.

In a normal and healthy gut epithelium fully functional enterocytes have the capability to sense damage and send as-yet-uncharacterised signals to the intestinal stem cells that more enterocytes are needed. This can be demonstrated by an immense increase in stem cell proliferation caused by substantial damage to the enterocytes caused by feeding with a DNA damaging agent (bleomycin), enteric infection by pathogens or forced cell death in ECs (Amcheslavsky et al., 2009; Jiang et al., 2009), (Chatterjee and Ip, 2009). Hence, it is quite surprising that \( cdk4^3 \) mutant enterocytes do not seem to be capable of initiating stem cell proliferation, as a response to their reduced number. We have been able to demonstrate that mutant intestines have significantly lower rates of stem cell proliferation as shown by BrdU incorporation assay. In order to investigate if loss of ISC proliferation could be specifically due to absence of Cdk4 in ECs, we performed another BrdU feeding experiment with \( cdk4^3 \) mutant flies ectopically expressing Cdk4 using \( NP1-gal4 \). Re-introduction of Cdk4 into mutant enterocytes completely reverses the decrease in stem cell proliferation and shows no differences compared to control intestines (Fig. 36). Additionally, it also abolished the dramatic increase in BrdU incorporation in mutant ECs. Since the intestinal stem cells and enteroblasts still lack Cdk4, these data demonstrate that rescuing Cdk4 in enterocytes supports proliferation of stem cells that are mutant for \( cdk4^3 \). This would speak in favour of the existence of an epithelial niche where functional ECs are important constituents required for maintaining stem cell proliferation and most likely also for regulating stem cell differentiation and renewal.
2.3.12 Intestinal clearance rates are restored in cdk4$^3$ mutant flies expressing Cdk4 in their enterocytes

Coming back to the results showing that cdk4$^3$ mutants have decreased enteroendocrine cell number, reduced gene expression of a neuropeptide controlling gut contraction and reduced gut clearance rates, we were wondering if all these phenotypes are interconnected and/or dependent on each other. Mutant flies re-expressing Cdk4 only in their enterocytes did not have dramatically increased EE cell number (Fig. 33d), however they displayed completely restored gut clearance rates (Fig. 37). These results suggest that the previously observed gut clearance phenotype in mutant flies is most likely not due to reduced numbers of EEs, supporting the hypothesis that cdk4$^3$ flies suffer from slower food passage due to impaired EC absorbance.

2.3.13 Esg-gal4 specific Cdk4 over-expression only partially rescues the mutant lipid phenotype

In an attempt to uncover whether Cdk4 is required exclusively in enterocytes or if it is simultaneously necessary in enterocytes and stem cells, rescue experiments were performed where Cdk4 was ectopically expressed in cdk4$^3$ mutants using esg-gal4.
Lipid quantification of experimental flies showed that escargot driven Cdk4 expression only partially rescues the lipid phenotype in mutant flies (Fig. 38). These results point towards Cdk4 primarily being needed in enterocytes and that the requirement in stem cells is secondary. Considering that esg-gal4 is expressed strongly in stem cells and enteroblasts and only extremely weakly in enterocytes, we were surprised that even a partial lipid rescue was obtained. In theory, it is possible that Cdk4 is over-expressed to such an extent in the stem cells and enteroblasts that the protein is very abundant and in addition long-lived and persists throughout enterocyte maturation, thus partially supporting EC growth. Experiments are currently ongoing to characterise the effects of escargot driven Cdk4 expression, on epithelial homeostasis to determine to what extent the mutant phenotypes are rescued using this driver.

Figure 37. Ectopic expression of Cdk4 in mutant enterocytes restores the impaired intestinal clearance in mutant flies. 20-21 day old flies were fed over-night with food containing Allura Red. After gut clearance on normal food for the indicated time period, remaining Allura Red was quantified spectrophotometrically in homogenates of whole flies. Shown is mean and SD.

Figure 38. Ectopic expression of Cdk4 with esg-gal4 partially restores lipid levels in cdk4^{3} mutant animals. esg-gal4 is strongly expressed in ISC and EBs and weakly in immature enterocytes. Shown in mean and SD from biological triplicates. Age of flies: 7-8 days old
Results

2.4 Effects of ectopic expression of Cyclin D/Cdk4 on gut homeostasis

2.4.1 Enterocyte specific over-expression of Cyclin D/Cdk4 causes obesity in adult flies

As a complement to the loss of function studies with respect to adult intestinal homeostasis, we wanted to investigate the consequence of Cyclin D/Cdk4 gain-of-function in the same setting. Ectopic expression of Drosophila CycD/Cdk4 has been shown to induce overgrowth phenotypes in the postmitotic cells of the adult eye and the endoreplicating cells in larval salivary gland, although not in an identical manner as the classical growth promoters Myc and InR (Datar et al., 2000) (Brogiolo et al., 2001), (Pierce et al., 2004). The gain of function experiments were performed by NP1-gal4 driven co-overexpression of both Cyclin D and Cdk4, since Cdk4 is a poor growth driver on its own. Cyclin D/Cdk4 over-expression in enterocytes resulted in considerably increased enterocyte nuclear and cellular area (Fig. 37a and Fig. 37b respectively). Additionally, the flies appeared rather obese with dramatically increased total body lipids and displaying high starvation resistance (Fig. 37c and 37f). The metabolic aberrance also included at least partially affected stored/circulating carbohydrate storage, with the experimental flies suffering from severe hypertrehalosemia (Fig. 37e). I hypothesise that the immense expansion in cellular area and absorptive surface of enterocytes correlates directly with increased nutrient uptake and that this is sufficient to cause a severe obesity phenotype in adult flies.
**Results**

**Figure 39.** Enterocyte-specific ectopic expression of Cyclin D/Cdk4 drives growth in enterocytes and causes obesity in flies.

a) Enterocytes in experimental flies show a dramatic increase in cellular and nuclear size (shown is posterior midgut). Cellular size is significantly larger compared to control ECs (b) (n= 24 for control and n= 28 for experimental. Shown as mean + SEM). Scale bar: 20 µm.

c) and d) CycD/Cdk4 EC-specific over-expression leads to increased stored lipids in adult flies, which is even more dramatic when total lipids are normalized per fly. e) The obese flies have notably, but not significantly higher levels of circulating/stored trehalose and are additionally starvation resistant compared to control flies (f). All graphs are shown as mean and SD. f) significance between the curves was analyzed with a Log-rank survival test. Number of flies used 32-32 per genotype.
Results

2.4.2 Induction of EC over-growth, but not lipid accumulation, caused by Cyclin D/Cdk4 is kinase dependent

Since we could show that the lipid phenotype in cdk4\(^3\) mutant adults is kinase independent, it was highly relevant to investigate if the metabolic and cellular phenotypes caused by CycD/Cdk4 over-expression also depend on Cdk4 kinase function. Using the NP1-gal4 driver CycD/Cdk4 was co-over-expressed with Cdk4 or with the Cdk4 kinase-dead transgene (UAS-Cdk4\(^{D175N}\)) and the metabolic and cellular status of the flies and their intestines was evaluated. Lipid quantification showed that over-expression of the kinase-dead Cdk4 leads to significantly elevated lipids compared to control, but only partially when compared to expression of kinase active Cdk4 (Fig. 40a). A similar partial increase was observed with regard to trehalose levels (Fig. 40b), whereas glycogen levels were as high in kinase-inactive compared to kinase-active flies (Fig. 40c). These results demonstrated that ectopic expression of CycD/Cdk4 not only leads to increased lipid and trehalose levels, but additionally to an increase in stored glycogen levels. Quantification of cellular area showed that induction of enterocyte growth is completely abolished in kinase-dead intestines (Fig. 40d) and in accordance with this, the increase in enterocyte nuclear area is also suppressed (Fig. 41). These studies suggest that there are two functions of Cdk4 that may contribute to nutrient uptake, one being kinase-dependent effect of EC growth and the second an unidentified kinase-independent and growth independent function.

![Figure 41](image_url). Ectopic co-expression of Cyclin D with a kinase inactive Cdk4 does not increase enterocyte nuclear size compared to kinase active Cdk4, as shown by lamin and DAPI staining. Scale bar: 30 \(\mu\)m. Age of flies: 8-9 days old.
Results

Figure 40. a) Co-overexpression of kinase inactive Cdk4 with Cyclin D can partially cause similar lipid phenotype as co-over-expression with kinase active Cdk4. Similar results were obtained when trehalose levels were measured (b).

b) Kinase dead Cdk4 can cause same increase in lipids as kinase active.

d) Cell area shown as Mean +SEM, Control n=58, Cyclin D/Cdk4 n=32, Kinase dead n=52

e) Staining of guts of indicated genotypes with Armadillo and DAPI. Age of flies: 8-9 days. Scale bar: 20 µm
2.4.3 Ectopic expression of Cyclin D/Cdk4 in intestinal stem cells mildly promotes cell division

It has been previously reported that over-expression of CycD/Cdk4 in wing imaginal disc clones increases the cellular growth and rate of cell division (Datar et al., 2000). Similar results were obtained when we over-expressed CycD/Cdk4 using esg-gal4 in the adult intestine, meaning that an increase in GFP positive cells could be observed presumably reflecting more stem cell division (Fig. 42, far right). This was not detected in intestines ectopically expressing Cdk4 in the absence of ectopic expression of Cyclin D (Fig. 42, middle). Despite the fact that the increase in cell division is notable in intestines over-expressing CycD/Cdk4, it is rather insignificant when compared to previously characterized regulators of stem cell proliferation, such as Notch and JAK/STAT signalling (Beebe et al., 2010; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Taken together, our data show that ectopic expression of CycD/Cdk4 has dramatic effects on post-mitotic cells and only slight effects on the division rate of mitotic cells, suggesting predominant role in growth control of enterocytes and a more minor, but significant, control of proliferation of intestinal stem cells.

Figure 42. Ectopic co-expression of Cyclin D/Cdk4 marginally increases proliferation when over-expressed in intestinal stem cells. Over-expression of Cdk4 alone had no effect. esg-gal4 expresses GFP specifically in ISCs and EBs. Scale bar: 30 µm. Age of flies: 8-9 days old.
2.4.4 Effects of ectopic expression of Myc and Tsc1/Tsc2 on EC growth and adult metabolism

Since the results presented so far highlighted an interesting link between regulation of intestinal homeostasis and nutrient/energy storage at the organismal level, we sought to modulate EC growth using other known activators and repressors of cellular growth. Drosophila Myc is a transcription factor that regulates several cellular processes, ranging from control of ribosomal biogenesis, translation and metabolism to control of cell competition, proliferation and growth (Furrer et al., 2010; Grewal et al., 2005; Johnston et al., 1999; Li et al., 2010; Pierce et al., 2008), (Moreno and Basler, 2004). Additionally it controls endoreplication of polyploid tissues such as the larval fat body and salivary gland, demonstrated though dramatic increase in nuclear volume when over-expressed (Pierce et al., 2004).

The Tumour Sclerosis Complex 1 and 2 (Tsc1/2) is known to be a negative regulator of the Target of Rapamycin (mTOR) signalling pathway and is downstream of Akt, but upstream of Rheb, S6K and 4E-BP (Napolioni and Curatolo, 2008). Loss of Tsc1 and Tsc2 leads to growth induction but normal ploidy and inappropriate entry into S-phase. Conversely, ectopic expression results in repressed growth of the entire adult eye and wing, due to the cells becoming smaller. Tsc1/2 over-expression in wing imaginal disc clones caused an increase in cell cycle length compared to wild-type cells, concluding that cell proliferation is restricted. Moreover, it was shown in the same study that co-overexpression of Tsc1/2 together with Cyclin D/Cdk4 in the Drosophila eye abolishes the induction of growth by CycD/Cdk4 through a currently unknown mechanism (Hariharan and Bilder, 2006; Tapon et al., 2001).

The questions we wanted to address were: a) does over-expression of Myc in enterocytes cause similar cellular and metabolic phenotypes as Cyclin D/Cdk4 over-expression? b) are Cyclin D/Cdk4 growth promoting signals in the enterocytes repressed by Tsc1/2 over-expression as in the eye? And c) can ectopic Tsc1 and Tsc2 expression in ECs repress their growth and cause similar metabolic phenotypes as loss of cdk4?
Results

2.4.5 NP1-gal4 driven Myc over-expression dramatically increases EC nuclear size and induces proliferation of ISCs

Ectopic expression of Myc in enterocytes substantially increased their nuclear size while there was no corresponding increase in cytoplasmic size, however the overall size of the ECs appeared greatly enlarged, even when compared to Cyclin D/Cdk4 over-expression (Fig. 43a). Unfortunately, reliable quantifications of nuclear and cellular size were made unfeasible by the severe distortion of the lamin and the armadillo staining, respectively. Yet another important observation was the remarkable increase in numbers of smaller cells whose identity is unknown due to lack of cell type specific staining. Despite the undetermined identity of these cells, as they could either be stem cells, enterblasts or enteroendocrine cells, it can be presumed that such an increase in cell number was preceded by increased ISC proliferation. What further supports this idea is the observation that EC specific Myc gain-of-function causes tumour-like congregations of cells, which has previously been described in other settings as being indicative of more stem cell division (Lin et al., 2008; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Surprisingly, in the case of ectopic Myc expression the clustering cells had nuclei far larger than those of normal diploid cells, suggesting they might in fact be immature enterocytes (Fig. 43a).

In parallel with the microscopical examination, lipid and carbohydrate storage were quantified in flies over-expressing Myc. Lipid levels in experimental animals were slightly but significantly higher compared to control (6.5 percent), however the increase in lipids was not as large as in flies over-expressing CycD/Cdk4 (13.6 percent) (Fig. 43b). Unexpectedly, the carbohydrate storage, measured as stored glycogen and circulating/stored trehalose, was significantly reduced in the Myc over-expressing animals, indicating that the extreme epithelial disorganisation has no severe consequence on dietary lipid uptake and storage, however it potentially disturbs carbohydrate breakdown, uptake or storage (Fig 43c and Fig 43d).

I therefore concluded that induction of Myc expression in the Drosophila enterocytes has a more dramatic affect on the growth and endoreplication of enterocytes than CycD/Cdk4 over-expression and also most likely stem cell proliferation. However, it
Results

induces only a partially overlapping set of phenotypes as Cyclin D/Cdk4 over-expression.

2.4.6 Ectopic expression of Tsc1/2 in ECs does not reverse the cell size increase caused by Cyclin D/Cdk4 overexpression

A transgenic construct expressing Tsc1 and Tsc2 was ectopically expressed in enterocytes and the consequences were examined with regard to cell and nuclear size in the adult midgut, as well as assessment of lipid and carbohydrate storage in the whole animal. The assessment of Tsc1/2 effect on cellular size was not conclusive because the strength of the Armadillo staining was insufficient throughout all intestine examined, for the cell size to be reliably quantified. It appears that Tsc1/2 over-
expression weakens Armadillo expression (Fig. 44a). Nonetheless, analysis of DAPI staining (Fig. 44a) allows the conclusion that Tsc1/2 over-expression does not have dramatic effect on enterocyte number or size. A more precise conclusion about the effects of Tsc1/2 over-expression on EC cellular and nuclear size, as well as on epithelial homeostasis awaits the outcome of ongoing experiments. Interestingly, lipid and glycogen stores were significantly increased in experimental animals compared to control (9.6 and 22.9 percent respectively, Fig. 44b and 44d). On the other hand, the levels of circulating and stored trehalose were unchange (Fig. 44c).

**Figure 44.** Tsc1 and Tsc2 over-expression does not cause noticeable decrease in enterocyte cellular size (a), however it causes changes in nutrient storage levels (b and d). 

b) Ectopic expression of Tsc1/2 in enterocytes leads to increased lipid storage. 
c) Levels of circulating/stored trehalose are unchanged in experimental Tsc1/2 flies. 
d) Levels of stored glycogen are significantly increased.

Age of flies: 8-9 days. Scale bar: 20µm.
Results

It was previously shown that co-overexpression of Cyclin D/Cdk4 in combination with Tsc1/2 overexpression represses the growth induction caused by CycD/Cdk4, however the mechanism behind this was never understood. When both of these complexes were over-expressed specifically in the enterocytes, a significant increase in whole body lipid levels could be observed, both compared to control and to flies

![Image of DAPI and Armadillo staining of indicated genotypes](image)

**Figure 45.** Simultaneous over-expression of Cyclin D/Cdk4 and Tsc1/2 has an additive effect on lipid levels.

- **a)** DAPI and Armadillo staining of the indicated genotypes
- **b)** Quantification of total lipids normalized against body weight.

Experiment done with 4 biological replicates (9 days old adult males).

- Control compared to Cdk4 over-expression p=0.0054.
- Control compared to Cyclin D/Cdk4 over-expression p=0.001
- Control compared to Tsc1/2;Cyclin D/Cdk4 over-expression p<0.0001
Results

over-expressing only CycD/Cdk4 (Fig. 45b). This was not accompanied by an additional increase in EC cellular size (Fig. 45a), meaning that the additive increase in lipids could possibly be caused by a mechanism that favours lipid accumulation independent of increased EC cell size. Importantly these results clearly show that over-expression of Tsc1 and Tsc2 in enterocytes does not suppress the cellular overgrowth phenotype caused by CycD/Cdk4, as was shown to be the case in the adult eye.

2.5 Characterisation of the effects of ubiquitous and tissue specific Cyclin D and Cdk4 knock-down

In order to be able to phenocopy the various mutant phenotypes tissue-specifically, Cyclin D and Cdk4 RNAi lines were characterised, initially using ubiquitous Gal4 drivers. The UAS/Gal4 system was employed to drive the expression of a hairpin RNA, giving rise to double stranded RNAs later processed by Dicer into siRNAs (Dietzl et al., 2007). The direct sequence-specific degradation of CycD or Cdk4 mRNA was confirmed by qRT-PCR.

2.5.1 Ubiquitous knock-down of Cdk4 or Cyclin D phenocopies size and weight reduction, but not lipid reduction.

RNAi knock-down of Cdk4 using da-gal4 could phenocopy the weight reduction (Fig. 46a) and to a certain extent size reduction in mature adult male flies (Fig. 46c), however it could not reproduce the lipid phenotype observed in null mutant flies (Fig. 46b). Analysis of the knockdown efficiency on gene expression level revealed that more than 60 percent of the Cdk4 transcript was still present (Fig. 46d), indicating potentially insufficient knockdown to copy the lipid phenotype. Moreover, off-target gene expression was examined as well, showing that three out of four predicted off-targets exhibited changed mRNA levels (Fig. 46d). One more attempt was made to phenocopy the lipid phenotype, this time using another ubiquitous driver Actin5C-gal4 (Act5C-gal4). Unfortunately the trial was unsuccessful as the adult flies displayed a mere 5 percent reduction in weight (compared to 15 percent with da-gal4) and even showed an increase in lipid levels (data not shown). This could possibly be attributed to the fact that Act5C-g4 is supposedly a stronger inducer of expression compared to
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da-gal4 and that the lack of reduction in weight and increase in lipid levels is due to strong off-target effects.

Since the Cdk4-RNAi line had several off-targets and insufficient efficiency, we attempted using a CycD-RNAi line with no known off-targets. Da-gal4 driven knockdown produced significantly lighter flies (13 percent, Fig. 47a), but without any reduction in lipid levels (Fig. 47b). Older flies showed a slight, but not statistically significant, reduction in lipids (Fig. 47c). Quantitative RT-PCR performed on whole adult flies from the same parental cross showed that approximately 50 percent of CycD transcript remained (Fig. 47e). Similarly to the RNAi experiments with the Cdk4-

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**Figure 46.** a) and c) RNAi knock-down of Cdk4 can phenocopy the weight and size reduction similar to cdk4^3 mutant adults, but not the mutant lipid phenotype, as shown in (b). Quantitative RT-PCR shows that approximately 60 percent of the Cdk4 transcript is still present. Although the decrease is statistically significant it might not be biologically sufficient to mimic a null mutant phenotype. Png, PhK and CG5182 are predicted off-targets of the Cdk4 dsRNA line.
Results

RNAi line, Act5C-gal4 was again used to achieve a stronger knock-down effect. This resulted in a 17 percent decrease in lipid levels in the experimental flies (Fig. 47d), however this decrease was not statistically significant despite being noteworthy. Additionally, tissue specific RNAi experiments were performed using the EC specific Gal4 driver NP1 and the ISC specific esg-gal4, unfortunately without yielding any affect on total lipid levels in adult males (Fig. 47f and 47g).

The lack of highly efficient knock-down of Cdk4 or Cyclin D makes conclusions from these studies difficult. Knock-down experiments are currently being repeated in a cdk4<sup>3</sup> heterozygous mutant background to attempt to sensitise to RNAi and hopefully phenocopy mutant cellular and metabolic phenotypes. Interestingly in RNAi experiments where Cdk4 is specifically knocked-down in wild-type flies using esg-gal4, a similar loss of intestinal stem cells and enteroblasts in mature and aging adults was observed, at least partially reproducing the mutant gut phenotype (data not shown). We are currently testing whether it is necessary to drive knock-down simultaneously in enterocytes and intestinal stem cells (with NP1-gal4 and esg-gal4) to fully reproduce the mutant phenotypes. This would be consistent with our loss- and gain-of-function studies that indicate that there is a dual requirement of CycD/Cdk4 for both ISC proliferation and EC growth.
Results

Figure 47. Upper panels are showing ubiquitous Cyclin D knock-down using da-gal4. Shown in a) weight of experimental flies 7-8 days old flies is significantly less compared to control, however there is no effect on lipid levels at this age (b).

c) Lipid levels are slightly, but not significantly (6.6 percent) reduced in adult flies at 19 days of age. Flies in a) were used for lipid quantification in b) and originate from same parental cross as flies in c) and e).

d) RNAi knock-down using Act5C-gal4 caused a larger (17 percent), however not significant, decrease in lipids. mRNA expression level of Cyclin D in experimental flies was not quantified.

e) Quantitative RT-PCR showed approximately 50% decrease in Cyclin D transcript using da-gal4.

f) Enterocyte specific knock-down of Cyclin D has no affect on whole body lipid levels.

g) Intestinal stem cell specific Cyclin D knock-down has no affect on whole body lipid levels.
3. DISCUSSION

3.1 Metabolic phenotypes caused by Cyclin D and Cdk4 loss-of-function

The initial general aim of this doctoral thesis was to investigate if the *Drosophila* Cyclin D/Cdk4 complex has any role in regulating larval or adult energy homeostasis. After analysing the contributions of this complex in larval metabolism, it can be concluded that Cyclin D/Cdk4 most likely does not play any significant role in this context. However, in the case of adult energy homeostasis, Cyclin D/Cdk4 does seem to play a distinct role, demonstrated through adversely affected nutrient storage in *cdk4* homoygous mutant adult male flies. These flies displayed a significant reduction in stored levels of dietary lipids and glycogen and were starvation sensitive. Lipid quantification in *cycd* mutants showed the same reduction in stored lipids, implying that this phenotype is Cyclin D dependent. Through rescue experiments with a ubiquitous Gal4 driver, it was shown that the lipid and glycogen phenotypes in *cdk4* mutant flies are specifically due to absence of Cdk4 and that this particular function of Cdk4 does not require its kinase activity. The previously described functions of *Drosophila* Cdk4 and Cyclin D are induction and control of cellular growth in post-mitotic cells and tissues. Additionally, it was demonstrated that the Cdk4 kinase domain is essential in this setting, since the ectopic ubiquitous expression of a kinase inactive Cdk4 suppressed growth in heterozygous mutant flies resulting in lighter flies with smaller tissues. Simultaneously, the same transgene caused a partial rescue of adult weight and tissue size when expressed in homozygous mutants, explained by possible titration of putative INK family members (Meyer et al., 2000). In our experiments, we observed the same partial rescue of weight in homozygous mutants, however the lipid levels were completely restored, arguing for a kinase-independent, but Cyclin D-dependent function of Cdk4 with respect to this particular phenotype.

In addition to the lipid phenotype, *cdk4* mutant flies displayed a reduction in gene-expression of several lipases raising the possibility that repressed lipase expression could contribute to the observed metabolic phenotype. Lipases are enzymes that function to break down dietary lipids before they can be absorbed, thus reduced lipase
expression could be linked to less storage of dietary fat. Therefore, I designed a feeding experiment where the requirement for lipases was partially circumvented. Mature adult flies were fed with food supplemented with a free fatty acid (oleic acid) that should be easily taken up and stored as TAG. Since the mutant flies did not accumulate more lipids as a result of the feeding, it appeared that they might in fact be impaired in intestinal absorption and not dietary lipid breakdown. This idea was addressed indirectly through an intestinal gut clearance assay, which has previously been shown to correlate with absorptive capacity of the gut (Bland et al., 2010). Mutant flies had higher initial levels of the non-absorbed feeding dye Allura Red before the start of gut clearance, which in reality could be a sign of excessive or aberrant feeding, as dye feeding is also used for this purpose (Phillips and Thomas, 2006; Wong et al., 2009). After 60 minutes, control flies had cleared 37.5 percent of their starting dye content compared to the mutants, which had only cleared 7.7 percent during the same time-period, indicating significantly slower clearance rates. These results insinuated that cdk4 animals have issues with food passage, encouraging a closer analysis of the mutant intestine. Regarding the data showing higher initial accumulation of dye in mutants, we speculate that this is directly correlated with slower passage of nutrients, as dissections and microscopical examinations of AR fed flies demonstrated that mutant animals have excessively enlarged crops containing AR dyed food. There is still the possibility that cdk4 mutants feed more, however this is quite unlikely, as the meal size kept in the crop is regulated by the absorptive rates of nutrients in the anterior midgut in other insects (Nation, 2002).

### 3.2 Flies mutant for Cdk4 are defective in their intestinal cellular composition

Examination of control and cdk4 mutant intestines of mature male flies revealed that mutant intestines have dramatically decreased numbers of the absorptive enterocytes and the secreting enteroendocrine cells. Moreover, the reduction in enterocytes and enteroendocrine cells was accompanied by a substantial decrease in intestinal stem cells and enteroblasts, demonstrated by a decrease in numbers of cells expressing GFP when driven by the ISC and EB specific Gal4 driver esgGFP. This decrease was equally noticeable in newly eclosed flies, indicating that mutant flies lose ISCs and EBs already before they are born. This implies that the decrease in enteroblast and eventually enterocyte and enteroendocrine cell number is most likely due to insufficient
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proliferation and/or growth of adult midgut progenitor cells during the larval and pupal stages. The exceptional regenerative abilities of intestinal stem cells have been demonstrated in several studies, shown through induction of ISC proliferation in damaged intestines where substantial loss of enterocytes is observed through bacterial infection, DNA damage or ectopic induction of cell death (Amcheslavsky et al., 2009; Buchon et al., 2009; Jiang et al., 2009). Stem cell proliferation can also be induced through damage to the basement membrane of the gut epithelium, most likely through stem cells sensing a disruption in epithelial organisation (Amcheslavsky et al., 2009). Therefore it is quite remarkable that the existing stem cells in mutant intestines do not seem to be able to compensate for the absence of EEc and EEs.

BrdU feeding experiments revealed that mutant stem cells proliferate less frequently than in control guts, while the BrdU incorporation is dramatically increased in mutant enterocytes (Fig. 30). A normal healthy intestine has a certain level of stem cell proliferation; this has been shown in intestines of female flies, which replace all their enterocytes on a weekly basis, demonstrated through high BrdU incorporation into both stem cells and enterocytes (Micchelli and Perrimon, 2006). In our experiments, the BrdU incorporation into enterocytes was very low in male control flies, most likely reflecting very little damage in intestines of very young flies and thus reduced need to produce new enterocytes.

The observation that stem cells in mutant intestines proliferate to a lesser degree compared to control was somewhat surprising and could have several plausible causes. One possibility is that mutant ISCs are simply not receiving the right cues from their surrounding to induce proliferation. On the other hand, the low rate of stem cell proliferation seen in controls is partially absent in cdk4\(^3\) intestines, arguing that the ISCs cannot even maintain basal levels of turnover. Furthermore, it needs to be mentioned that reduced proliferative rates have been observed in other cdk4\(^3\) mutant epithelia such as the wing, which also resulted in fewer cell numbers albeit not as dramatically as seen for the numbers of EC and EE cells. The slower proliferation was attributed to the mutant cells progressing slower through the entire cell cycle, which here cannot be excluded as being a possible mechanism as well (Meyer et al., 2000).
At present, it is not exactly known what controls ISC proliferation and differentiation, nevertheless two key pathways involved are the Notch and Wg/Wnt signalling pathways (Lin et al., 2008; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Loss of Wg components leads to reduced ISC proliferation and loss of stem cells over time, however without affecting the overall numbers of EC and EE cells (Lin et al., 2008). Part of the mechanism behind differentiation defects in stem cells is aberrant Notch/Delta signalling in enteroblasts, which interferes with the differentiation of the EBs and accumulation of polyploid EC-like cells that do not express EC markers (Biteau et al., 2008).

In connection to these studies, we wanted to investigate if the reduction in stem cell numbers might partially occur through terminal or disrupted differentiation of ISCs. The Notch signalling ligand Delta is expressed specifically in ISCs and is equally distributed between the ISC and the daughter cell after division, followed by rapid down-regulation and removal of Delta from the EB. A subsequent event after Delta removal is activation of Notch signalling in the EB, which depending on the levels of Notch activation commits either towards an EC or EE fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Control and mutant intestines expressing esgGFP in ISCs and EBs were co-stained with Delta, in order to recapitulate previous published experiments showing mis-differentiation events. In mutant animals we found several different combinations of esgGFP and Delta expression within the same intestine, as shown in figure 29. The cell denoted by arrow d has a strong GFP signal usually observed in EBs and a nucleus too large to belong to a stem cell, however it is expressing cytoplasmic Delta. The presence of Delta in cells other than stem cells is believed to be a sign of disrupted differentiation. At present, given the absence of specific intestinal cell markers characterisation of cell identity must be based on the nuclear size and/or presence of a reporter for Notch activity. Enteroblasts with Delta still present presumably have disturbed activation of Notch and no longer have a clear path of commitment, thus possibly remaining as semi-differentiated enteroblasts. It is tempting to speculate that this occurs in cdk43 intestines, as it would partially explain loss of stem cells and simultaneous lack of increased numbers of mature enterocytes. One important future avenue of research will be to monitor the status of Notch activation in ISCs and EBs combined with Delta staining, to gain further insight into the nature of the differentiation defect. Additionally, it will be important to perform MARCM
based clonal analysis to definitely analyse potential differentiation defects. These experiments involve labelling and tracing of one particular stem cell and its daughter cell as it commits to a particular cell fate (Micchelli and Perrimon, 2006). Several attempts were made to mark stem cells in control and mutant intestines, however no GFP marked clones were ever obtained in the mutant guts, yet they were obtained in the control guts. The crucial step in these experiments is the timing and length of heat-shock, which induces mitotic recombination and thus marks the clones with GFP. It is possible that since the \( cdk4^3 \) mutant flies exhibit dramatically reduced rates of stem cell division/proliferation that the appropriate time window for heat-shock in the mutant differs from that in control animals. A systematic series of studies will be required to attempt to optimise the experimental setup.

Aside from the already mentioned decrease in ISC, EB, EC and EE numbers in \( cdk4^3 \) mutant intestines, additional changes and defects were discovered in these tissues. The mutant enterocytes displayed a larger cellular area without a corresponding increase in nuclear area; it was in fact significantly decreased and these nuclei most often stained weaker for DAPI. Taken together, the reduced nuclear size and weaker DAPI hinted towards endoreplication defects in mutant enterocytes, which is quite contradictory as the cells reasonably should be smaller if they have endoreplicated insufficiently. I propose that the enterocytes appear larger because they are compensating for the lack of sufficient cell numbers by stretching to maintain epithelial integrity.

In addition to the microscopical examination of mutant intestines, I attempted to investigate indirect functional consequences of absence of Cdk4, through examining gene expression levels of a set of lipases specifically in the midgut. The results showed that several lipase genes are in fact significantly down regulated in mutant compared to control midguts, most likely correlating with reduced abundance or functionality of the mutant enterocytes.
3.3 Enterocyte-specific expression of Cdk4 rescues mutant gut and metabolic phenotypes

Since considerable changes in the cellular composition of mutant guts were observed, especially in the number and appearance of the main intestinal cell type, it was of interest to determine whether these changes may be the cause of the metabolic phenotypes of mutant flies. Since all dietary nutrients are absorbed through the intestinal enterocytes, this would be a rather probable primary site of defect leading to nutrient storage defects. Rescue experiments were performed where Cdk4 was over-expressed using an enterocyte-specific Gal4 driver (NP1-gal4) in cdk4^{3} homozygous mutant animals. This resulted in a complete rescue of both the lipid and glycogen phenotype, demonstrating that the absence of Cdk4 specifically in the enterocytes indeed is the main cause behind reduced nutrient storage (Fig. 33). Importantly, NP1-gal4 driven Cdk4 re-expression fully restored the reduction in EC numbers, as well as their cellular and nuclear size (Fig 33). These results clearly show that presence of Cdk4 in enterocytes is absolutely required for their growth and most likely also endoreplication, as these events are tightly coupled. The assumption that cdk4^{3} mutants have endoreplication defects in their ECs is plausible and is supported by a study showing that cdk4^{3} mutant embryos display reduced rates of BrdU incorporation in the embryonic middle midgut (Meyer et al., 2002). Conversely, cdk4^{3} mutant larvae do not have any metabolic defects, questioning a potential need for Cyclin D/Cdk4 in regulation of endoreplication and growth of larval enterocytes. Since the larval gut was not examined, we cannot speculate if the complex is in fact required during this developmental stage or not. The attempts to directly address endoreplication issues yielded quite unexpected results since an increase in BrdU incorporation was observed in mutant enterocytes, directly opposing our hypothesis (Fig. 36). Endoreplication events leading to BrdU incorporation in enterocytes are usually caused by enterocyte renewal, meaning that a new enterocyte has been created to replace one that has been removed (Micchelli and Perrimon, 2006). If increased enterocyte renewal truly is the cause of more BrdU incorporation in mutant intestines, increased stem cell division should have preceded this event. As demonstrated in the same BrdU assay, this was not the case as mutant stem cells proliferate significantly less frequently than in control flies. Another explanation for increased BrdU incorporation could be increased DNA synthesis in the mutant enterocytes due to DNA
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damage. Since we concluded that the mutant ECs are impaired in their growth and function, it is possible that they are more susceptible to environmental damage/influence, thereby causing more reactive oxygen species and more DNA damage. Another possibility is that DNA synthesis occurs very slowly in cdk43 mutant enterocytes, therefore it takes the cells longer to even partially complete endoreplication.

To investigate if there is increased stem cell proliferation in NP1-gal4 rescued mutant flies, another BrdU incorporation assay was performed and again BrdU positive small and large nuclei were scored separately. The BrdU quantification clearly demonstrates that rescued mutants have normal proliferation rates comparable to controls and that the dramatic increase in ECs with strong BrdU incorporation has been completely restored. Interestingly, these data demonstrate that re-expression of Cdk4 in mutant ECs supports the proliferation of stem cells still lacking Cdk4. This speaks against the hypothesis that cdk43 mutant intestines have lower stem cell proliferation due to an ISC cell autonomous defect leading to slower rates of cell cycle progression. It remains to be definitely confirmed using the stem cell specific marker Delta, that intestinal stem cell numbers are indeed restored in NP1-gal4 rescued mutant intestines. Nevertheless, microscopical examination of preliminary Delta staining experiments suggests that this is indeed the case, providing further evidence for the hypothesis that functional enterocytes are required for supporting intestinal stem cell proliferation (data not shown).

One cellular phenotype that was not fully rescued is the reduction in EE numbers, which increased only marginally in mutant intestines with EC-specific Cdk4 expression compared to mutant intestines not expressing Cdk4 (Fig. 33). Absence of restored EE cell numbers is quite puzzling as there is an increase in EC numbers, implying that this increase was most likely proceeded by more stem cell proliferation. Furthermore, it also means that not only was there more proliferation, but that the daughter cells were probably encouraged to favour committing along an EC rather than EE lineage.

Additionally, since I postulated the possibility that reduced intestinal clearance rates in mutant animals are due to decreased absorbance in the anterior midgut, it was of interest to determine if this particular phenotype had also been restored with NP1-
gal4? Indeed, mutant animals re-expressing Cdk4 in their enterocytes had practically identical clearance rates of Allura Red compared to control animals, supporting the notion of an existing link between functional absorbance and intestinal clearance rates (Fig. 37).

In summary, these data demonstrated that the Drosophila Cyclin D/Cdk4 complex has an important role in directly regulating enterocyte growth and through this possibly indirectly affecting stem cell proliferation. Moreover, via control of growth, Cyclin D/Cdk4 also regulates the function of enterocytes shown through decreased nutrient storage most likely caused by reduced enterocyte absorption.

### 3.4 Role of Cyclin D/Cdk4 in intestinal stem cells

In order to investigate if the main cause of the phenotypes described above is indeed absence of Cdk4 primarily in enterocytes, or if Cdk4 function is required simultaneously in enterocytes and intestinal stem cells, additional rescue experiments were performed using esg-gal4. esg-gal4 is expressed strongly in intestinal stem cells and enteroblasts and very weakly in immature enterocytes (Micchelli and Perrimon, 2006). Surprisingly, ectopic expression of Cdk4 with esg-gal4 partially rescued the lipid levels in mutant flies, possibly due to Cdk4 still being moderately over-expressed in immature enterocyte and thus partly restoring their growth defects. The complete analysis of these flies is currently ongoing. It will therefore be intriguing to investigate the effect of esg-gal4 driven Cdk4 on ISC, EB, EC and EE cell numbers and whether EC cellular and nuclear size are also rescued. The initial preliminary microscopical examination hinted towards increased EC numbers but not exceptionally larger EC nuclear area, however all these parameters remain to be quantified (data not shown). Similarly, analysis of a preliminary BrdU feeding experiment showed that proliferation of intestinal stem cells is increased in mutant flies re-expressing Cdk4 with esg-gal4 and the vast abundance of BrdU positive enterocytes is abolished (data not shown). These data would argue that Cdk4 is needed for normal proliferation of intestinal stem cells, however it is not clear if this is a cell-autonomous or non-autonomous requirement, the latter potentially being derived from restored and sufficient EC cell numbers.
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3.5 Does a permanent intestinal stem cell niche exist and is it controlled partially by Cyclin D/Cdk4?

The discovery that cdk4^3 mutant intestines display reduced stem cell numbers in immature and mature adults and in addition proliferate less, prompted us to investigate potential causes behind these observations. The control of asymmetrical stem cell division through cell-autonomous decisions and non-autonomously through influence of the surrounding stem cell niche, has been well studied and described in the *Drosophila* ovaries, testis and neuroblasts (Egger et al., 2008; Kiger et al., 2001; Kiger et al., 2000; Tulina and Matunis, 2001; Xie and Spradling, 2000). A stem cell niche is defined by a restricted position in an organ that supports the self-renewing division of stem cells and also prevents them from differentiating (Lin, 2002). Stem cells usually require polarity cues for the initiation and completion of cell division and these cues can be determined by planar polarity, epithelial polarity and through niche architecture. In the *Drosophila* intestine, no permanent stem cell niche has yet been found or defined, nor is it known how stem cells generally are established. One study demonstrated that paracrine Wingless signalling from the intestinal muscles controls self-renewal of *Drosophila* intestinal stem cells (Lin et al., 2008) and another study showed that there is a transient stem cell niche in the larval gut (Mathur et al., 2010), however none of these studies indicated the existence of a permanent niche. Several studies have shown that the *Drosophila* ovarian germline stem cells require strict niche architecture for their asymmetrical cell division, therefore we wanted to investigate if similar circumstances are needed for cell division of intestinal stem cells. The fruit fly intestinal epithelium has a very organised structure and the cells adhere to one another through cellular junctions, as seen in many other *Drosophila*, insect and mammalian epithelia. Electron microscopy studies of *Drosophila* gut epithelia in both larvae and adults have shown the existence of adherens and septate junctions and immunofluorescence microscopy studies further confirmed these findings by pinpointing specific intestinal adherens proteins (armadillo) and proteins of the brush border and basement membrane (α and β-spectrin) (Amcheslavsky et al., 2009; Biteau et al., 2008; Ohlstein and Spradling, 2006), (Phillips and Thomas, 2006).

I employed the current existing knowledge about intestinal junction proteins combined with general knowledge about structural proteins in *Drosophila* epithelia, in order to
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answer questions regarding the epithelial organisation in mutant intestines. Adherens junctions were detected with Armadillo (Arm) protein, septate junctions with Discs large (Dlg) and basement membrane cytoskeleton with α-spectrin. As shown in figure 32, the mutant intestines display a weaker Arm staining and stronger Dlg and spectrin staining, implying altered abundance of these proteins. Bearing in mind that the mutant epithelium has fewer enterocytes that appear larger but are most likely thinner, we wondered if having approximately a third fewer ECs could create excessive mechanical tension on the existing ECs? The reduction in Arm protein could be interpreted as this perhaps being the case. Arm is a cytoplasmic protein that binds directly to the transmembrane DE-cadherin and is an essential part of the cadherin-catenin (CC) complex found in epithelial junctions. Moreover, disturbed DE-cadherin causes reduced cytoplasmic Arm levels. To date, no staining against DE-cadherin has been performed on mutant intestines, however it could be a more direct way of assessing the status of the CC complex in adherens junctions, perhaps combined with Western Blot quantification of these proteins. Observing the decrease in adherens junction staining, I asked the question if this is interconnected with the increase in septate junction staining observed in mutant intestines? The Dlg staining is dramatically more intense throughout the entire length of the mutant gut and especially in the anterior midgut. One plausible explanation could be that mutant ECs compensate for the reduced abundance of the CC complex by depositing higher levels of Dlg in the septate junctions, residing directly underneath the adherens junctions. Another interesting observation is that the pattern of Dlg staining at certain locations between ECs forms Dlg rich nodules and the vast majority of cells with small nuclei (ISC and EBs presumably) reside underneath these nodules. However, the mutant intestines with their intense Dlg staining actually appear to have reduced abundance of “nodular” Dlg. It is difficult to interpret these data since no function for Dlg has ever been described in the intestinal epithelium, however its function as a tumour suppressor and in control of cell proliferation is well established (Humbert et al., 2008; Woods et al., 1996). It would be interesting to investigate whether the absence of Dlg nodules in mutant epithelia is a cause or a consequence of fewer stem cells.

Similarly to the results of the intestinal Dlg staining, it is not straightforward to interpret the observed increase in α-spectrin staining in mutant guts. α-Spectrin protein is
believed to reside in adherens junctions and basolateral junctions (septate junctions) (Dubreuil et al., 1997; Thomas, 2001; Thomas and Kiehart, 1994). What is clearly visible is that α-spectrin is more densely deposited between basal extracellular matrix and the basement membrane in mutant intestinal epithelium compared to control epithelium. The functional purpose of basement membranes and extracellular matrices surrounding the midgut has been more extensively studied in insects other than Drosophila, however the general primary function seems to be mechanical support and anchorage of nerves and muscles surrounding the midgut. In addition, all the molecules that pass between the epithelial cells and the haemolymph have to pass through the basement membrane and other connective tissue. It could be imagined that abundant α-spectrin protein possibly indicates a compensation of some kind, as in the case with Dlg. However, in order to gain more insight and get a clearer overview, it would be important to stain for other components of the basement membrane, such as type IV collagen, Laminin and position specific integrins (Lehane and Billingsley, 1996).

In summary, I hypothesize that the epithelial organisation of enterocytes and other cell types in the gut, as well as junctional proteins comprises an intestinal stem cell niche. This may explain the significant stem cells loss in cdk4 mutant intestines where the abundance of all other epithelial cellular components is dramatically reduced and the abundance of epithelial junction and basement membrane proteins in mutant guts are altered.

3.6 Ectopic expression of Cyclin D/Cdk4 drives EC growth and causes obesity in adult flies.

Loss of Cdk4 results in lean flies with dramatically decreased cell numbers of all intestinal cellular components, pointing to an essential requirement for this gene primarily in the maintenance of normal intestinal homeostasis and nutrient absorption. To complement the loss-of-function analyses, the effects of Cdk4 gain-of-function on cellular gut homeostasis and overall fly metabolism. Ectopic co-expression of Cyclin D/Cdk4 specifically in the enterocytes caused a significant increase in enterocyte cellular and nuclear size, which most likely vastly expands their absorptive area, likely leading to greater nutrient uptake, making the flies obese with increased lipid levels
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and increased levels of stored and circulating carbohydrates (glycogen and trehalose) (Fig. 39). In addition, these flies are starvation insensitive and can survive significantly longer compared to control flies when completely deprived of food. The observation that ectopic expression of *Drosophila* Cyclin D/Cdk4 can drive growth has been observed in other tissues, such as the adult eye and endoreplicative tissues such as the larval salivary gland, fat body and gut (Emmerich et al., 2004; Frei et al., 2005), C. Baltzer, personal communication). Moreover, we could show that the increase in EC cellular area is dependent on Cdk4 kinase activity, as the EC growth is completely abolished when Cyclin D is over-expressed with a kinase-dead Cdk4 transgene (Fig. 40). However, the kinase inactive Cdk4 still caused a partial increase in total lipid levels compared to control levels and an almost equal increase in trehalose and glycogen levels compared to flies over-expressing Cyclin D with a kinase active Cdk4. These data demonstrate that at least some of the functions of Cyclin D/Cdk4 in enterocytes could be kinase independent. In comparison, the ectopic expression of the kinase inactive transgene in enterocytes of cdk43 mutant flies can fully rescue the decrease in enterocyte nuclear size, further supporting the notion that Cyclin D/Cdk4 complex has some kinase independent modes of action. It is difficult to speculate what the mechanism of this kinase independent function might be, as there are no known kinase independent activities of *Drosophila* Cdk4. Possibilities might include binding of Cyclin D/Cdk4 complex to transcription factors or to components of other signal transducing pathways such as JAK/STAT and Hh, with which CycD/Cdk4 has been shown to interact.

3.7 Other modulations of epithelial growth and homeostasis

Since disruption of intestinal epithelial homeostasis by Cdk4 loss-and gain-of-function has effects on whole organism energy storage, it was interesting to investigate the generality of my findings by perturbing epithelial homeostasis with other growth and proliferation inducers. Enterocyte specific over-expression of dMyc dramatically increased EC nuclear size, although without a corresponding proportional increase in cytoplasmic volume. In comparison to growth induction by CycD/Cdk4 that increases nuclear size and cytoplasmic volume proportionally, dMyc is known to induce mainly nuclear and not cytoplasmic growth (Saucedo and Edgar, 2002). Additionally, dMyc
over-expression was accompanied by a dramatic increase in intestinal stem cell proliferation, shown by the appearance of multiple cell clusters containing cells that could be enteroblasts or immature enterocytes. The analysis of the metabolic consequence of ectopic dMyc expression demonstrated a slight but significant increase in lipid levels, however the levels of trehalose were significantly reduced (Fig. 43). The glycogen levels were considerably, although not statistically significantly, reduced as well.

In summary, these data demonstrate that ectopic expression of Drosophila Cyclin D/Cdk4 complex in enterocytes has a dramatic effect on the growth and endoreplication of these cells and in addition causes significant metabolic changes through increased storage of carbohydrates and lipids. In comparison, over-expression of Drosophila Myc in ECs has an even more substantial effect on the endoreplication and nuclear size of enterocytes. Surprisingly though, in this setting the immense distortion of EC nuclei and the entire cell seems to have no or little effect on the capacity of these cells to absorb dietary lipids, on the contrary, the flies even display moderately increased lipid levels. Nevertheless, we observed a decrease in glycogen and trehalose levels suggesting that dMyc over-expression interferes with breakdown, absorbance or storage of dietary carbohydrates. Furthermore, forced expression of dMyc in ECs drives proliferation of intestinal stem cells, further supporting the notion that there is a crucial role of ECs in investigating intestinal stem cell proliferation.

In the Drosophila eye, growth induction by ectopic expression of Cyclin D/Cdk4 is dependent on the mTOR signalling pathway (Tapon et al., 2001). Simultaneous ectopic expression of Tsc1 and Tsc2, the negative regulators of mTORC1 complex activity and CycD/Cdk4 in the adult eye abolishes the growth induction usually caused by CycD/Cdk4 expression in this tissue. To determine whether comparable requirement exists in the gut, a similar experiment was performed in adult enterocytes. Surprisingly, Tsc1/2 over-expression failed to repress the growth promoting effects of Cyclin D/Cdk4 on ECs (Fig. 45). Enterocytes co-over-expressing Tsc1/2 and CycD/Cdk4 appeared equally large as enterocytes only over-expressing CycD/Cdk4, the only differences being that nuclei seemed less “dense” in comparison and changed pattern of Armadillo staining. Lipid quantification revealed that flies over-expressing both CycD/Cdk4 and Tsc1/2 had significantly increased lipid levels.
compared to flies only over-expressing CycD/Cdk4 (Fig. 45). Tsc1/2 over-expression alone caused an increase in lipids as well as substantially elevated glycogen levels, implying increased uptake and/or storage of dietary carbohydrates (Fig. 44). Taken together, these data demonstrate that simultaneous ectopic expression of Tsc1/2 with Cyclin D/Cdk4 causes an additive effect on the lipid levels caused by Cyclin D/Cdk4 over-expression. It is possible that this increase in lipids might occur via mechanisms other than the ones employed by CycD/Cdk4 driven lipid accumulation, possibly through increased dietary glucose uptake. Recent studies of mammalian TSC1 and TSC2 function may provide some intriguing hints in this respect. Loss of TSC1 or TSC2 function leads to hyperactivation of signalling by the mTORC1 complex, which in turn induces HIF1α and SREBP transcription factor activities that coordinate an increase in cellular glucose uptake/glycolysis and de novo lipid biosynthesis, respectively (Duvel et al., 2010). At the same time, these cells also exhibit decreased signalling by PI3K and Akt upstream of mTORC1 due to up-regulation of p70S6K-mediated negative feedback loops and a direct requirement of the TSC1/2 complex for activation of mTORC2, an important activator of Akt (Huang et al., 2009). Akt is well known to induce glucose uptake by promoting the translocation of glucose transporters from the cytosol to the plasma membrane (Rathmell et al., 2003). With this in mind, it is possible to speculate that over-expression of Drosophila Tsc1 and Tsc2 may act to inhibit dTOR activity, removing the p70S6K-regulated negative feedback loops that normally serve to inhibit Akt, thereby allowing Akt-mediated glucose uptake. Thus, it is conceivable that, via different mechanisms, either loss or over-expression of Tsc1 or Tsc2 could cause an increase in glucose uptake. It would be interesting in future experiments to further investigate genetically the possible enterocyte-specific role of the PI3K-Akt-TSC-mTOR pathway in regulating glucose and nutrient uptake and thereby regulating whole body nutrient homeostasis.

3.8 Conclusions

In summary, through the work in this doctoral thesis, it was shown that the Drosophila Cyclin D/Cdk4 complex plays a major role in indirectly regulating stem cell proliferation and adult intestinal homeostasis through control of enterocyte growth and function. As depicted in the summary scheme and model presented in Figure 48, loss of Cdk4
leads to reduced numbers of all intestinal cellular components, impaired growth and presumably function of enterocytes, leading to substantial metabolic consequences in adult flies. We hypothesize that Cdk4 function is primarily required for control of enterocyte growth, however it also seems to have a function in regulating intestinal stem cell proliferation. It is currently unclear if the regulation of stem cell proliferation is cell autonomous or non-autonomous, as rescue experiments using a stem cell specific or an enterocyte specific Gal4 driver both restore the stem cell proliferation defects in $cdk4^3$ mutant flies. It is possible that there is a requirement for Cdk4 in both stem cells and enterocytes. One important aspect of how Cdk4 might regulate intestinal physiology and function not covered in this thesis is the connection and convergence of Cdk4 regulation with other signalling pathways. As mentioned in the introduction, Cdk4 and Cyclin D do have links to pathways such as Hedhehog and JAK/STAT, therefore it would be interesting to explore if intestinal roles of CycD/Cdk4 act in concert with these pathways. For instance, our preliminary data has shown changes in the JAK/STAT signalling cascade in $cdk4^3$ mutant intestines, making it a primary target for future investigations.

In general terms this study has provided increased insight into how adult flies control their normal intestinal homeostasis and stem cell proliferation, by demonstrating that sufficient enterocyte numbers and function are prerequisites for this homeostasis. It also shows that disruptions of epithelial homeostasis can have profound effects on the energy metabolism and storage of the whole animal.
**Figure 48.** Proposed model of how *Drosophila* Cyclin D/Cdk4 controls multiple aspects of adult intestinal homeostasis. In green are conclusion based on experimental evidence. In red are hypothetical assumptions. Numbers is red specify reduction in cell number.
4. Materials and methods

4.1 Fly work

4.1.1 Fly stocks

Cdk4 stocks used: cdk4\(^{3}\) (null allele, (Meyer et al., 2000), cdk4\(^{4}\) (C(2)SH0671, here called cdk4\(^{SH0671}\), hypomorph, (Chen et al., 2003), UAS-Cdk4 (Datar et al., 2000), UAS-Cdk4\(^{D175N}\) (Meyer et al, 2000), UAS-CycD-UAS-Cdk4\(^{D175N}\)

For rescue experiments with NP1-gal4 and esg-gal4, cdk4\(^{3}\) was recombined on the same chromosome as the respective Gal4 driver. For induction of cdk4\(^{3}\) mutant clones, cdk4\(^{3}\) was recombined on a FRT42 chromosome and crossed to produce the final genotype: Flp/Flp;FRT-cdk4\(^{3}\)/FRT42-GFP. Fat body clones were induced by 1 hour heat-shock at 37°C at 8-9 hours after egg deposition.

Ubiquitous Gal4 drivers used: Act5C-gal4 and da-gal4 (Bloomington Stock Center, Indiana University, USA).

Tissue specific Gal4 drivers: 3.1Lsp-gal4 (adult fat body, (Lazareva et al., 2007), kindly provided by B. Dauwalder, University of Houston, Houston, USA), NP1-gal4 and esg-gal4-UAS-GFP-UAS-lacZ (enterocyte and intestinal stem cell/enteroblast specific respectively, kindly provided by N. Buchon, EPFL, Lausanne, Switzerland)

RNAi lines: CycD-RNAi (KK library), Cdk4-RNAi (GD library), both from the Vienna Drosophila RNAi Center (IMP, Vienna, Austria).

Other transgenes: UAS-dMyc (Schreiber-Agus et al., 1997), UAS-Tsc1/2 (Tapon et al., 2001).

In lipid quantification assays, genetic background of the experimental flies has an influence on the final total lipid levels, therefore all flies in every set of experiments were either in an yw or w\(^{iso}\) background. Experiments done with cdk4\(^{3}\) hetero- or homozygous animals were all done in an yw background and experiments with Cyclin D/Cdk4 over-expression were performed in a w\(^{iso}\) background.
4.1.2 Developing conditions

All flies were kept at 25°C, 60% humidity and a 12 hours day-night light cycle. For all experiments, flies developed under non-crowded conditions at a density of 50 larvae/vial. Females were allowed to lay eggs on apple agar plates for 2-6 hours and the hatched larvae were transferred to vials with standard fly food after 20-24 hours. Flies were collected within 6 hours of eclosing, separated by sex and divided into batches of maximum 30 animals per vial and transferred into vials with fresh food every 2-3 days prior to start of experiments. Mature, adult males at least 6 days of age were used for all experiments if not otherwise stated.

4.1.3 Fly weighing

Flies were weighed on a MX5 micro-balancer (Mettler Toledo) either in batches or individually and wet weight was recorded.

4.1.4 Determination of larval stage

Egg-depositions were made the same way as described above, with the exception that a pre-egg deposition was made on a plate to be discarded and the actual experimental egg-deposition being maximum 1-1.5 hours long. The purpose of the pre-egg deposition is to encourage females to lay older eggs, in order to ensure precise age of the future progeny deriving from the experimental egg-deposition. Larvae were extracted from the food either before molting from L2 to L3 or just after L2 to L3 molting. The stage was determined by morphology of the posterior spiracles, as they change noticeably after molting. Most larvae of the Cdk4 and Cyclin D mutant alleles molted 2-3 hours later compared to their respective controls.

4.2 Fly food and feeding experiments

1 liter of normal fly food contains: 100 g yeast, 75 g sugar, 55 g corn, 10 g flour and 8 g agar. For feeding experiments, standard fly food was re-melted and either Oleic Acid, Allura Red or BrdU were added to food when cooled down to 40-45°C under
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constant stirring. The mixture was added to new food vials on ice at 4°C to prevent sedimentation and aid in quick solidifying. The food vials were stored at 4°C for maximum one week.

4.2.1 Oleic Acid feeding

Normal fly food was re-melted and Oleic Acid (Sigma) dissolved in ethanol was added to a final concentration of 400 µM. Control food was also re-melted and corresponding volume of only ethanol was added. The food was allowed to cool and re-solidify and was used for feeding experiments for 8, 15 or 16 days. Flies were put on fresh food every 2-3 days.

4.2.2 BrdU incorporation assay

Adult male flies of different starting ages (usually 2-3 days or as indicated) were fed 5-bromo-2-deoxyuridine (BrdU, dissolved in DMSO, final concentration in fly food 0.2mg/ml, Calbiochem) mixed into standard fly food for varying lengths of time (2, 4, 6 days) or as indicated. The flies were dissected in 1x PBS, fixed in 4% Paraformaldehyde (PFA)/PBS (Electron Microscopy Sciences) for 45 minutes and treated with 3M HCl for 30 min, followed by washing in PBS. After HCl treatment, samples were stained against anti-BrdU antibody (1:1000 dilution, monoclonal rat, Oxford Biosystems) following standard antibody staining protocol. Anti-rat Alexa488 was used as a secondary antibody (1:5000 dilution, Invitrogen).

4.3 Gut function assays

Normal fly food was re-melted and Allura Red (2% stock solution, Sigma) dissolved in water was added to a final concentration of 1.3 percent. Batches of 10-15 male flies in triplicates were kept on the experimental food for 12 hours to over-night (during the night-cycle). One batch of approximately 10 flies was kept on normal food in parallel, to be used as control. Following morning flies were anesthetized and shock-frozen in liquid nitrogen or dry ice. Flies were weighed in batches and homogenized in 1 ml 1x PBS using a Retsch MM2 homogenizing machine (Schieritz & Hauenstein AG),
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following centrifugation for 10 minutes at 13,000 rpm. Nine hundred and fifty µl of the supernatant was transferred to a new Eppendorf tube and centrifuged for additional 5 minutes. After centrifugation 900 µl were transferred into plastic cuvettes and the volume was adjusted to 1 ml with PBS. Two blanks were used, one being 1 ml of PBS and the other flies not fed on Allura Red food. Absorbance was read using an Ultrospec 3100 spectrophotometer (Amersham Biosciences) at 500 nm and the absorbance of control flies was subtracted from the total absorbance of AR fed flies.

4.4 Starvation experiments

Starvation medium was made with 1xPBS mixed with agarose (1%) and poured into empty food vials. Flies in batches of 15-30 flies were used per biological replicate; three biological replicates were used per genotype. Starvation was always started in the afternoon and the numbers of dead/alive flies was recorded at 1-hour intervals until all the flies were dead. Starvation of larvae was done as follows: larvae were taken out of the food, washed twice in 1xPBS, transferred to an empty petri-dish and placed in a drop of PBS (one larva/drop) for set number of hours before dissection, fixation and staining.

4.5 Stainings

4.5.1 Antibody stainings for immunofluorescence

Flies were dissected in 1xPBS, fixed in 4% PFA/PBS (Electron Microscopy Sciences) for 45 minutes and washed in 0.2% Triton X-100/PBS (PBST) for 20-60 minutes. Samples were washed in increasing concentrations of PBST (0.5% 20 min, 1% one to several hours) followed by blocking with 0.2 % milk powder for 2 hours at room temperature (RT) or over-night (O/N) at 4°C. After blocking, samples were washed down to 0.2% PBST (1%, 0.5% for 20 minutes each) and incubated with primary antibody in 0.2% PBST 2-3 hours at RT or O/N at 4°C, followed by washing in 0.2% PBST for 2-3 hours. Samples were incubated with secondary antibody for 3 hours at RT or O/N at 4°C and incubated with DAPI (0.5mg/ml, 1:1000 dilution, Molecular Probes) for 5 minutes, washed 2-3 hours in 0.2% PBST, rinsed in 1xPBS for 5
minutes, mounted on slides and covered with Vectashield mounting medium (Vector Labs). All steps were done with mechanical agitation.

Primary antibodies used: monoclonal anti-mouse Delta (1:100), anti-Prospero (1:100), anti-Armadillo (1:50), anti-Dlg (1:100), anti-Lamin (1:100), anti-α-Spectrin (1:100). All from Developmental Studies Hybridoma Bank.

Secondary antibodies used: goat anti-mouse Alexa Flour 568 (1:5000 dilution, Invitrogen), anti-mouse Alexa Flour 488 (1:5000 dilution, Invitrogen).

4.5.2 Cryosectioning and Oil Red O staining

Mature adult flies were anaesthetized and transferred to 15 ml Falcon tubes containing 3% PFA and 2% sucrose and were fixed at 4°C over-night while rotating. After subsequent washing in PBS for 10 minutes and draining, the flies were embedded in moulds containing OCT embedding medium (Tissue-Tek, Sakura) and placed on dry ice to solidify. Frozen blocks were cryo-sectioned on a HM560 Cryostat (Microm AG) with a thickness of 7 µm per cut slice and lifted onto Super Frost Plus Slides (Thermo Scientific). Slides were stored at -20 °C prior to staining procedure.

Slides with the sections were thawed at room temperature, washed 10 minutes with distilled H₂O, shake-dried, placed in a humidity chamber following addition of 0.2 percent ORO (Sigma) staining solution. Slides were incubated over-night at 4°C, subsequently washed 3 times 10 minutes with distilled H₂O, shake-dried and mounted in Mowiol (Calbiochem).

4.5.3 Nile Red staining

For Nile Red (Molecular Probes) staining, five to eight larvae were inverted and fixed in 8% PFA/PBS for 45 minutes, followed by washing in 0.2% Triton/PBS (PBST) (3 x 30 minutes) and Nile Red (1:5000 dilution of a 1% stock in DMSO) incubation for 45 minutes. Samples were stained with DAPI for 5 minutes (1:1000 dilution) and after subsequent washing in PBST (3 x 30 minutes) fat bodies were mounted in Vectashield mounting medium (Vector Labs).
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4.5.4 Phalloidin staining

Five to ten adult male flies were dissected in 1xPBS and fixed in 8% PFA/PBS for 45 min at RT, following washing in 0.2 % PBST and incubation with Alexa Flour546-phalloidin (1:50 dilution, 300U, Invitrogen), for 60 minutes at room temperature. Samples were stained with DAPI for 5 minutes (1:1000 dilution), washed O/N, rinsed 5 minutes in 1xPBS and mounted in Vectashield.

4.5.5 Antibody and Phalloidin staining of FRT clones

Female larvae were inverted, fixed for 1 hour in 8% PFA/PBS at RT and treated the same way as adult tissues for immunoflourescent staining. To enhance the signal of the endogenous GFP, samples were incubated with mouse α-GFP primary antibody (1:1000 dilution, Roche), followed by incubation with Alexa Flour 488 secondary antibody (anti mouse, Invitrogen) for 3 hours and phalloidin (1:50 dilution, 300U, Invitrogen) for 60 minutes. Samples were stained with DAPI (5 minutes, 1:1000 dilution), washed O/N in PBST and mounted as described.

4.6 Microscopy

Images of all larval fat body tissues were acquired on a Deltavision Olympus K70 microscope, using CoolSnap HQ camera (Photometrics) and a 40x objective. Serial Z-sections were taken at 0.2 μm distance and deconvoluted using Softworx software (Applied Precision). Shown are the projections of the maximal intensity from 4 subsequent sections. Images of Oil Red O stained adult tissues were acquired on a Zeiss AxioImager A1 microscope, using a Zeiss AxioCam MRc5 camera (Zeiss) and with AxioVision software (Zeiss). Images of adult intestines with immunofluorescent staining were acquired using a Leica SP2-AOBS laser scanning confocal, with a 63x Oil Objective. Serial Z sections were taken between 0.244 or 0.3 μm distance and pictures are shown as snapshots of the average intensity from all taken sections (Leica Confocal Software). The laser intensity and background filtering was always set according to the control samples and remained the same for all subsequent samples, if not otherwise stated. Pictures of midguts were taken with strict distinction with
respect to the anterior and posterior part of the gut. All pictures displayed are acquired in the anterior midgut, if not otherwise stated.

4.7 Image analysis and quantification

The colour intensity of most images has been enhanced equally for all images within the same experiment, using linear adjustments. The quantification of various stainings (lamin: nuclear area, armadillo: cellular area) was done in ImageJ by manually tracing the outline of the staining using the built in measuring tool (Polygon selection) and the results are shown as area in pixel$^2$. Counting of DAPI stained nuclei was done in ImageJ using the snapshot pictures generated with confocal microscopy. Counting of BrdU, Prospero and esgGFP positive cells was performed manually on an AxioPlan2 Imaging microscope (Zeiss). Analysis was done in blind.

4.8 RNA extraction, cDNA synthesis and Quantitative Real Time PCR

Five to ten whole adult males were shock-frozen in liquid nitrogen, crushed in an Eppendorf tube using a plastic pestle and lysed using TriZol (Sigma). RNA was extracted using RNA Extraction Kit (Macherey-Nagel) following the manufacturers recommendations. For qRT-PCR on adult tissues, 15-40 male adult guts were dissected in 1xPBS for each genotype and transferred to RNA later (Sigma). Tissues were lysed with TriZol (Sigma) and subsequent RNA extraction was performed using RNA Extraction Kit (Macherey-Nagel) following the manufacturers recommendations. RNA concentration was quantified using NanoDrop (Thermo Scientific). cDNA synthesis for all qRT-PCR experiments was done with Ready-To-Go-Prime-Beads (GE Healthcare) and using oligo(dT)15 primers (Promega), following the manufacturers recommendations. Quantitative Real-Time PCR was performed using Roche SYBR Green Mix and using the Light Cycler 480 (Roche). All experiments were done in biological triplicates and the results are shown as mean and standard deviation. The ribosomal gene rp49 was used as internal control.
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List of primer sequences used:

<table>
<thead>
<tr>
<th>CG NUMBER/Gene name</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| Rp49 ((CG7939, RpL32)) | forw: GCAAGCCCAAGGGGTATCGA  
rev: CGATGTTGGGCGATCAGATCTAG |
| FAS: | forw: CTTTGGCATCGATGTCCTTT  
rev: GCTCCAGACTCTGTCCTG |
| ACC (CG11198) | forw: GATCGAGCGAATGAAGAAGC  
rev: GTACGATCTGGAGCTCAGG |
| Lip3 (CG8823) | forw: GCCGGATCTCGGCCTATTT  
rev: AATACGGGATGCGGTGATA |
| Lip4 (CG6113) | forw: CACCAATTTTGAGCAGCTGAG  
rev: TTCTCCAGGTGGTAGGATGG |
| CG8093 | forw: ACCGCATACGCGCAGGAGGT  
rev: CACTGTGAGGTCCAGCAAGA |
| CG6277 | forw: ACCAAGGGCGAGACTACTGGA  
rev: CAATTTTCTCGTCCCTTGA |
| CG6271 | forw: GGATATCCCAACGGGAACACTCA  
rev: CCCATCATCACCCAGGTATT |
| Brummer (CG5295) | forw: ATTTAGTCCGCGGACCTCCTC  
rev: CTCAGCTCCGACGGGTACCA |
| β-trypsin | forw: TCATGGCTCTCTGATTTGTTCCG  
rev: TCGTCGCCAAGGTTTCCTT |
| ε-trypsin | forw: TGAGTTCCAGGGCACTTAATTGGA  
rev: TTTCCCTGCTCACAAGGGGTATT |
| γ trypsin | forw: CG13633 Allatostatin A | forw: ATTTAGTCCGCGGACCTCCTC  
rev: CTCAGCTCCGACGGGTACCA |
| CG6456 Allatostatin B | forw: AACAGCCAGAACAGGACATC  
rev: GTGAATTTACGGGCACGAGT |
| CG14919 Allatostatin C | forw: CCCAATATTCCAATGGATCG  
rev: CATTCAATCTCCGTAAGT |
| CG14734 Tachykinin | forw: ATCCATGGCTCTACCTGGTG  
rev: ATCCGCTCACAATCGCCCTAC |
| CG10342 Neuropeptide F | forw: GGCTGATGCCTACAAGTTCC  
rev: CCTCCATTGTTGGGATTTT |
| Cyclin D (CG9096) | forw: TCACCGGCAGATCATC  
rev: CCGGATGGAATACGGATT |
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*Cdk4 (CG5072)*

\[ \begin{align*}
\text{forw:} & \quad \text{CTGGTGCAACTAACGGTTTC} \\
\text{rev:} & \quad \text{CCTGGTAGTTGAACGGATCG}
\end{align*} \]

### 4.9 Lipid measurement

Flies were homogenized in 0.05% Tween/PBS using a Retsch MM2 homogenizing machine. All larval samples were homogenized in 0.05% Tween/PBS using a Mikro-Dismembrator homogenizing machine (Braun Biotech). Samples and standards were heated to 70 °C for 5 minutes to inactivate all enzymes. Samples were added uncentrifuged to 1ml cuvettes containing 800ul of Triglyceride Reagent (Sigma) and incubated at 37°C for 5 minutes, following initial absorbance reading at 540nm. Two hundred µl Triglyceride lipase (Sigma) was added, samples and standards were incubated at 37°C for 5 minutes and final absorbance was recorded. Proteins were quantified using a Bradford assay (BioRad). Measured data was interpolated from a standard curve.

### 4.10 Glycogen/trehalose measurement

Flies were homogenized in 250 µl Na$_2$CO$_3$ (0.25M) for 2 minutes using a Retsch MM2 homogenizing machine, following 2h incubation at 95°C. After incubation 150 µl 1M Acetic Acid was added together with 650 µl Na-acetate (0.2M, pH 5.2) and samples were centrifuged at 13 000 rpm for 10 minutes. Nine Hundred µl of the supernatant were transferred to a new Eppendorf tube and centrifuged 13 000 rpm for 5 minutes. One hundred µl of the supernatant was transferred to an Eppendorf tube where 2 µl of Amyloglucosidase enzyme (67.4U/mg, Sigma) was added, followed by an overnight incubation at 56°C. Two hundred µl of the supernatant was transferred into another Eppendorf tube where 2 µl of Trehalase Porcine Kidney enzyme (1U, Sigma) was added, followed by an overnight incubation at 37°C. After incubation, glycogen and trehalose in the samples are converted to glucose, which was quantified in 96-well plates using 100 µl of Glucose Reagent (Sigma). (20 µl sample for glycogen, 50 µl sample for trehalose quantification) after 30 min incubation at RT. Absorbance was read at 340 nm using a SpectraMax 190 96-well spectrophotometer. For each measurement, separate glycogen (glycogen from bovine liver type IX) and trehalose
(D(+)-Trehalose dihydrase) standards were made and incubated together with respective samples with addition of either Amyloglucosidase or Trehalase enzyme. Measured data was interpolated from a standard curve.

4.11 Statistical analysis

For all experiments, statistical significance was calculated in Prism 5 for Mac OS X (GraphPad Software, Inc.) using the Student’s t-test (two-tailed, two sample equal variance). * equals p<0.05, ** equals p<0.01 and *** equals p<0.001, n.s: not significant. For survival curves, the Log-rank test was performed.
5. REFERENCES


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