Analysis of miR-277 in *Drosophila melanogaster* and its role for metabolism and lifespan

Stephanie Maria Esslinger

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Summary

My PhD-thesis deals with the analysis of the microRNA-277 in *Drosophila melanogaster*. Our aim was to examine the expression and the role of miR-277 for metabolism and lifespan of the fly.

First I was able to determine that miR-277 is predominantly expressed in the thoracic muscles, the fat body, but not the gonads of adult flies.

Further I analysed the expression profiles of *D.melanogaster* microRNAs during ageing. I found out, that the profiles of the 40 most abdundant *Drosophila* microRNAs are distinct in young and aged flies; and that in particular the expression of miR-277 decreases with age.

To examine the relevance of this downregulation for the lifespan of the fly, I impaired down-regulation of miR-277 by transgenic expression with a constitutive promotor on different food regimes with various contents of sugar and protein. Flies were short-lived on all food regimes, but the effect was most pronounced on food with a low sugar content and a high protein content.

It has already been proposed, that miR-277 in *Drosophila* has a role for downregulation of the branched chain amino-acid (BCAA) degradation enzymes. To confirm this, I analyzed miR-277 overexpressing flies and classified the effects of constitutive expression of miR-277 into Gene Ontology terms (GO-terms). The majority of the GO-terms that were significantly enriched in the down-regulated mRNAs were related to metabolic processes, among them also terms that describe the metabolism of BCAAs or terms that include BCAA metabolism. These results suggest that miR-277 controls genes involved in the degradation of BCAAs, consistent with previous predictions.

Next I profiled mRNA levels after inhibition of endogenous miR-277 in *Drosophila* Schneider S2 cells and use of a pulse-labeling technique of newly synthesized mRNA. 8 genes - all of them enzymes of the degradation pathway of BCAAs - were detected as upregulated. Upon fractionation I determined that the increased steady-state level was due to a stabilization of the corresponding mRNAs. Thus the changing genes represented direct miR-277 targets and regulation by miR-277 can occur on a post-transcriptional level.

It was already reported that the BCAAs stimulate the TOR (target of rapamycine) kinase in *Drosophila*. Overexpression of miR-277 in *Drosophila* Schneider S2 cells led to increased phosphorylation of eIF4E-binding protein (4EBP), an established substrate of

the TOR kinase. I therefore propose that constitutive expression of miR-277 shortens life span via inappropriate activation of the TOR kinase.

Interestingly metabolite measurements revealed that overexpression of miR-277 did not lead to increasing levels of BCAAs, neither in *Drosophila* Schneider S2 cells nor in flies .

Notably the first enzyme of the BCAA degradation cascade - CG1673, a transaminase that converts e.g. leucine to α-keto isocaproic acid (KIC) - changed neither in our transgenic flies with constitutive miR-277 expression, nor in the *Drosophila* Schneider S2 cells upon miR-277 inhibition. If transamination occurs but further degradation is diminished, then branched chain α-keto acids (BCKAs), like KIC, should build up.

KIC was also known to be a strong activator of the TOR kinase in mammalian cells. I could show that KIC can activate *Drosophila* TOR even more persistently and more potently than leucine. Hence miR-277 modulates TOR activity by adjusting the clearance of BCKAs rather than BCAAs.

The TOR pathway interacts with the insulin signaling pathway in *Drosophila*. The combination of the transgenic miR-277 construct was synthetic lethal with a homozygous mutation of the insulin receptor substrate *chico*.

In summary, I could demonstrate that miR-277 controls metabolism through the BCAA degradation pathway. Since the first enzyme of the cascade, the transaminase CG1673, is not efficiently repressed by miR-277, the result of regulation by miR-277 is an increase of BCKA concentration followed by an increase of TOR kinase activity. This signaling event is likely of physiological importance since miR-277 is down-regulated with age and constitutive expression of miR-277 shortens life span.

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1 Introduction

1.1 Two classes of small RNAs: microRNAs and siRNAs

A variety of small RNA pathways exist in animals. Two of the most important ones are micro RNAs (miRNAS) and small-interfering RNAs (siRNAS), the latter can be divided into exogenous and endogenous siRNAs.

Lee et al. and Dalmay et al. were the first that described small RNA species (Dalmay et al., 2000; Lee et al., 1993).The group around Lee identified two small lin-4 transcripts in *C. elegans*, that did not encode a protein. They contained sequences complementary to a repeated sequence element in the 3' untranslated region (3' UTR) of lin-14 mRNA, suggesting that lin-4 regulates lin-14 translation via an antisense RNA-RNA interaction. Several years later Dalmay et al.described four genetic loci that are required for post-transcriptional silencing in *Arabidopsis.*They proposed that one of these, SDE1, encoding an RNA-dependent RNA polymerase has the role to synthesize a double-stranded RNA initiator of posttranscriptional gene silencing. Most of the small RNA silencing pathways are highly conserved and can be found in all eukaryotic phyla from the yeast *S. pombe* to plants and animals, but not in bacteria or archea (reviewed in Ghildiyal and Zamore, 2009).

Drosophila melanogaster is one of the best studied model-systems for small RNA silencing. A variety of established genetic tools exist, and immortalized cell lines are available and large amounts of material for biochemical analyses can be produced.

1.1.1 Biogenesis

MiRNAs are 21- to 23-nucleotide single stranded RNAs that are encoded in the chromosomal DNA and repress cognate mRNA targets (Bartel, 2004; He and Hannon, 2004). They are transcribed as long, hairpin-containing precursors with imperfect complementarity by RNA polymerase II (Lee et al., 2002; Bracht et al., 2004; Cai et al., 2004; Lee et al., 2004; Parizotto et al., 2004; Baskerville and Bartel, 2005). These primary transcripts are called pri-miRNAs. They are then processed in the nucleus by the multidomain RNase III endonuclease Drosha (Lee et al., 2003). Drosha is assisted with a double-stranded RNA-binding domain (dsRBD) protein partner, known as Pasha (Denli et al., 2004). The resulting double-stranded precursor miRNA (pre-miRNA) is transported to the cytoplasm. Exportin-5 (Ranbp 21 in *Drosophila*) binds the pre-miRNA and transports it via the Ran-GDP-Ran-GTP transport system. In the cytoplasm, a second RNase III endonuclease, Dicer, converts pre-miRNA into mature miRNA (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Park et al., 2002).

In *Drosophila*, two Dicer paralogs define parallel pathways for small RNA biogenesis, Dicer-1 (Dcr-1) and Dicer-2 (Dcr-2). Dicer-1 (Dcr-1) liberates miRNA from pre-miRNA. The double-stranded precursor is then loaded into an effector complex, termed RNA-

induced silencing complex (RISC). Loquacious (Loqs), more precisely the isoform Loqs-PB, is the dsRBD partner protein of Dcr-1. MiRNAs (and also siRNAs) function in RISC as single-stranded RNA guides for members of the Argonaute family of proteins (Hutvágner and Zamore, 2002; Martinez et al., 2002).

In *Drosophila* there are five Argonaute proteins. Argonaute 1 (Ago1) and Ago2 bind miRNAs and siRNAs, respectively. Piwi, Aub and Ago3 bind another class of small RNAs, piRNAs (Piwi-interacting RNAs). Most miRNAs are loaded into the effector endonuclease Ago1 (Lee et al., 2004; Okamura et al., 2004)(Fig. 1.1). Mature miR-277 is, untypical for microRNAs, loaded to 2/3 into an Ago2-RISC rather than into Ago1-RISC (Förstemann et al., 2007).

Exogenous siRNAs are derived from long double-stranded precursors. They are either introduced into the cell with the purpose of inducing RNAi experimentally or appear during replication of certain RNA viruses (reviewed in Golden et al., 2008). SiRNAs follow a similar biogenesis pathway as miRNAs. They are excised by an RNAseIII enzyme, Dcr-2, with the help of a dsRBP, R2D2, a paralog of Loquacious; this yields a double-stranded precursor (Liu et al., 2003), which is perfect complementary due to the origin of siRNAs. They are preferentially loaded into a RISC that contains the endonuclease Ago2 (Liu et al., 2003; Pham et al., 2004) (Fig. 1.1).

Endo-siRNAs are derived from long double-stranded RNA precursors with endogenous origin.They can be produced from long hairpin structures with extensive stretches of complementarity (Okamura et al., 2008a), from convergent transcription (Czech et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura and Lai, 2008), or potentially by low levels of cryptic antisense transcription throughout the genome (reviewed in Berretta and Morillon (2009)). Biogenesis of endo-siRNAs depends, as mentioned before for exogenous siRNAs, on Dicer-2 and Ago2 (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008). But several studies showed that instead of R2D2, the dsRBP acting with Dcr-2 in the exo-siRNA pathway (Czech et al., 2008; Hartig et al., 2009; Kawamura et al., 2008; Okamura et al., 2008b), Loqs (the isoform Loqs-PD) is involved (Fig. 1.1).

1.1.2 Role of small RNAs

One strand of the precursor, the miRNA* strand, is expelled from the complex. After that, complementary base-pairing with an mRNA can silence gene expression by inhibiting translation or favoring degradation of the message (Okamura et al., 2004) (Fig. 1.2). SiRNAs silence their targets by cleavage of the corresponding message (Okamura et al., 2004; Tuschl et al., 1999). MiRNAs regulate gene expression and are important in development, oncogenesis (reviewed in Ghildiyal and Zamore, 2009; Kim et al., 2009), differentiation, metabolic homeostasis, and memory (Ashraf and Kunes, 2006; Bernstein

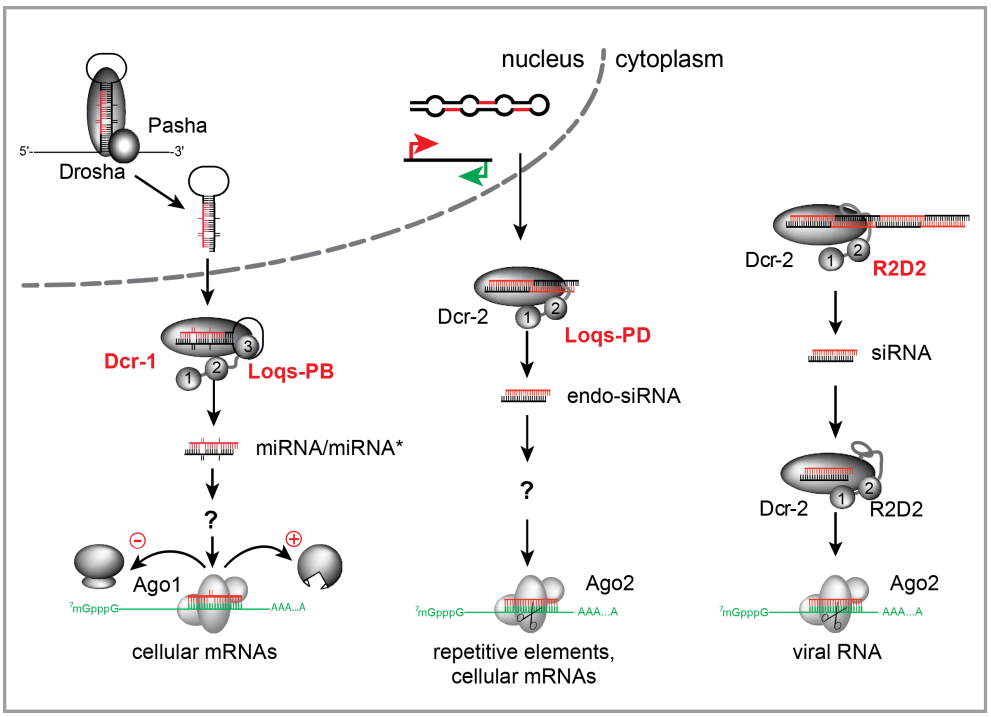

Figure 1.1: **Small RNA biogenesis pathways and generation of endo-siRNA precursors.** All somatic small RNA biogenesis pahtways can be distinguished by, at least, one specific component. The model represents important biogenesis steps for miRNAs, endo-siRNAs and siRNAs. Pathway specific components are labelled in red. For the Loqs isoforms and R2D2, the individual dsRBDs are numbered to make the differences more obvious. Although a RISC-loading complex (RLC) hast been discovered for siRNAs (Pham et al., 2004; Tomari et al., 2004), analogous complexes for endo-siRNAs and miRNAs are yet to be identified (adapted from Hartig et al. (2009)).

et al., 2003; Grishok et al., 2001; Harfe et al., 2005; Kanellopoulou et al., 2005; Ketting et al., 2001; Lee et al., 2004; Li and Carthew, 2005; Poy et al., 2004; Reinhart et al., 2000; Schratt et al., 2006; Teleman et al., 2006). About 30% of the protein coding genes in *Drosophila* and humans are predicted to be regulated by miRNAs (Krek et al., 2005; Lewis et al., 2005, 2003; Rajewsky and Socci, 2004; Stark et al., 2003). Stark et al. predicted 2003 for the first time a role for miR-277 in degradation of branched-chain amino acids and thus for regulation of fly metabolism.

Exo-siRNAs provide a defense against RNA viruses that involve the production of long dsRNA in their amplification cycle. Endo-siRNAs suppress transposable elements in the soma to reduce the possibility of transition into the germ cell lineage (Chalvet et al., 1999; Pelisson et al., 2002) and regulate expression of endogenous genes (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008).

In my work I was able to describe a cell-culture model in which an artificial plasmid sequence, integrated at high-copy number, has become subject to endosiRNA-mediated repression. This artificial target for endo-siRNAs is silenced through a post-transcriptional

mechanism, indicating that repression is direct. The repressive response also occurred in transiently transfected cells; thus, *Drosophila* endo-siRNAs can mount a *de novo* response.

1.2 Ageing

Ageing is a complex process of accumulation of molecular, cellular and organ damage, leading to loss of function and increased vulnerability to disease and death and concomitant decline in reproductive output. The fruit fly *Drosophila melanogaster* as a quite simple organism with a relatively short lifespan has been extensively used as a model organism for ageing studies. Early reports revealed reduced mitochondrial number and mitochondrial structural changes in the aged *Drosophila* (Sohal, 1975).

Ageing is characterized by highly dynamic changes in the expression of many genes; this provides a powerful molecular description of the ageing process and its underlying mechanisms. In several studies (Girardot et al., 2006; Pletcher et al., 2002; Zhan et al., 2007) RNA transcript profiles for the whole *Drosophila* genome during normal ageing were generated by examining whole body samples or different tissues at several time points during adult fly life. They described the changes in transcript representation using the annotation project directed by the Gene Ontology (GO) Consortium. Three seperate ontologies, comprising biological process, molecular function and cellular component, define a set of terms and relationships through which the role of a particular gene, gene product, or gene-product group can be interpreted. They were able to show that for example the functions of cytochrome P450s and antibacterial peptides are associated with the ageing process, as a significant number of corresponding genes showed an increased transcript

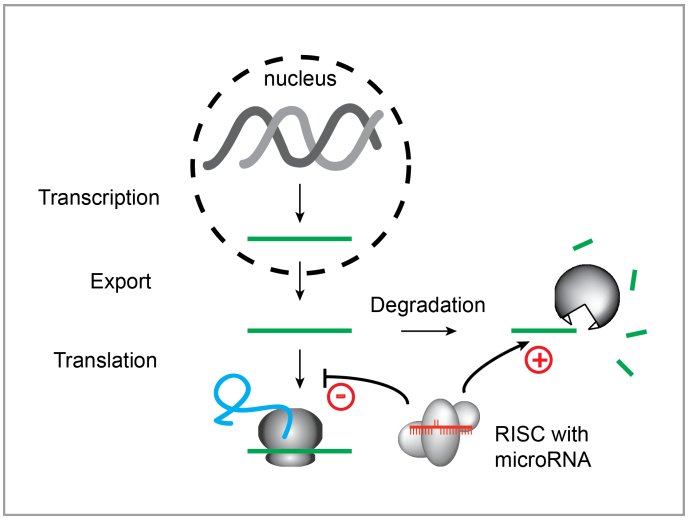

Figure 1.2: **MicroRNAs repress the expression of corresponding target mRNAS.** MicroRNAs can stiumlate the degradation of direct target mRNAs or incluence the rate of transcription of indirect target mRNAs.

representation with age. Nearly all forms of stress response (GO:0006950), including response to pathogens, and genes involved in perception of biotic stimulus (GO:0009595), such as bacteria, increased with age. In addition transcript levels of genes involved in the response to external stimulus, especially response to bacteria (GO: 0009617), were highly influcenced by age. Well-known immune defense genes like Cecropins, Attackins, Defensin and the NF-KB homolog Relish were present in this group.

By contrast genes whose product is localized to the mitochondrion, especially those found on the inner membrane and involved in electron transport (GO:0005746) showed strong declines in expression with age; as do genes whose products localize to the nucleus (GO:0005730). Furthermore aged flies have reduced transcript levels of genes of the tricarboxylic acid cycle (TCA) (Morrow and Tanguay, 2008). Nearly all genes involved in protein metabolism and modification are (GO0006411) maintained at relatively constant levels of transcription throughout life span.

Despite the obvious complexity of ageing, recent work has shown that dietary and genetic alterations can substantially increase healthy life span of laboratory model organisms, like yeast, worms, flies, mice and monkeys (Fontana et al., 2010).

1.3 Effect of caloric intake on lifespan

Dietary restriction (DR), a reduction in food intake without malnutrition, extends median life span and survivorship (i.e. reduced mortality) of diverse organisms, including yeast, flies, worms, fish, rodents, and rhesus monkeys (Fontana et al., 2010).

It can protect against an age-related decline in function (e.g. reproductive output) and disease and reduces risk factors for diabetes, cardiovascular disease, and cancer in humans. A body of evidence in several organisms demonstrates that an increase in mitochondrial activity, together with activation of the reactive oxigen species (ROS) defense system, is associated with the salutary effects of the DR regimen (Guarente, 2008; Nisoli et al., 2005).

Chippindale et al. reported 1993 unambigously DR in *D. melanogaster*. Adults maintained on sugar medium with yeast lived longer and laid fewer eggs when yeast was scarce rather than abundant (Chippindale et al., 1997). In mammal studies the level of restriction usually ranges from 10% to 50% below the level fed ad libitum, but longevity extension can be achieved by complete starvation in yeast and worms (Fontana et al., 2010). In the experiments of Chippindale et al. the median life span was greatest upon a diet with 5% SY (sugar, yeast), more diluted diets reduced both survival and fecundity, and richer diets reduced survival but increased fecundity.

The mechanisms that affect life span during starvation level diets differs from those that affect life span when DR slows ageing (Chapman and Partridge, 1996; Partridge et al.,

2005).When malnourished, both fecundity and survival decline as diet is progressively restricted, followed by impaired fitness; furthermore physiology that is activated to ensure survival, must be directed at coping with the effects of starvation. In constrast, in the physiological range of nutrients that maintain sustenance, a trade-off occurs between repoduction and somatic survival; thus restricted diet increases survival and decreases fecundity (Fig. 1.3).

Numerous studies demonstrate different approaches to put flies on diet (reviewed in Tatar, 2007). A design with only dilution of the concentration of nutrient yeast while holding the sugar concentration constant was able to extend life span. It can be concluded that it was sufficient to limit a specific nutrient component from yeast rather than caloric intake per se to retard fly sencescence (Mair et al., 2005). Thereby essential amino acids are mediating most of the response (Grandison et al., 2009). Importantly flies do not compensate the nutrient dilution by increased food intake (Wong et al., 2009).

We wanted to examine the role of miR-277 for life span under different dietary regiments. For this we set up life span analysis series of male flies overexpressing miR-277 (that means downregulation during life was impaired by transgenic expression of miR-277 with a constitutive promoter) and chronicled the survivors compared to control flies. I chose the diet design applied in the work of Mair et al.(Mair et al., 2005).

Grandison et al. were able to show that an amino-acid imbalance explains the extension of life span by DR in *Drosophila*. Lifespan was decreased by the addition of amino acids, with an interaction between methionine and other essential amino acids having a key role. Hence, an imbalance in dietary amino acids away from the ratio optimal for reproduction shortens life span during full feeding and limits fecundity during DR.

Previous observations in yeast suggested that the branched-chain amino acids (BCAAs)

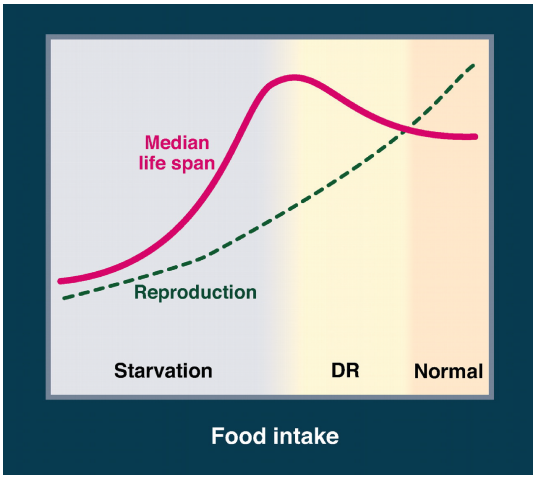

Figure 1.3: The median life span and fecundity of higher eucaryotes are negatively affected by a very low food intake. However, life span but not fecundity is optimized by dietary restriction (DR). (adapted from Fontana et al. (2010))

leucine, isoleucine and valine might be potential candidates in promoting survival (Alvers et al., 2009). Furthermore it was recently demonstrated by D'Antona et al. that long-term dietary supplementation with a specific BCAA-enriched amino acid mixture (BCAAem) increased average lifespan of male mice.

It has already been published that miR-277 has a predicted role for downregulation of the enzymes of the degradation pathway of the BCAAs (Stark et al., 2003; see Fig. 1.4). As I wanted to know which genes are responsible for the short-lived phenotype of the miR-277 overexpressing flies I tested on the one hand the samples of the life span analysis series for effects of miR-277 overexpression (male flies with constitutive expression of miR-277 on day 1 and that day x when 50% of the cohort were dead) and on the other hand I profiled mRNA levels of *Drosophila* Schneider S2 cells after inhibition of miR-277. Genes classified as valine metabolic process (GO:0006573), leucine metabolic process (GO:0006551) and branched-chain family amino acid metabolic process (GO: 0009081) were significantly downregulated upon miR-277 overexpression. By using a pulse labelling technique of newly synthesized RNA after inhibition of miR-277 I was able to confirm that miR-277 targets directly the enzymes of the degradation pathway of branched-chain amino acids.

The human autosomal recessive metabolic disorder Maple syrup urine disease (MSUD) that is caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKDC), is characterized by a buildup of the BCCAs (leucine, isoleucine, and valine) and α-keto acids in the blood and urine.

As mentioned before, Pletcher et al. characterized RNA transcript levels for the whole *Drosophila* genome during normal ageing; they compared age-dependent profiles from animals aged under full-nutrient conditions with profiles obtained from animals maintained on a low-calorie medium. They wanted to determine if caloric restriction (CR) slows the ageing process. Indeed, caloric restriction was accompanied by a slowing of the progression of normal, age-related changes in transcript levels. CR resulted in downregulation of genes primarily involved in cell growth, metabolism, and reproduction. For expample genes classified as DNA repair proteins (GO:0006281) and DNA replication factors (GO:0006260), along with genes whose products are involved in cell cycle control, DNA replication, chromosome condensation, chromosome segregation, and other cell cycle processes (GO:0007049). Localization of products from downregulated genes strongly identified the nucleus as a primary site of downregulation. Many developmental processes were significantly repressed in the reduced-calorie environment. Protein metabolism and ubiquitin-dependent protein degradation (GO:0006511) were downregulated, despite protein biosynthesis (GO:0006412) that was significantly unchanged. Upregulation was much less focused, with a small number of genes involved in response to external stimulus showing significant diet-mediated expression.

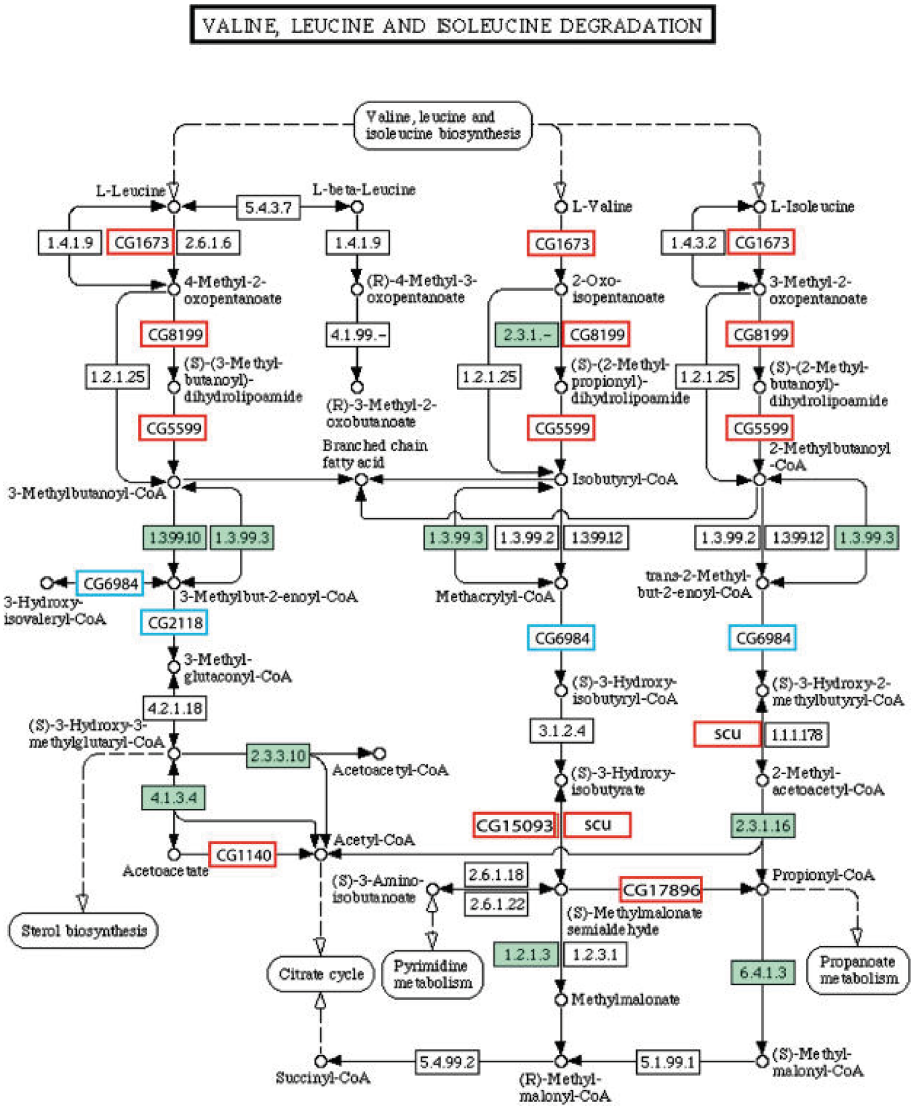

Figure 1.4: **Valine, leucine and isoleucine catabolic pathway.** Enzymes identified as miR-277 targets are boxed and identified by CG number. In addition to the predicted targets, the other enzymes for which the gene has been identified in *Drosophila* are shaded in green. The metabolic pathway chart is from www.genome.ad.jp/kegg/pathway/map/map00280.html. (adapted from Stark et al. (2003))

Based on these results I want to demonstrate the role of nutrient-sensing pathways in mediating the beneficial effects of dietary restriction with the help of two examples: the insulin/insulin-like growth factor-like (IIS) signalling pathway and the target of rapamycine (TOR) pathway.

1.4 Role of nutrient sensing pathways: The TOR (target of rapamycin)-pathway and insulin/IGF (IIS) signaling

Nutrient-sensing pathways are fundamental to the ageing process. Different nutrients in conditions of dietary restriction (DR) or others can activate different pathways directly or indirectly (Fontana et al., 2010). Nutrition modulates the current level of susceptibility to fail in response to underlying systems that degenerate with age (Tatar, 2007). Dietary restriction partially inactivates one or several nutrient signaling pathways through the reduced levels of growth factors such as IGF-1, thereby causing life span extension in model organisms. The reason why these pathways are inactivated or partially inactivated by reduced nutrients is apparently simple: During periods of food scarcity, cells and organisms must be able to enter a standby mode in which cell division and reproduction are halted or minimized to allow energy to be available to maintenance systems. The conserved composition and function of anti-ageing pathways in the different organisms indicate that most species have developed anti-ageing systems to overcome periods of starvation (Fig. 1.5).

Involvement of pathways in dietary restriction response is usually tested by generating mutant animals or screening mutant animals to determine if a genetic mutation alters the response. The focus of current work is on candidate genes, testing whether misexpression reduces the longevity gain induced by DR in matched wildtype controls. When they do, functions associated with the gene are considered to overlap with mechanisms by which diet modulates ageing. This approach has been applied to genes that retard ageing when misexpressed. The mutations that extend life span decrease for example the activity of nutrient-signaling pathways, suggesting that they may induce a physiological state similar to that resulting from periods of food shortage.

Reduced activity of IIS and TOR can extend lifespan, by protecting against life span shortening by increased food intake in *Drosophila* (Bjedov et al., 2010; Kapahi et al., 2004; Piper et al., 2008). The role of TOR in promoting ageing seems to be also conserved in yeast, worms and mice (Fig. 1.5).

There are a number of studies that have tested the interaction of mutations in the IIS pathway that extend lifespan (Clancy et al., 2002; Giannakou et al., 2008; Min et al., 2008). The mutant lines are in all cases long-lived in a food concentration-dependent manner. The *Drosophila* genome contains seven genes encoding *Drosophila* insulin-like peptides (dilps), that are predicted structurally to be similar to human insulin, and genetic deletion of three of them made in neuroendocrine cells in the brain extends life span (Grönke et al., 2010). The response to dietary restriction may be mediated by Dilps. Prominent among these approaches is also $chico^1$. Flies with a mutant allele $chico^1$, the *Drosophila* orthologue of the insulin receptor substrate, are less than half the size of wild-

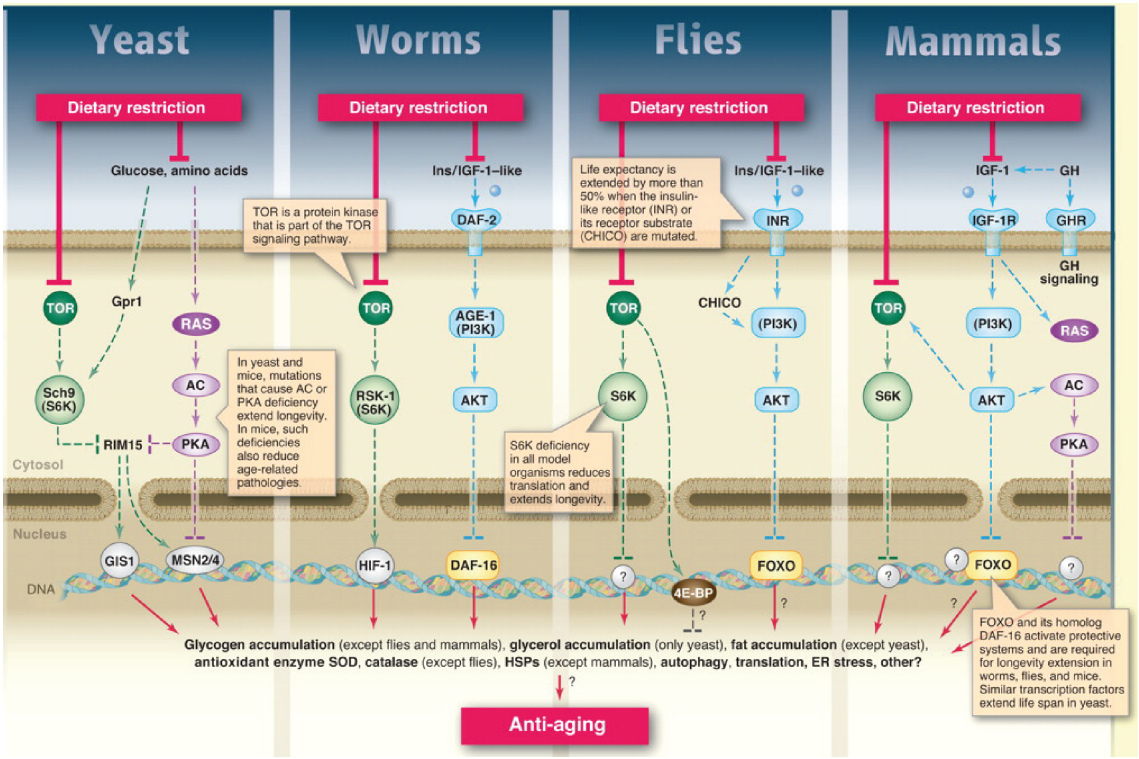

Figure 1.5: **A model for the conserved nutrient signaling pathways that regulate longevity in various organisms and mammals.** In flies DR reduces the activity of various signal transduction pathways indirectly through the reduced levels of growth factors such as IGF-1. The role of TOR and S6K in promoting ageing appears to be conserved in yeast, worms, flies and mice. Insulin/IGF-1-like receptor accelerates ageing in worms, flies and mice. In flies, reduced activity of both Ins/IGF-1 and TOR can protect against shortening of life span by increased food intake, although deletion of FOXO shortens life span, but the animal continues to respond to DR. (adapted from Fontana et al. (2010))

type flies, have elevated lipid levels and a reduced age-dependent mortality (Clancy et al., 2002). Thus, *chico*[1] induces a stage equivalent to submaximal, DR-induced slowing of ageing.

Identification of the tissues mediating the response to reduced IIS activity, by overexpression of the FOXO transcription factor, which is negatively regulated by IIS, has implicated tissues similar to those in *C. elegans*, namely, the fat body (equivalent of mammalian white adipose tissue and liver) and/or gut.

The TOR pathway is activated by several signals, but amino acid levels (in particular the availability of the branched-chain amino acid leucine) may be the primary efferent (Stanfel et al., 2009).An essential step of the activation appears to be membrane recruitment of Rheb and the Ragulator complex to the surface of lysosomes (Sancak et al., 2010). A continous import of leucine, coupled to efflux of glutamine, is required for sustained activation of TORC1 (Nicklin et al., 2009), but the direct sensor of leucine concentration is currently unknown.

Down-regulation of the TOR pathway activity by rapamycin extends life span in yeast and *C. elegans*; there exist controversal results about the effect of rapamycin in *Drosophila* (Bjedov et al., 2010; Harrison et al., 2010). Extension of life span by rapamycin requires reduced activity of the two different downstream targets of TOR: S6K1 (S6 kinase 1) and eukarytic initiation factor 4E (eIF4E) binding protein (4E-BP). Phosphorylation by TOR inhibits the activity of 4E-BP, which enables the mRNA cap-binding protein eIF4E to bind mRNAs and this leads to increased translation and shortened lifespan. Also a genetic downregulation of TOR, as shown by Kapahi et al., through ubiquitous upregulation of the negative regulators of TOR activity, dTsc1 and dTsc2, or expression of a dominant-negative TOR variant or a mutation in the downstream kinase dS6K increased *Drosophila* lifespan by up to 24%. Kapahi et al. also looked at the tissue specifity of the reduced TOR signalling for increased longevity. They found that nervous-system-specific reduction of TOR activity had no effect on lifespan, whereas reduction of TOR signalling by increased expression of dTsc2 in the muscle and fat, or muscle, fat and gut did.

It is likely that both TOR and IIS function together in the adult fat body to regulate lifespan. Its known that deletion of *Drosophila* FOXO shortens life span, but the flies continue to respond to dietary restriction (Giannakou et al., 2008; Min et al., 2008). The IIS and TOR pathways interact at the level of PKB, which phosphorylates Tsc2, in addition to dFOXO, thereby acitvating the TOR pathway (Fig. 1.6). Thus loss of FOXO can be compensated by the TOR pathway. Géminard et al. showed that the larval fat body couples the level of circulating Dilps with dietary amino acid levels by remotely controlling Dilp release through a TOR-dependent mechanism. A humoral signal emitted by the fat body transits through the hemolymph and activates Dilp secretion in the IPCs (brain insulin-producing cells). This is also a hint that ageing may be promoted within the cells in which the pathway genes are expressed but also in other cells through the regulation of circulating factors (Fig. 1.7).

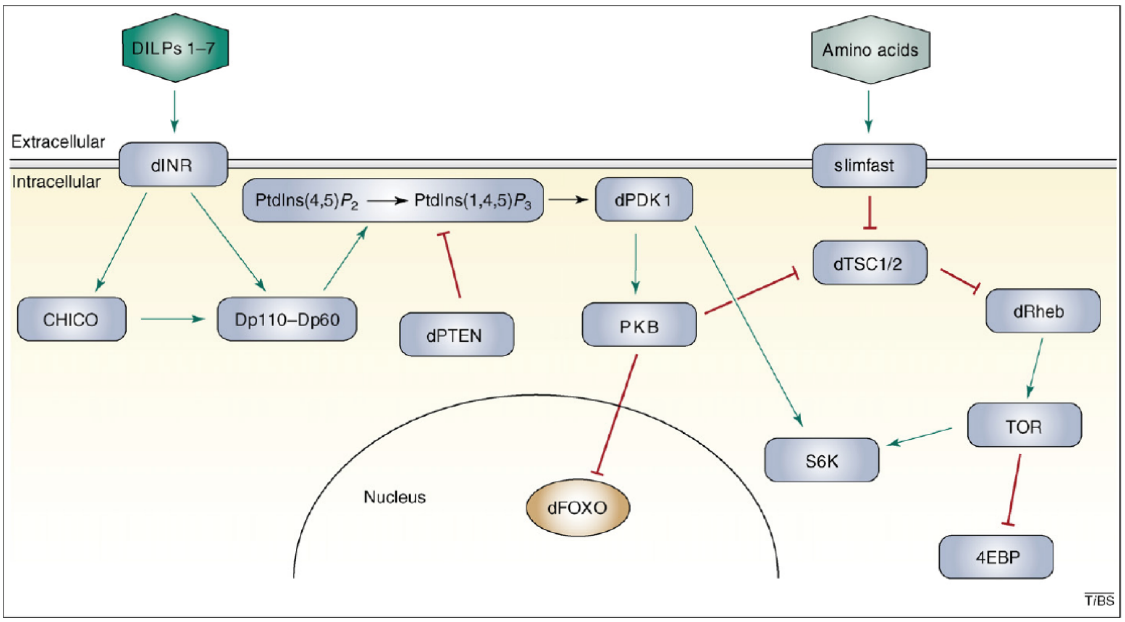

Figure 1.6: **The *Drosophila* IIS and TOR pathway.** Shown are the known components of the *Drosophila* IIS and TOR pathway. For example Chico (insulin receptor substrate), dFoxo (transcription factor), TOR kinase, S6 kinase (phosphorylates the small ribosomal subunit S6), 4EBP (eukaryotic initiation factor 4E-binding protein), PKB. (adapted from Giannakou and Partridge (2007))

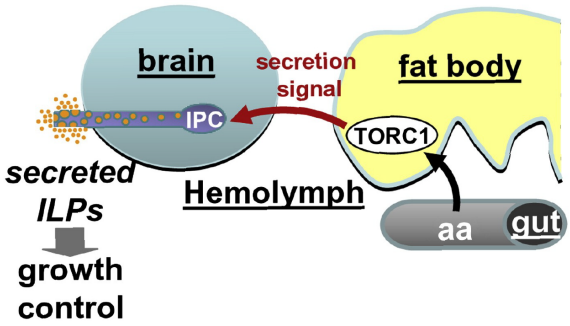

Figure 1.7: **A humoral link between the fat body and the brain remotely controls insulin secretion.** Amino acids are delivered by the gut and taken up by fat body cells. In the presence of amino acids in the diet, Torc1 signaling in fat cells generates a positive messenger that is released into the hemolymph. This signal reaches the brain IPCs, where it remotely controls Dilp secretion. (adapted from Géminard et al. (2009))

2 Material and Methods

2.1 Materials

2.1.1 Laboratory hardware

ABI PRISM 7000 qPCR cycler Applied	Biosystems; Foster City, USA
Agarose gel running chamber (H1-Set)	Carl Roth GmbH; Karlsruhe, Germany
Flow buddy CO_2 -distributer	Genesee Scientific; San Diego, USA
Fly anesthetic pad/pistol	Genesee Scientific; San Diego, USA
INTAS UV Imaging System	INTAS; Göttingen, Germany
LAS 3000 mini Western Imager	Fujifilm; Tokyo, Japan
Leica MZ7 stereomicroscope	Leica Microsystems; Wetzlar, Germany
Magnetic Stand	Miltenyi Biotec GmbH; Bergisch Gladbach, Germany
PAGE-electrophoresis material	BioRad; Hercules, USA
Power supply for electrophoresis	BioRad; Hercules, USA
Rotanta 460R centrifuge	Hettich; Tuttlingen, Germany
Semi-dry blotter	BioRad; Hercules, USA
SpectroLinker XL1500 UV Crosslinker	Spectronics Corporation; Westbury, USA
SterilGARD cell culture workbench	The Baker Company; Sanford, USA
Super Roller	Kisker; Steinfurt, Germany
Table top centrifuge (5417R and 5415R)	Eppendorf AG; Hamburg, Germany
Tank-blotting chamber	BioRad; Hercules, USA
Thermocycler	Eppendorf AG; Hamburg, Germany
Typhoon 9400 Variable Mode Imager	GE Healthcare; Freiburg, Germany

2.1.2 Analysis software

Multi Gauge V3.0	Fujifilm; Tokyo, Japan
OriginLab	Additive GmbH; Friedrichsdorf, Germany
R, Bioconductor	Fred Hutchinson Cancer Research Center; Seattle, USA
BOWTIE	Hartig et al. (2009)
PERL	Hartig et al. (2009)

2.1.3 Laboratory chemicals

Acrylamide 40%	Carl Roth GmbH; Karlsruhe, Germany
Agarose	Biozym Scientific GmbH; Oldendorf, Germany
Amino acids	Sigma Aldrich; Taufkirchen, Germany
Ampicillin	Carl Roth GmbH; Karlsruhe, Germany
APS (ammonium peroxodisulfate)	Carl Roth GmbH; Karlsruhe, Germany
BactoTM Agar	Becton, Dickinson; Franklin Lakes, USA

Bradford Assay	BioRad; Hercules, USA
Biotin-HPDP	Pierce, Thermo Fisher Scientific; Waltham, USA
BSA (bovine serum albumin)	Fermentas; St. Leon-Rot, Germany
Chloroform	Merck Biosciences GmbH; Schwalbach, Germany
Complete ®without EDTA	Roche Diagnostics; Mannheim, Germany
Dimethylformamide (DMF)	Pierce, Thermo Fisher Scientific; Waltham, USA
Desoxyribonucleotides	Sigma Aldrich; Taufkirchen, Germany
DTT (dithiothreitol)	Carl Roth GmbH; Karlsruhe, Germany
Ethanol (p.a.)	Merck Biosciences GmbH; Schwalbach, Germany
Ethanol (tech.)	VWR; Ismaning, Germany
Fetal bovine serum (FBS)	Thermo Fisher Scientific; Waltham, USA
Fugene ®HD transfection reagent	Roche Diagnostics GmbH; Mannheim, Germany
H_2O HPLC quality	VWR; Ismaning, Germany
Hepes	Carl Roth GmbH; Karlsruhe, Germany
Isopropanol (p.a.)	Merck Biosciences GmbH; Schwalbach, Germany
Methanol (p.a.)	Merck Biosciences GmbH; Schwalbach, Germany
Methanol (tech.)	VWR; Ismaning, Germany
Nipagin	Sigma Aldrich; Taufkirchen, Germany
PhosSTOP	Roche Diagnostics; Mannheim, Germany
Powdered milk	Rapilait Migros; Zürich, Switzerland
Propionic acid	Carl Roth GmbH; Karlsruhe, Germany
RiboLock RNase Inhibitor	Fermentas; St. Leon-Rot, Germany
Roti ®Aqua Phenol/C/I	Carl Roth GmbH; Karlsruhe, Germany
SDS (sodium dodecyl sulfate)	Merck Biosciences GmbH; Schwalbach, Germany
Syber Safe/Gold	Invitrogen; Karlsruhe, Germany
TEMED	Carl Roth GmbH; Karlsruhe, Germany
4-Thiouridine (T4509, 100 mg)	Sigma Aldrich; Taufkirchen, Germany
Triton X-100	Sigma Aldrich; Taufkirchen, Germany
Trizol	Invitrogen; Karlsruhe, Germany
Tween 20	Carl Roth GmbH; Karlsruhe, Germany

Other standard laboratory chemicals were obtained from the in-house supply system.

2.1.4 Radiochemicals

[γ^{32}P] ATP (SRP 501) 10 mCi/ml; 6000 Ci/mmol; 250 μCi
Hartmann Analytic; Braunschweig, Germany

2.1.5 Enzymes

2.1.5.1 General Enzymes

FastAP Thermosensitive Alkaline Phosphatase	Fermentas; St. Leon-Rot, Germany
Polynucleotidekinase (PNK) with Buffer A	Fermentas; St. Leon-Rot, Germany
Proteinase K	Fermentas; St. Leon-Rot, Germany
RNAse A	Fermentas; St. Leon-Rot, Germany
T4-DNA Ligase	New England Biolabs; Ipswich, USA

2.1.5.2 Polymerases

Pfu DNA Polymerase	Fermentas; St. Leon-Rot, Germany
Phusion Hot Start DNA Polymerase	Finnzymes via New England Biolabs
Superscript II, Reverse Transcriptase	Invitrogen; Karlsruhe, Germany
Taq DNA Polymerase	laboratory stock

2.1.5.3 Restriction Enzymes

SpeI (BfuCI)
HinPI
MspI
BamHI
NotI
XbaI
Fermentas; St. Leon-Rot, Germany and New England Biolabs; Ipswich, USA

2.1.6 Kits

CloneJet PCR Cloning Kit (TA-cloning)	Fermentas; St. Leon-Roth, Germany
DyNAmo Flash SYBR Green qPCR Kit	Finnzymes via New England Biolabs
μMacs Streptavidin Kit (130-074-101)	Miltenyi Biotec GmbH Bergisch Gladbach, Germany
miScript SYBR Green PCR Kit	Qiagen; Hilden, Germany
QIAGEN Gel extraction Kit	Qiagen; Hilden, Germany
QIAGEN miRNeasy Mini-Kit	Qiagen; Hilden, Germany
QIAGEN RNeasy MinElute Cleanup Kit	Qiagen; Hilden, Germany
QIAGEn PCR Cloning Kit	Qiagen; Hilden, Germany
QIAGEN PCR Purification Kit	Qiagen; Hilden, Germany
QIAGEN Plasmid Midi Kit	Qiagen; Hilden, Germany
QIAGEN Plasmid Mini Kit	Qiagen; Hilden, Germany

2.1.7 Other materials

α-Flag affinity Agarose (A2220)	Sigma Aldrich; Taufkirchen, Germany
Blotting paper	Machery-Nagel; Düren, Germany
Cell culture materials	Bio & Sell; Nürnberg, Germany Sarstedt; Nümbrecht, Germany
ECL substrate	Thermo Fisher Scientific Waltham, USA
GeneChip Drosophila Genome 2.0 Array	Affymetrix; Santa Clara, USA
Gene Ruler DNA Ladder Mix	Fermentas; St. Leon-Rot, Germany
Infinity Triglycerides Liquid Stable Reagent	Thermo Fisher Scientific; Waltham, USA
Nitrocellulose membrane (Protan BA 83)	Schleicher & Sch üll; Dassel, Germany
Nylon membrane, positively charged	Roche Diagnostics GmbH Mannheim, Germany
Parafilm	Carl Roth GmbH; Karlsruhe, Germany
Phase Lock Gel Heavy Tubes	Eppendorf AG; Hamburg, Germany
Phosphoimager Screen	Fujifilm; Tokyo, Japan
Pistils for fly lysis	Sigma Aldrich; Taufkirchen, Germany
Polyvinylidenfluoride (PVDF) membrane	Milipore; Billerica, USA
Prestained Protein Ladder	Fermentas; St. Leon-Rot, Germany
Protein G Plus/Protein A Agarose beads	Calbiochem via Merck
qPCR plates	Biozym Scientific GmbH Oldendorf, Germany
RestoreTM Western Blot Stripping Buffer	Thermo Fisher Scientific Waltham, USA
Sephadex spin column (G25)	Roche Diagnostics GmbH; Mannheim, Germany
Spin column for IP	MoBiTec; Göttingen, Germany
SuperSignal West Dura Extended Duration	Thermo Fisher Scientific; Waltham, USA Waltham, USA
Whatman 595 ®Folded Filters	Whatman GmbH; Dassel, Germany

2.1.8 Plasmids

Plasmid	**Description**	**Reference**
pKF63	constitutive myc-GFP expression; ubiquitin promotor	Förstemann and Lingner (2005)
pKF67	constitutive myc-GFP expression; ubiquitin promotor; miR-277 dependent regulation (2 perfect matches)	Förstemann et al. (2007)

pKF68	constitutive myc-GFP expression; ubiquitin promoter; miR-277 dependent regulation (4 buldged matches)	Förstemann et al. (2007)
pKF84	constitutive pre-miR-277 expression (fragment of mature miR-277); ubiquitin promotor	Förstemann et al. (2007)
pUASP	conditional expression under Gal4-control	Rørth (1998)
pPTGal-4	Gal4-controlled - expression vector	Sharma et al. (2002)
pSE1	conditional expression of miR-277 under Gal4-control	S. Esslinger
pSE2	conditional expression of miR-34 under Gal4-control	S. Esslinger
pSE3	Gal4-4Kb miR-277 promoter controlled expression vector	S. Esslinger
pSE4	Gal4-2Kb miR-277 promoter controlled expression vector	S. Esslinger
pKM3	constitutive expression of miR-277; mutated *strand; 'super-miRNA-like' ; ubiquitin promotor	K. Michalik
pKM4	constitutive expression of miR-277; mutated *strand; 'siRNA-like'; ubiquitin promotor	K. Michalik

2.1.9 Cells

2.1.9.1 Bacterial stocks

XL2-blue CaCl2-competent cells
laboratory stock

2.1.9.2 Cell lines

Cell line	Description	Comments
S2B2	parental cell line	laboratory stock
63N1	myc-GFP; no miRNA binding sites	endo-siRNA cell culture reporter Hartig et al. (2009)

2.1.10 Fly stocks

In the following table #BL refers to Bloomington Stock Center number, #VDRC refers to Vienna Drosophila RNAi Center number and #LSL refers to laboratory stock list number.

For genotype information see laboratory stock list, http://flystocks.bio.indiana.edu/ (Bloomington Stock Center) or http://stockcenter.vdrc.at/control/main (Vienna Drosophila RNAi Center).

For each construct were generated several independent transgenic fly lines with different P-element insertions. Lines in the table (generated by K. Förstemann or S. Esslinger) marked with the laboratory stock number were used for experiments.

#BL #VDRC #LSL	**Description**	**Origin**
BL189	*yw*, recessive *white* mutation	Bloomington Stock Center
BL6326	w^{1118}, recessive *white* mutation	Bloomington Stock Center
BL10738	p-element insertion in *chico*	Bloomington Stock Center
BL4533	p-element insertion for actin-GFP expression; cyo marker	Bloomington Stock Center
BL1521	conditional expression of GFP under Gal4 control	Bloomington Stock Center
BL6280	conditional expression of RFP under Gal4 control	Bloomington Stock Center
BL7732	deficiency for miR-277, miR-34 and miR-317	Bloomington Stock Center
BL7199	double balancer	Bloomington Stock Center
BL5138	tubulin-Gal4 driver line	Bloomington Stock Center
BL3954	actin5C-Gal4 driver line	Bloomington Stock Center
BL6984	fat body-Gal4 driver line	Bloomington Stock Center
BL8169	oenocytes-Gal4 driver line	Bloomington Stock Center
BL8760	nervous system-Gal4 driver line	Bloomington Stock Center
LSL 265 and 257 (VDRC)	conditional expression of hairpin of CG5599 under Gal4 control	Vienna Drosophila RNAi Center
LSL 263 (VDRC)	conditional expression of hairpin of CG5599 under Gal4 control	Vienna Drosophila RNAi Center
LSL 256 (VDRC)	conditional expression of hairpin of CG6984 under Gal4 control	Vienna Drosophila RNAi Center
LSL 260 (VDRC)	conditional expression of hairpin of CG1673 under Gal4 control	Vienna Drosophila RNAi Center
LSL 261 (VDRC)	conditional expression of hairpin of CG1140 under Gal4 control	Vienna Drosophila RNAi Center

fmr1 delta2 LSL 209	*fmr1* null mutation	T. Jongens
fmr1 delta3 LSL 39	*fmr1* null mutation	T. Jongens
63-L10 LSL 124	myc-GFP	K. Förstemann
67-L1 LSL 125	myc-GFP; 2x perfect match miR-277 reporter	K. Förstemann
68-L6 LSL 140	myc-GFP; 4x bulged match miR-277 reporter	K. Förstemann
84-3 LSL 107	constitutive pre-miR-277 expression (part of mature miR-277); *yw* - background	K. Förstemann
84-3 LSL 90	ubiquitous constitutive pre-miR-277 expression (part of mature miR-277); w^- - background	S. Esslinger
SE1 LSL 220 and 224	conditional expression of miR-277 under Gal4-control	S. Esslinger
SE2	conditional expression of miR-34 under Gal4-control	S. Esslinger
SE3 LSL 41, 42	Gal4-4Kb-miR-277-promotor driver line	S. Esslinger
SE 4 LSL 209, 228	Gal4-2Kb-miR-277-promotor driver line	S. Esslinger
KM3	ubiquitous constitutive expression of miR-277; mutated *strand; 'super-miRNA-like'	S. Esslinger
KM4 LSL 100	ubiquitous constitutive expression of miR-277; mutated *strand; 'siRNA-like'	S. Esslinger

2.1.11 PCR oligonucleotides

2.1.11.1 Cloning

Name	**Sequence 5' - 3'**
upst_277_as_A	GGATGCATTTTATCGTTAGGCCTC
ups_277_as_B	CATAAATCATATTCGGCACGGC
mir277ups1 fwd	ATCCTAGGTGGCAGACTGCATAACTCATC
mir277ups2 fwd	ATCCTAGGTAAAGAATCGAATAGCAAACA
mir277pr rev	ATCCTAGGGCTGCGTATACGCGATGTGTC
miR277Not_s	GTGCGGCCGCATACATATATAACGAGGCCTAACG
miR277Xba_as	CGTCTAGAAAAAACAGTGTCTTACAAACAAGTGG
miR34Not_s	GTGCGGCCGCGTAACCGTTACACACGACTATTCT
miR34Xba_as	CGTCTAGAATTATAGATATGATAACTACTGCC
mir34 hp NotI s	ATGCGGCCGCTCAAGGAGAATTAACCAAATTAGTGA ATTAATCCAAAC
mir34 hp Bam as	ATGGATCCTCCGATGTGATTATGAGCCAGTTCC
BamH 277 4kb as	ATGGATCCATTCGGCACGGCGCGGCGTAAG
Not 277 4kb s	ATGCGGCCGCACATCATCGTTGGCGGCATTCTGTG
4Kb277Pr3547out	TATCTGCCCCTTGGAAAGTG
4Kb277Pr373out	TATTGACGGTATCGCAACCC

2.1.11.2 qPCR

name	**sequence 5' - 3'**
hsp70 s	AGGACTTTGACAACCGGCTA
hsp70 as	ACAGTGCGTCAATCTCGATG
dcr-2 s	GTTCCGCTTTGGTCAACAAT
dcr-2 as	GGCTGAACATCAGCTTCCTC
white s	CTAATATCCTGCGCCAGCTC
white as	ACGGAACCATGAGAGGTACG
GFP s	ACGTAAACGGCCACAAGTTC
GFP as	AAGTCGTGCTGCTTCATGTG
GAPDH s	CTTCTTCAGCGACACCCATT
GAPDH as	ACCGAACTCGTTGTCGTACC
297 s	Chung et al. (2008)
297 as	Chung et al. (2008)
mdg1 s	Chung et al. (2008)
mdg1 as	Chung et al. (2008)
rp49 s	Chung et al. (2008)

rp49 as	Chung et al. (2008)
upiquitin promoter s	GCCGGTAGAGAAGACAGTGC
ubiquitin promoter as	ACTGACTTGACCGGCTGAAT
CG5599 s	CTCCCGGTACTAACGTTCCA
CG5599 as	TTGCATCAACTGGGTCATGT
CG1673 s	ATGAACATGAACCGCATGAA
CG1673 as	GGCTGAGGATCGTGTAGAGC

MicroRNA Profiling; Integrated DNA Technologies; Coralville, USA

Pos.	**Name**	**Sequence 5' - 3'**
A1	scrambled_bantam	AGTGCTAGTATTTACAGCTATAT
A2	dme-bantam	TGAGATCATTTTGAAAGCTGATT
A3	dme-let-7	TGAGGTAGTAGGTTGTATAGT
A4	dme-miR-1	TGGAATGTAAAGAAGTATGGAG
A5	dme-miR-1	TGGAATGTAAAGAAGTATGGAG
A6	dme-miR-10	ACCCTGTAGATCCGAATTTGT
A7	dme-miR-10*	AAATTCGGTTCTAGTGTGGTT
A8	dme-miR-1002	TTAAGTAGTGGATACAAAGGGCGA
A9	dme-miR-1003	TCTCACATTTACATATTCACAG
A10	dme-miR-1012	TTAGTCAAAGATTTTCCCCATAG
A11	dme-miR-1017	GAAAGCTCTACCCAAACTCATCC
A12	scrambled_dme-miR-184	AGTAGCGAGATGACATGCGGAC
B1	dme-miR-11	CATCACAGTCTGAGTTCTTGC
B2	dme-miR-12	TGAGTATTACATCAGGTACTGGT
B3	dme-miR-124	TAAGGCACGCGGTGAATGCCAAG
B4	dme-miR-125	TCCCTGAGACCCTAACTTGTGA
B5	dme-miR-133	TTGGTCCCCTTCAACCAGCTGT
B6	dme-miR-13a	TATCACAGCCATTTTGATGAGT
B7	dme-miR-13b	TATCACAGCCATTTTGACGAGT
B8	dme-miR-14	TCAGTCTTTTTCTCTCTCCTA
B9	dme-miR-184	TGGACGGAGAACTGATAAGGGC
B10	dme-miR-184*	CCTTATCATTCTCTCGCCCCG
B11	dme-miR-193	TACTGGCCTACTAAGTCCCAAC
B12	dme-miR-219	TGATTGTCCAAACGCAATTCTTG
C1	dme-miR-252	CTAAGTACTAGTGCCGCAGGAG
C2	dme-miR-263a	GTTAATGGCACTGGAAGAATTCAC
C3	dme-miR-274	TTTTGTGACCGACACTAACGGGT

C4	dme-miR-275	TCAGGTACCTGAAGTAGCGCGCG
C5	dme-miR-276*	CAGCGAGGTATAGAGTTCCTACG
C6	dme-miR-276a	TAGGAACTTCATACCGTGCTCT
C7	dme-miR-276b	TAGGAACTTAATACCGTGCTCT
C8	dme-miR-277	TAAATGCACTATCTGGTACGACA
C9	dme-miR-278	TCGGTGGGACTTTCGTCCGTTT
C10	dme-miR-279	TGACTAGATCCACACTCATTAA
C11	dme-miR-281	TGTCATGGAATTGCTCTCTTTGT
C12	dme-miR-282	AATCTAGCCTCTACTAGGCTTTG
D1	dme-miR-284	TGAAGTCAGCAACTTGATTCCAG
D2	dme-miR-285	TAGCACCATTCGAAATCAGTGC
D3	dme-miR-286	TGACTAGACCGAACACTCGTGCT
D4	dme-miR-289	TAAATATTTAAGTGGAGCCTGCG
D5	dme-miR-2a	TATCACAGCCAGCTTTGATGAGC
D6	dme-miR-2b	TATCACAGCCAGCTTTGAGGAGC
D7	dme-miR-2c	TATCACAGCCAGCTTTGATGGGC
D8	dme-miR-3	TCACTGGGCAAAGTGTGTCTCA
D9	dme-miR-305	ATTGTACTTCATCAGGTGCTCTG
D10	dme-miR-306	TCAGGTACTTAGTGACTCTCAA
D11	dme-miR-306*	GGGGGTCACTCTGTGCCTGTGC
D12	dme-miR-308	AATCACAGGATTATACTGTGAG
E1	dme-miR-309	GCACTGGGTAAAGTTTGTCCTA
E2	dme-miR-310	TATTGCACACTTCCCGGCCTTT
E3	dme-miR-311	TATTGCACATTCACCGGCCTGA
E4	dme-miR-312	TATTGCACTTGAGACGGCCTGA
E5	dme-miR-316	TGTCTTTTTCCGCTTACTGGCG
E6	dme-miR-317	TGAACACAGCTGGTGGTATCCAGT
E7	dme-miR-318	TCACTGGGCTTTGTTTATCTCA
E8	dme-miR-31a	TGGCAAGATGTCGGCATAGCTGA
E9	dme-miR-34	TGGCAGTGTGGTTAGCTGGTTGTG
E10	dme-miR-375	TTTGTTCGTTTGGCTTAAGTTA
E11	dme-miR-4	ATAAAGCTAGACAACCATTGA
E12	dme-miR-5	AAAGGAACGATCGTTGTGATATG
F1	dme-miR-7	TGGAAGACTAGTGATTTTGTTGT
F2	dme-miR-79	TAAAGCTAGATTACCAAAGCAT
F3	dme-miR-8	TAATACTGTCAGGTAAAGATGTC
F4	dme-miR-927	TTTAGAATTCCTACGCTTTACC

F5	dme-miR-92a	CATTGCACTTGTCCCGGCCTAT
F6	dme-miR-92b	AATTGCACTAGTCCCGGCCTGC
F7	dme-miR-932	TCAATTCCGTAGTGCATTGCAG
F8	dme-miR-956	TTTCGAGACCACTCTAATCCATT
F9	dme-miR-958	TGAGATTCTTCTATTCTACTTT
F10	dme-miR-965	TAAGCGTATAGCTTTTCCCCTT
F11	dme-miR-970	TCATAAGACACACGCGGCTAT
F12	dme-miR-977	TGAGATATTCACGTTGTCTAA
G1	dme-miR-980	TAGCTGCCTTGTGAAGGGCTTA
G2	dme-miR-981	TTCGTTGTCGACGAAACCTGCA
G3	dme-miR-984	TGAGGTAAATACGGTTGGAATTT
G4	dme-miR-986	TCTCGAATAGCGTTGTGACTGA
G5	dme-miR-987	TAAAGTAAATAGTCTGGATTGATG
G6	dme-miR-988	CCCCTTGTTGCAAACCTCACGC
G7	dme-miR-989	TGTGATGTGACGTAGTGGAAC
G8	dme-miR-992	AGTACACGTTTCTGGTACTAAG
G9	dme-miR-993	GAAGCTCGTCTCTACAGGTATCT
G10	dme-miR-994	CTAAGGAAATAGTAGCCGTGAT
G11	dme-miR-995	TAGCACCACATGATTCGGCTT
G12	dme-miR-996	TGACTAGATTTCATGCTCGTCT
H1	dme_mdg1	AACAGAAACGCCAGCAACAGC
H2	dme-miR-998	TAGCACCATGAGATTCAGCTC
H3	dme-miR-999	TGTTAACTGTAAGACTGTGTCT
H4	dme-miR-9a	TCTTTGGTTATCTAGCTGTATGA
H5	dme-miR-9b	TCTTTGGTGATTTTAGCTGTATG
H6	dme-miR-9c	TCTTTGGTATTCTAGCTGTAGA
H7	dme-CG4068_B	TTGACTCCAACAAGTTCGCTC
H8	dme-2S-rRNA	ACTACATATGGTTGAGGGTTG
H9	dme-tRNA-CR32359	CGTGGGTTCGAATCCCACTTC
H10	dme_snRNA_U6	CAAAATCGTGAAGCGTTCCAC
H11	dme_RP49	ATCGGTTACGGATCGAACA
H12	as_dme_2S-rRNA	CAACCCTCAACCATATGTGT

MRNA-'Ageing'-Profiling; Integrated DNA Technologies; Coralville, USA

Pos.	**Name**	**Sequence** $\frac{\textbf{sense}}{\textbf{antisense}}$
A1	GAPDH H.sapiens	ACCCAGAAGACTGTGGATGG TTCTAGACGGCAGGTCAGGT
A2	AttA	ACAATCTGGATGCCAAGGTC TCCCGTGAGATCCAAGGTAG
A3	cactin	GCGCTATATACGCGAAGAGG TGGTCTCCACACTGAACTCG
A4	CecC	CAGCATTGGACAATCGGAAG TTCCCAGTCCTTGAATGGTT
A5	CG10433	GAGAAGGAGCTTGCTGTGCT ACAATCCCTTGTTCGAGGTG
A6	CG10535	AAGAAGGAGACGCAGTTCCA CACTCTTTTCGGCTGTGTGA
A7	CG15678	AAGAGCACGAGCAGGGTAAA GTCTGGTGCTATTGCCGATT
A8	CG2736	TAGCACCCGACGTCCTTAAC GCGACGTTGGGTCTTATGTT
A9	CG31217	GGGGAAAACGACTGCTATGA TCTCCCTCCAGCACATATCC
A10	CG9733	AATCGGCGATATGTCCTCAC CGAATGAGACCAATGTCGTG
A11	PGK E. coli	GTCTGATGTTCGCGTAGCAA CGATAGCGTTAGCCACGATT
A12	PGK S. cerevisiae	TTCCAGAAAGGTCGATGGTC GTCTGGTTGGGTTCTCCAAA
B1	DptB	TGTGAATCTGCAGCCTGAAC ATAGGGTCCACCAAGGTGCT
B2	dro5	TGCAGATCAAGTTCCTGTACCT CTCGCACCAGCACTTCAGAC
B3	Drs	AGTACTTGTTCGCCCTCTTCG TTAGCATCCTTCGCACCAG
B4	fon	GCCAGAACTTCCAGCAGAAC GGAGCTGGCAGAGTTCAAAG
B5	GNB-P2	CCCGGCTCTAATGGACTACA CACTCGCCCTCTCCTGTAAG

B6	IM10	GCATGTGTACACCGATGAGG GGCCACGCTGAATGTAGAAT
B7	IM4	CCCTTCTCTTGGCCATGTT GTACGAATGTACTGGGTGTTGTC
B8	LysX	GGAATCTGTGTCCTGGCACT TGGCACCAGTACATGTCGTT
B9	Mtk	TACATCAGTGCTGGCAGAGC AATAAATTGGACCCGGTCTTG
B10	nec	TCTCCTGCTAACCGTCCATC ACCACATCGCTTATGGCTTC
B11	PEK	AATCTCGTCCAGCATCCATC AACGGAAACACCGTATGAGC
B12	PGRP	CCCTGACCAACTCCACAGAT GTTGGGCAGAAAAGGACAAA
C1	Rel	TACAAGAGCGAGATGCATGG ATAAATTGCGCCACATAGCC
C2	Snmp	TCATTTTCAACCCGAAGGAG CTTCCGAGGAGCAATCTACG
C3	Sp7	GCTGCCTTTCACCGTTCTAC ATCCAAAGCTGCGATCACTT
C4	Spn27A	GATCCGTTCTCATGGCATCT GTCTCGTGGAGCTGGTTCTC
C5	TepII	TGGATCAGAGTGTCCTGCTG ATATCTTGGGCGGGTAATCC
C6	TepIV	GAAACGAAACGTGTGGACCT TCTTGATCTCCAGTGCAACG
C7	Thor	CCATGATCACCAGGAAGGTT ACTTGCGGAAGGGAGTACG
C8	Tsf1	CCATTTATCGCCTGTGTGTG GCGATAGTCCTCGTTCTTGC
C9	ATPsyn	GGTCTGTCGCTCGGTATCAT CAGCTGCAATGCAATGTTCT
C10	blw	GACTGGTAAGACCGCTCTGG GGCCAAGTACTGCAGAGGAG
C11	CoVa	AGGAGTTCGACAAGCGCTAC ATAGAGGGTGGCCTTTTGGT
C12	cype	ACACTCCAGCCACCTCCTC CTAGCAGGACTGGAAACGAC

D1	Cyt-c	TTCTGGTGATGCAGAGAACG ACTCGTCCAAATTCCCCTCT
D2	Gdh	GCACACCACTCGCTATCTGA GGGAATGAAGATGTCGCACT
D3	GlyP	ACCTGCACTACACCCTGGTC CAACTGGTACATGGCCTCCT
D4	Gpdh	GCAAGCTGTCCACCTTCTTC TGTCCTCCAGACCCTTGTTC
D5	Gpo-1	ATACATCGATGCCGAGATCC GTTGACCGAGTGTCCCATCT
D6	kdn	AATGTGGGAGCCTATGTTCG ATACCCTCGTCAGCATCCAG
D7	levy	TCTGGTGGCTACAAGGTGTG GGGTTGTGGAACAGGCTCT
D8	mtacp1	GCCAGTTGAGCCAAAAGTTC AGCTTCTCGGCATCAGAGTC
D9	ND42	GTCTTCATGGAGGCCATGTT TGTACAGCTGCTCCAAATCG
D10	ND75	GCACCTACGTGGAGAAGCTC ATTTGTCAGCAAGCCATTCC
D11	Oscp	TCAATCTGTTGGGTCTGCTG GCGGGAAGTGATCTTCAGAG
D12	ox	TCTACAACACCCTGTTCAAGC CGTATTTGCCCTTGATGTCC
E1	Pdsw	GACGATCGACCAGTGCTACA CCCAAGTCGCCATACTTGAT
E2	Scsalpha	AGCCAACAGGCTCTGGAGTA ACACCCTCCGTAATGCAAAC
E3	Vha14	GACACCTGTGTGGGCTTTCT ATGGTCCTTGGAGGGAATCT
E4	l(1)G0156	GAGTACGCCTTCCAGTACGC ACCAGCACGTCGTATTTTCC
E5	CG3621	CGTGATGTGTCGCCTTTAAT CTGCTGCTCCACCAGGTC
E6	CG3446	GCCCAGAATGTCATCTTTCC GTGGGCGTAGAACTCCTTGA
E7	CG3192	CCGGAGGAGTACAAACCGTA CCAGGAAGCAGGCAAAGTAG

E8	CG18624	GGACTGGATAAGCGAGCACT GTCCCGGAACATGGTCATAC
E9	CG32230	GCAAGGTCTTGGTCTGCAA TGGGGCAGCACTCTTAGTTT
E10	CG5548	CTACATGAAACCGGACGTGA ACTCGCAGGTGAGGTACTCG
E11	CG9172	CTCCAAAAGGCGACTCAGAC AAGTCAGTGGCCAGATCGAG
E12	CG11455	ATGTCGCTTACCCCCTTTCT AGCCACTGCTTGTAGCGTTC
F1	CG3214	CCACGTCAACATGGACTACG CTACTGCTTCTTCGCCTTGG
F2	CG12400	TTTTCATCAACTGGGGCTTC CACCCAACTCTCCAGCACTT
F3	Foxo	GAACCAGTCGGAATGCAAGT ACCCTCATAAAGCGGTTGTG
F4	Tor	AGTCGCTTCCAAGTCAGCAT AAACATGCCCTTTACGTTGC
F5	S6K	CGGCACAATTGAGTACATGG GAGCTTGGCTTTCAGAATGG
F6	Akt	ATAGCAGCGGCGTTAAGAAA TGGACTTGAGCACACGACTC
F7	Tsc1	AAGCAGCCTGCCAGAAATTA GCACAGGAACCATTTCCACT
F8	chico	ATGAAGCCAGTCGGTAATGG TGGCGAGTTCGACTTCTTTT
F9	CG1640	CTCCAATTGGCAGGACATCT CTTCTTCGCCTCATCGTAGG
F10	CG8745	TGGGCGATTATCTTTTGGAG ACATGGGTGGCTTTAGCTTG
F11	CG10399	TTCAAAACGAGCCCAAACTC AGCACTCTCGAATCCCTTCA
F12	CG3267	GTGATAATCGGCGGTTCCTA AGCTTTTGGGCTTCTTCCTC
G1	CG6543	TCCTCCACCAACAACAACTG GAGTAGGTGTTGCCCACCAT
G2	CG15093	GTCTTCGCCGAGATCATCA TTGTTGCCCAGACCCTTATC

G3	CG6638	CTCATGTCGGGATTGGACTT CAATCTTTGGGACTGCGATT
G4	CG8199	TTTGAAGCAGATCGCAGTGT TCGAGTCTCTCGGTTCGTTT
G5	CG2188	CAAAATTGGCTTTCCCTTGA TCACCATACTGATCCGCAAA
G6	CG5599	CTCCCGGTACTAACGTTCCA TTGCATCAACTGGGTCATGT
G7	actin 2	GAGCGCGGTTACTCTTTCAC GCCATCTCCTGCTCAAAGTC
G8	tubulin	TACGAGAAGCCAGCCTTTGT CGTTCCAGTACGGCGATATT
G9	dynein	GGTGATCAAGAACGCTGACA TGGCCCAGGTAGAAATAGATG
G10	ncd	GGTGATCAAGAACGCTGACA TGGCCCAGGTAGAAATAGATG
G11	EGFR	CTATGCAAGTTGCGCATTGT CAGCAGATTAACGTGCTCCA
G12	Ras 64B	ATCCCACCATTGAAGACTCG ATCCTTGACGCGCAGTATCT
H1	H_2O	
H2	MapK/Erk	CTGGGCGAAACTATTTCCAA ATCAACGACTTCAGGGCATC
H3	sec5	GCTGCGATAATTTGGTTGGT TCTTGGCAGTCTCATTGACG
H4	sec15	ACGGAGAGGAGCGTATCTCA GAAGGCAAAGCTGTCAAAGG
H5	sec22	CTGGCCTTCAATTACCTGGA TCGTCAATGTTTTGCACCAT
H6	Elo68alpha	TCGGGTTAGCTTTGATCCAC ATGATGATGTGCACGAAGGA
H7	scu	CGTTCAATGTGATCCGTCTG GCATAGGCGTGTTGAACAAA
H8	Acox57D-d	TAGTTAAGGCATGGGCTTCG TGCACGAGTCAGTTCGATTC

H9	Rm62	TTGTGACGAGTTCAGCAAGG GCCACCAGAATGTTGGACTT
H10	Polo	CAGGTGCCAACAATGTGAAC GTGCGGAAGTTCTTCTCCTG
H11	Ago1	CAATTTGGAGGAAGCTCTCG TGAGCATCATCTTCCACTGC
H12	H_2O	

2.1.11.3 Mapping P-element insertions in transgenic flies

	Primer sequence 5' - 3'	**5' or 3' end**	**Annealing**
Plac4	ACTGTGCGTTAGGTCCTGTTCATTGTT	5' end	60°C
Plac1	CACCCAAGGCTCTGCTCCCACAAT		
Pry4	CAATCATATCGCTGTCTCACTCA	3' end	55°C
Pry1	CCTTAGCATGTCCGTGGGGTTTGAAT		
Pwht1	GTAACGCTAATCACTCCGAACAGGTCACA	5' end	60°C
Plac1	CACCCAAGGCTCTGCTCCCACAAT		

2.1.11.4 Fragments for dsRNA

Name	**Sequence 5' - 3'**
dsDsRed	CGTAATACGACTCACTATAGGAGGACGGCTGCTTCATCTAC
dsDsRed	CGTAATACGACTCACTATAGGTGGTGTAGTCCTCGTTGTGG
dsDcr-2	CTGCCCATTTGCTCGACATCCCTCC
dsDcr-2	TTACAGAGGTCAAATCCAAGCTTG

2.1.12 Media

2.1.12.1 Bacterial stocks

All *E. coli* strains were cultivated in LB-medium or in SOC-medium following transformation. Agarose plates were obtained from in-house supply.

- **SOB-medium**
  - 0.5% (w/v) yeast extract
  - 2% (w/v) Tryptone
  - 10 mM NaCl

  - 2.5 mM KCl
  - 10 mM $MgCl_2$
  - 10 mM $MgSO_4$
  - pH7

- **SOC-medium**
  - SOB-medium
  - 20mM glucose

- **LB-medium**
  - 1% (w/v) Tryptone
  - 0.5% (w/v) yeast extract
  - 1% (w/v) NaCl
  - pH 7.2

Antibiotics added to medium after autoclaving: 100 μg/ml ampicillin (100 mg/ml stock)

2.1.12.2 Cell culture

Cell culture medium and additives for *Drosophila* Schneider cells was obtained from Bio & Sell (Nürnberg, Germany) and supplemented with 10% temperature-inactivated Fetal Bovine Serum (FBS; Thermo Fisher; Waltham, USA).

2.1.13 Fly food

- **Standard fly food was obtained from in-house supply:**
  - 5.8% corn meal
  - 5.5% molasses
  - 2.4% yeast extract

- **Food for lifespan analysis (1l):**
  1. **Food with dietary restriction for sugar and for protein (LSLP):**
     - 10 g agar
     - 65 g sugar
     - 65 g yeast extract
     - 6.4 ml propionic acid

- 0.76 g in 5 ml nipagin in EtOH
- **according to analysis setup, additives per l:**
- OEAAs: His 5.3g, Phe 3.8g, Thr 6.2g
- BCAAs: Ileu 6g, Leu 3g, Val 3g
- glutamine: 6g
- methionine: Grandison et al. (2009)

2. **Food with dietary restriction for sugar, but excess protein (LSHP):**
   - 10 g agar
   - 65 g sugar
   - 150 g yeast extract
   - 6.4 ml propionic acid
   - 0.76 g in 5 ml nipagin in EtOH
3. **Food with excess sugar and protein (HSHP):**
   - 10 g agar
   - 150 g sugar
   - 150 g yeast extract
   - 6.4 ml propionic acid
   - 0.76 g in 5 ml nipagin in EtOH

2.1.14 Antibodies

2.1.14.1 Primary antibodies

Antibody	**Organism**	**Dilution**	**Reference/Catalog #**
α-phospho 4E-BP1	rabbit	1:1000	Cell Signaling #9459
α-beta- tubulin	mouse	1:2000	DSHB, E7

2.1.14.2 Secondary antibodies

Antibody	**Dilution**	**Origin**
Goat Anti-Rabbit IgG (H+L) HRP-coupled	1:50000	Pierce 31210
Goat Anti-Mouse IgG (H+L) HRP-coupled	1:100000	Pierce 31160

2.1.15 Stock solutions and buffers

- **Acrylamide solution**
  - Acrylamide : Bisacrylamide = 37.5 : 1

- **Biotinylation Buffer**
  - 100 mM Tris pH 7.4
  - 10 mM EDTA
  - store in aliquots of 1 - 1.5 ml at 4°C
- **Biotin-HDDP**
  - 1 mg/ml dissolved in Dimethylformamide (DMF)
- **Buffer A for fly DNA extraction**
  - 100 mM Tris/HCl, pH 7.5
  - 100 mM EDTA
  - 100 mM NaCl
  - 0.5% SDS
- **Church buffer**
  - 1% (w/v) bovine serum albumine
  - 1 mM EDTA
  - 0.5 M phosphate buffer
  - 7% (w/v) SDS
  - pH 7.2
- **Colloidal Coomassie staining solution**
  - 50 g/l aluminum sulfate
  - 2% (v/v) H_3PO_4 (conc.)
  - 10% (v/v) 100% ethanol
  - 0.5% (v/v) Coomassie G250 stock solution
- **Coomassie G250 stock solution**
  - 0.5 g/l Coomassie G250 in 100% methanol
- **Coomassie staining solution**
  - 45% (v/v) methanol
  - 10% acetic acid
  - 0.25% (w/v) Coomassie Brilliant Blue

- **Coomassie destain**
  - 45% (v/v) methanol
  - 10% acetic acid
- **DNA loading buffer (6x)**
  - 0.25% (w/v) bromophenol blue
  - 0.25% (w/v) xylene cyanol
  - 30% (w/v) glycerol
- **Elution Buffer (for separation of labeled and unlabeled RNA)**
  - 100 mM Dithiothreitol (DTT) in Rnase free H_2O
- **Formamide loading dye (2x)**
  - 80% (w/v) formamide
  - 10 mM EDTA, pH 8
  - 1 mg/ml xylene cyanol
  - 1 mg/ml bromophenol blue
- **Laemmli SDS loading buffer (2x)**
  - 100 mM Tris/HCl, pH 6.8
  - 4% (w/v) SDS
  - 20% (v/v) glycerol
  - 0.2% (w/v) bromophenol blue
  - 200 mM freshly added DTT
- **LiCl/KOAc Solution**
  - 1 part 5 M KOAc stock : 2.5 parts 6 M LiCl stock

- **Lysis buffer for protein extraction**
  - 100 mM KOAc pH 7.4
  - 30 mM Hepes # KOH pH 7.4
  - 2 mM $MgCl_2$
  - 1 mM DTT
  - 1% (v/v) Triton X-100
  - 2x Complete # without EDTA (=protease inhibitor cocktail)
  - 5% Glycerin
- **PBS (10x)**
  - 137 mM NaCl
  - 2.7 mM KCl
  - 10 mM Na_2HPO_4
  - 2 mM KH_2PO_4, pH 7.4
- **PBS-T**
  - PBS supplemented with 0.05% Tween-20
- **SDS-running buffer (5x)**
  - 125 mM Tris/HCl, pH 7.5
  - 1.25 M glycine
  - 5% SDS
- **SSC (20x)**
  - 3 M NaCl
  - 0.3 M sodium citrate
- **TAE (50x)**
  - 2 M Tris-base
  - 5.71% acetic acid
  - 100 mM EDTA

- **TBE (10x)**
  - 0.9 M Tris base
  - 0.9 M boric acid
  - 0.5 M EDTA (pH 8)
- **TBS (10x)**
  - 50 mM Tris
  - 150 mM NaCl
  - pH 7.4
- **TBS-T**
  - TBS supplemented with 0.02% Tween-20
- **TE**
  - 10 mM Tris
  - 1 mM EDTA
- **Washing Buffer (for separation of labeled and unlabeled RNA)**
  - 100 mM Tris pH 7.5
  - 10 mM EDTA
  - 1 M NaCl
  - 0.1% Tween20
- **Western blotting stock (10x)**
  - 250 mM Tris/HCl, pH 7.5
  - 1.92 M glycine
- **Western blotting buffer (1x)**
  - 10% Western blotting stock (10x)
  - 20% methanol

2.2 Methods

2.2.1 Molecular Cloning

2.2.1.1 Amplification of DNA sequences by PCR

For molecular cloning purposes the standard reaction mix was as follows:

10x Taq-buffer (-$MgCl_2$, +$(NH_4)_2SO_4$)	5 μl
dNTP-mix (10 mM each)	1 μl
25 mM $MgSO_4$	4 μl (final conc. 1.5 mM)
100 nM primer fw	0.1 μl
100 nM primer rev	0.1 μl
template DNA	1 μl
Taq polymerase	0.3 μl
Pfu polymerase	0.3 μl
H_2O	38.2 μl
	50 μl

or

5 x Phusion HF or GC Buffer	10 μl
dNTP-mix (10 mM each)	1 μl
500 nM primer fw	0.5 μl
500 nM primer rev	0.5 μl
template DNA	1 μl
Phusion Hot Start DNA polymerase	0.5 μl
H_2O	36.5 μl
	50 μl

Reaction components were acquired from Fermentas (St. Leon-Rot, Germany) and Finnzymes (Espoo, Finland), Taq polymerase was taken from our own laboratory stock. PCR reactions were carried out on an automated thermal cycler (Eppendorf; Hamburg, Germany).

The following standard protocol for gradient PCR was used to determine the appropriate annealing temperatures. Conditions were then adjusted accordingly:

3 min	95°C	(initial denaturation)
37 cycles:		
30 sec	94°C	(denaturation)
30 sec	50-65°C	(primer annealing)
1 min per kb product size	72°C	(extension)
5 min	72°C	(final extension)
Hold	4°C	

or

30 sec	98°C	(initial denaturation)
24-34 cycles:		
5-10 sec	98°C	(denaturation)
10-30 sec	60-72°C	(primer annealing)
15-30 sec per kb product size	72°C	(extension)
5-10 min	72°C	(final extension)
Hold	4°C	

PCR products were separated by agarose gel electrophoresis, excised and purified by QIAGEN Gel Extraction Kit or directly treated with the QIAGEN PCR Purification Kit (Qiagen; Hilden, Germany).

2.2.1.2 Agarose gel electrophoresis

Appropriate for the length of nucleotides to be separated 0.5% - 2% agarose gels were prepared with 1x TAE buffer and stained with 1x SyberSafe (Invitrogen; Karlsruhe, Germany). Gels were run at 55V for 30 min and photographed in an Intas UV Imaging System. If higher sensitivity was required gels were re-stained in 1x SyberGold (Invitrogen; Karlsruhe, Germany) for maximal 20 min.

2.2.1.3 Specific restriction of DNA by restriction endonucleases

Endonucleolytic digestion of DNA was carried out with restriction endonucleases acquired from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Ipswich, USA) according to manufacturer's recommendations. Usually, reactions were incubated for minimal 1 hour up to over night incubation at 37°C. To prevent recircularization the cloning vector DNA was dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase (Fermentas; St. Leon-Rot, Germany) according to manufacturer's protocol.

2.2.1.4 Ligation of vector with insert DNA

Digested and purified insert and vector were combined according to the following formula in a molar ratio of 1 to 3:

$$\text{required mass (insert)} = \frac{\text{Mass(vector)} \times \text{length(vector)} \times 3}{\text{length(insert)}}$$

50- 200 ng	vector
+ required amount	insert
+ 2 μl	T4-buffer (10x)
+ 1 μl	T4-DNA Ligase
+ x μl	H_2O
20 μl	

Optimally samples were ligated over night at 18°C and used for bacterial transformation.

2.2.1.5 Bacterial transformation

Transformation of competent bacteria was carried out by standard heat shock procedures. Briefly, 50 μl XL2-blue CaCl2-competent cells were thawed on ice. 1-4 μl of ligation sample were added and the mixture was incubated on ice for 30 min, subjected to a 1 min heat shock at 42°C and returned to ice for 1 min. 500 μl SOC-medium was added and cells were allowed to grow for 1 h in a 37°C shaking incubator. Cells were then centrifuged for 30 sec at full speed in a table top centrifuge, the supernatant was poured off and the resuspended cell pellet was streaked out on agarose plates with appropriate antibiotics for selection of transformants.

2.2.1.6 Test for correct transformants by colony-PCR

Individual colonies were tested for correct integration of the insert by colony-PCR with suitable primer pairs. A standard PCR reaction mix was inoculated with a single colony, which was subsequently streaked onto a fresh plate and labeled for later recognition. Standard amplification was carried out with 10 min initial denaturing for cell lysis of bacteria.

2.2.1.7 Preparation of plasmid DNA

Plasmid DNA was prepared from over-night cultures of 2 ml or 100 ml LB-medium, respectively, supplemented with appropriate antibiotics (usually 100 μg/ml ampicillin). QIAGEN Mini or Midi Kits (Qiagen; Hilden, Germany) were used according to the manufacturer's protocols.

2.2.1.8 DNA sequencing

Sequencing was carried out by Eurofins/MWG (Ebersberg, Germany) according to the provider's specifications. Sequence analysis and alignments were performed with ApE (openly available online tool).

2.2.2 Methods of *Drosophila* S2 cell culture

2.2.2.1 Maintenance

Drosophila Schneider 2 (S2) cells were cultured in Schneider's medium (Bio & Sell, Nürnberg, Germany) supplemented with 10% heat inactivated fetal calf serum (Thermo Fisher Scientific; Waltham, USA) in 10 cm cell culture dishes. Cultures were split once a week into fresh medium within a period of maximal 2 month.

2.2.2.2 Transfection

Transfections of S2 cells were carried out essentially as described in (Shah and Förstemann, 2008). For each well of a 24-well cell culture dish 100-300 ng of the vector of interest or 0.1 μl of 2'-O-Methyl antisense oligo (100 nM) in 50 μl medium (without serum) and 4 μl of Fugene Transfection Reagent (Roche Diagnostics; Mannheim, Germany) in 46 μl of medium (without serum) were mixed and incubated at RT for 1 hour. Cells were added to the transfection mix at 0.5 - 1x 10^6 cells/ml medium (+10% FBS) and harvested on day 3 after transfection or split on day 3 after transfection and harvested on day 5 for further experiments. For transfections in 6-wells or 10 cm dishes all amounts of reagents were scaled up.

2.2.2.3 Depletion of individual genes by RNAi in cell culture

RNA interference by simply adding dsRNA to cell culture medium (soaking) was performed essentially as described (Shah and Förstemann, 2008). Briefly, gene specific primers for target genes were designed to introduce flanking T7-promotor fragments and amplified using standard PCR. The PCR products were precipitated with ethanol, the pellet was dissolved in 1/10 of the original volume and used directly for over-night in-vitro transcription at 37°C with the following specifications:

10 μl	10x T7-buffer
10 μl	re-dissolved DNA
0.5 μl	1 M DTT
5 μl	100 mM ATP
5 μl	100 mM CTP
5 μl	100 mM UTP
8 μl	100 mM GTP
54.5 μl	H_2O
2 μl	T7-polymerase
100 μl	

After in-vitro transcription 1 μl of DNAseI was added per 100 μl of reaction and incubated for 30 min at 37°C. White precipitate was pelleted and RNA was precipitated from supernatant with 1x volume of isopropanol and washed with 70% ethanol. The dried pellet was dissolved in 100 μl of H_2O . For proper strand annealing $MgCl_2$ was added to a final concentration of 5 mM, the sample was heated to 95°C for 3 min and allowed to slowly cool down to room temperature. Concentration of dsRNA was estimated from an agarose gel in comparison to a DNA Ladder Mix (Fermentas; St. Leon-Rot, Germany). Cells were seeded at 0.5 x 106 cells/ml and 20 μg/ml dsRNA were added to the medium. On day 2, the cells were split 1:5 into a fresh culture dish and the dsRNA treatment was repeated. On day 5 or 6, GFP fluorescence was quantified in a Becton Dickinson FACSCalibur flow cytometer.

2.2.3 Protein analysis

2.2.3.1 Protein extraction

Cells were harvested (1000 x g, 3 min) and washed twice in ice cold PBS. The pellet was resuspended in lysis buffer (30 mM Hepes, 100 mM KOAc pH7.4, 2 mM $MgCl_2$, 1 mM fresh DTT, 1% (v/v) Triton X-100, 2x protease inhibitor cocktail (Complete ®without EDTA, Roche Diagnostics), 1x phosphatase inhibitor (PhosSTOP, Roche Diagnostics) 5% Glycerin) and frozen in liquid nitrogen. Samples were thawed on ice for 30 min and cell debris was pelleted in a refrigerated microcentrifuge at full speed (Eppendorf; Hamburg, Germany) at 4°C for 20 min. Protein concentrations were determined by Bradford Assay (BioRad; Hercules, USA).

2.2.3.2 Immunoprecipitation

50 μl of Protein G Plus/Protein A Agarose beads (IP05, Calbiochem) per IP were washed two times in 1 ml Lysis buffer and agitated for minimal 30 min up to overnight incubation at 4°C with the respective antibody. Beads were then washed three times and incubated with total protein in Lysis buffer at 4°C for 1 h on an overhead-rotator. a-Flag affinity agarose beads were accordingly washed three trimes and then used as Protein G Plus/Protein A Agarose Beads. Flow-through and beads were separated by spin columns (MoBiTec; Göttingen, Germany) and washed three times with 500 μl lysis buffer. RNA was extracted by applying 200 μl Trizol (Invitrogen; Karlsruhe, Germany) and following the manufacturer's instructions. Before precipitation with isopropanol 1,5 μl of glycogen were added. RNA was solved in 10 μl H_2O.

2.2.3.3 Immunoblotting for detection of proteins

Western blotting was performed as previously described (Förstemann, 2007). In short, proteins were separated on 8-12% polyacrylamide gels (150 V; 1h) in a BioRad electrophoresis tank. Gels were incubated for 15 min in Western Blotting buffer under constant agitation; polyvinylidenfluoride (PVDF; Milipore; Billerica, USA) membranes were incubated for several seconds in methanol, then also for 15 min in Western Blotting buffer under constant agitation. Proteins were transferred to the membrane by tank blotting (300 mA; 1h). The membrane was incubated in methanol for 5 min under agitation, air-dryed for 5 min, followed by another 5 min incubation in methanol. After blocking in 5% milk for minimal 15 min at RT membranes were incubated under constant rolling in 50 ml tubes with 2 ml of primary antibody solution (5% milk, 1x TBS or PBS, 0,1 % Tween-20) over night at 4°C. PBS-T (0.05% Tween) was used for washing for rabbit antibodies, for mouse antibodies TBS-T (0.02% Tween) was employed. After primary antibody binding blotting membranes were washed three times for 10 min in buffer and incubated with

appropriate secondary antibodies for minimal 2 h at RT. After analogous washing, Enhanced Chemiluminescence (ECL) substrate (Thermo Fisher Scientific; Waltham, USA) was applied and the signal was measured in an LAS3000 mini Western Imager System (Fujifilm; Tokyo, Japan). Multi Gauge software (Fujifilm; Tokyo, Japan) was used for relative quantification of protein band intensities. Western blots were stripped with 10 ml of Restore Stripping Solution (Thermo Fisher Scientific; Waltham, USA) for 30 min at RT or 15 min at 37°C, washed extensively in water and buffer and blocked for new primary antibody incubation.

2.2.4 RNA analysis

2.2.4.1 RNA extraction

RNA was extracted either with Trizol (Invitrogen; Karlsruhe, Germany) according to the manufacturer's instructions or using the QIAGEN miRNeasy Kit (Quiagen; Hilden, Germany) and quantified using spectrophotometry.

2.2.4.2 Thiouridine-Labeling; Biotinylation of labeled mRNA; Separation of RNA fractions

Schneider S2 cells were transfected with the 2'-O-Methyl antisense oligo of interest. On day 2 after transfection cells were splitted in 3 x 10^6 cells/ml and incubated over night. 50 μM thiouridine was added to a final concentration of 100 μM. Cells were incubated at 25°C for 60 min, centrifuged at 4°C at 1200 rpm for 4 min and resuspended in 6 ml Trizol. An incubation at RT for 5 min allowed the nucleoprotein complexes to dissolve. 1 ml of chloroform was added and shaked vigorously for 15 sec. The mix was then incubated at RT for 2-3 min and centrifuged at 4500 rpm for 45 min at 4°C. The aqueous upper phase containing the RNA was transferred to a new Falcon tube. 1/2 the reaction volume of RNA precipitation solution (1.2 M NaCitrate, 0.8 M NaCl) and isopropanol were added, mixed well, incubated at RT for 10 min and afterwards centrifuged at 4500 rpm for 30 min at 4°C. The supernatant was removed and an equal volume of 75% EtOH added. The falcon tube was vortexed and centrifuged at 4500 rpm for 30 min at 4°C. Again the supernatant was removed, followed by a brief centrifugation to spin down remaining ethanol. The RNA pellet was dissolved in 100 μl of 1xTE (10 mM Tris, 1 mM EDTA) per 100 μg expected RNA yield by heating to 65°C for 10 min in a shaker. For biotinylation following contents were mixed an incubated at RT for 1.5 h with rotation:

30-100 μg	total RNA
2 μl	Biotin-HDDP (1 mg / ml DMF) per 1 μg RNA
1 μl	10 x Biotinylation Buffer per 1 μg RNA
	(100 mM Tris pH 7.4, 10 mM EDTA)
7 μl	RNase free H_2O per 1 μg RNA

For the following chloroform extraction the initial volume should be at least 500 μl. An equal volume of chloroform was added and the contents mixed vigorously. After an incubation of 2-3 min tubes were centrifuged at full speed for 5 min. Next the upper phase was transferred into new tubes and the extraction step was repeated.

For RNA precipitation 1/10 the reaction volume of 5M NaCl, an equal volume of isopropanol and 2 μl of glycogene were added. Next the tubes were centrifuged at 20,000 g for 20 min. The supernatant was removed and an equal volume of 75% ethanol added. The tubes were again centrifuged at 20,000 g for 10 min followed. Finally the RNA was resuspended in 100 μl 1 x TE.

For separation of labeled and unlabeled RNA the biotinylated RNA samples were heated to 65°C for 10 min and immediately placed on ice for 5 min. Up to 100 μg (max. 100 μl) of biotinylated RNA were added to 100 μl of streptavidin beads and incubated with rotation for 15 min. The μMacs columns were placed into the magnetic stand and 0.9 ml of washing buffer (100 mM Tris pH 7.5, 10 mM EDTA, 1 M NaCl, 0.1 % Tween20) were added to the columns for pre-running and equilibration. Next the beads (RNA) were applied to the columns. The columns were washed 3 x with 0.9 ml 65°C washing buffer and 3x with 0.9 ml room temperature washing buffer. As the unbound RNA needs to be recovered, the flow-through and the first wash were collected for subsequent precipitation. The two fractions were combined and the unbound RNA was recovered by using 200 μl of the sample with the RNeasy MinElute Cleanup Kit analog to the bound RNA.

Next 700 μl of Buffer RLT (RNeasy MinElute Cleanup Kit) were pipetted into new 2 ml Eppendorf cups. RNA was eluted directly into Buffer RLT by placing the Eppendorf cups underneath the columns and adding 100 μl Elution Buffer (100 mM DTT) to the columns. A second elution was performed round 3 min later. Next was continued with the RNeasy MinElute Cleanup Protocol following the manufacturer's instructions. 500 μl of 96-100 % ethanol were added to the diluted RNA and mixed thouroughly by pipetting. 700 μl of the sample were applied to an RNeasy MinElute spin column in a 2 ml collection tube. The tube was centrifuged for 15 s at >8000g. Next the flow-through was discarded and the remaining 700 μl of the sample were applied, the centrifugation repeated and the flow-through discarded again. The spin columns were transferred into a new 2 ml collection tube. 500 μl Buffer RPE were pipetted onto the spin column and centrifuged for 15 s at >8000g to wash the column. The flow-through was discarded. Next 500 μl of

80% ethanol were added to the spin column and centrifuged for 2 min at >8000g. The flow-through and the collection tube were discarded. The spin columns were transferred into new 2 ml collection tubes, the caps were opened and then the tubes were centrifuged at full speed for 5 min. The flow-through and the collection tube were discarded again. To elute, the spin column was transferred to a new 1.5 ml collection tube, 14-20 μl RNase free water was pipetted directly onto the center of the silica-gel membrane and the tube was centrifuged for 1 min at full speed. The RNA was now ready for microarray analysis.

2.2.4.3 Northern Blotting

2-10 μg of RNA were separated on a 20% Sequagel Acrylamide/Urea gel (National Diagnostics; Atlanta, USA) at 250V for 1.5 hours. RNA was then transferred to a positively charged Nylon membrane (Roche Diagnostics; Mannheim, Germany) by semi-dry blotting for 30 min at 20V. Membranes were transferred to hybridizing tubes and incubated in Church Buffer for prehybridization for at least 1h in a hybridization oven under constant rotation at 37°C. Probes were labeled by incubating following contents for 1 h at 37°C:

9 μl	H_2O
2 μl	10x PNK buffer
2 μl	5 mM probe oligonucleotide (=10 pmol)
1 μl	PNK (Fermentas)
6 μl	[γ^{32}P] ATP
20 μl	

Unbound radioactive nucleotides were removed using a Sephadex G-25 spin column (Roche Diagnostics; Mannheim, Germany). Labeled oligonucleotide anti-sense probes were added to 5 ml of Church Buffer for over-night hybridization. Hybridization was carried out at 37°C for DNA-probes or 65°C for 2'-OMe-probes. Membranes were washed three times for 15 min in 2x SSC + 0,1% SDS and exposed to Phosphoimager Screens (Fujifilm; Tokyo, Japan) for up to 1 week. Screens were scanned using a Typhoon scanner (Amersham Biosciences) and band intensities were quantified using Multi Gauge software (Fujifilm; Tokyo, Japan). Membranes were immersed in 1% SDS heated to boiling in a conventional microwave and allowed to sit there for 5 min before they could be reused for prehybridization.

Probe for Northern Blotting	**Sequence 5' - 3'**
miR-277 2'-OMe as probe	TGTCGTACCAGATAGTGCATTTA
bantam 2'-OMe as probe	AATCAGCTTTCAAAATGATCTCA
Probe B (CG4068 B) as probe	GGAGCGAACTTGTTGGAGTCAA

2.2.4.4 Analysis of mRNA levels and miRNA levels by qRT-PCR

100 ng - 1μg of total RNA extract was reverse transcribed according to the Superscript II Reverse Transcriptase protocol (Invitrogen; Karlsruhe, Germany):

1 μl	oligo(dT) (500 μg/ml) or 50-250 ng random primers
x μl	100 ng - 1μg RNA
1 μl	dNTP Mix (10 mM each)
x μl	H_2O
12 μl	

The mixture was heated to 65°C for 5 min and quick chilled on ice. The contents of the tube were briefly centrifuged. Then following components were added:

4 μl	5X First-Strand Buffer
2 μl	0.1 M DTT
1 μl	RiboLock RNase inhibitor
1 μl	SuperScript II RT

The contents of the tube were mixed gently. If oligo(dT) primers were used, an incubation at 42°C for 52 min followed. If random primers were used, an incubation at 25°C for 12 min followed, next an incubation at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. 100 μl of water were added to get a final volume of 120 μl. The qPCR reaction mix was as follows, according to the DyNAmo Flash SYBR Green qPCR Kit (Finnzymes; Finland).

Reaction mix for one well of a 96-well plate:

5 μl	Master Mix
0.5 μl	each Oligo (10 μM)
0.2 μl	ROX
2.8 μl	H_2O
9 μl	

9 μl of reaction mix and 1 μl of RT-reaction (or 10ng genomic DNA) per well was

amplified in an ABI PRISM 7000 qPCR cycler (Applied Biosystems; Foster City, USA) using the following conditions:

10 sec	50°C
3 min	94°C
40 cycles:	
30 sec	95°C
30 sec	59°C
30 sec	70°C

Cycle of Threshold values (CT-values) usually determined via the auto-CT function and manually adjusted if necessary.

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100 ng of total RNA extract was reverse transcribed according to the Qiagen miScript protocol:

4 μl	miScript RT buffer (5x)
0.3-0.7 μl	total RNA
14.3-14.7 μl	H_2O
1 μl	miScript enzyme mix
20 μl	

Samples were incubated at 37°C for 60 min and then inactivated at 95°C for 5 min. 100 μl of water were added to make a final volume of 120 μl. The qPCR reaction mix was as follows:

Reaction mixes for 14 reactions (for 1 row of 96-well plate):

70 μl	Quantitect SyBr-green mix (2x)
35 μl	H_2O
14 μl	miScript universal primer (5 μM)
7 μl	miScript specific primers (10 μM)

9 μl of reaction mix and 1 μl of RT-reaction per well was amplified in an ABI PRISM 7000 qPCR cycler (Applied Biosystems; Foster City, USA) using the following conditions:

15 min	94°C
40 cycles:	
20 sec	94°C
30 sec	55°C
30 sec	70°C

Cycle of Threshold values (CT-values) usually determined via the auto-CT function and manually adjusted if necessary. The 2S-1 primer from the miScript kit was used as a control.

2.2.4.5 Analysis of miRNA levels by microarrays

Labeling of samples for array analysis was performed using the GeneChip 3' IVT labeling assay (Affymetrix) with 100 ng input RNA by Kerstin Maier (AG Cramer). Samples were hybridized to GeneChip *Drosophila* Genome 2.0 microarrays following the instructions from the supplier (Affymetrix). Computational analysis of differential expression was done by Björn Schwald (AG Tresch).

2.2.4.6 Deep sequencing

Small RNAs were isolated and libraries for Solexa sequencing were prepared essentially as described by (Czech et al., 2008). Solexa sequencing was carried out at Fasteris (Plan-Les-Ouates, Switzerland). For previously published deep sequencing data, the FASTA files were retrieved from NCBI GEO (GSM239041, GSM239050, GSM239051, GSM239052, GSM280089) and mapped onto the target sequences using BOWTIE (http://bowtie-bio.sourceforge.net/) with the option -v0 to force selection of only perfectly matching sequences. Pre-processing of sequences and analysis of the BOWTIE output files were done using PERL scripts. The sequence data are available at NCBI GEO under the accession number GSE16958.

2.2.5 *Drosophila melanogaster* methods

2.2.5.1 Maintenance and holding

Flies were kept on standard fly food at 25°C and transferred to new food every 2 days if they had to be amplified for lifespan analysis. For all other experiments flies were transferred to new food every 2-3 weeks. For phenotype selection flies were anesthetized with CO_2 and sorted on a CO_2-emitting pad (Genesee Scientific; San Diego, USA) using a Leica MZ7 stereomicroscope (Leica Microsystems; Wetzlar, Germany). To slow proliferation by reducing metabolic rates flies were kept at 18°C if they were not used for a current experiment and were transferred to new food every 4 weeks.

2.2.5.2 Transgenic flies

The constructs of interest were sub-cloned into pUASP vector. The constructs were sequenced, tested in cell culture and sent for injection into w^{1118} fly embryos (Rainbow Transgenic Flies; Newbury Park, USA). Transgenic offspring, marked by red eye color, were twice crossed with w^{1118} flies to reduce the risk of secondary mutations. Siblings

were then mated to produce homozygous stable lines. The protocol for the mapping of P-element insertions was applied according to the Berkeley *Drosophila* Genome Project. In short, DNA was prepared from 30 anesthetized flies by freezing at -80°C and subsequent mechanical lysis in Buffer A. After incubation at 65°C for 30 min LiCl/KOAc-solution was added, the tube incubated on ice for at least 10 min, debris was pelleted and supernatant was precipitated with isopropanol. The genomic fly DNA pellet was washed with 70% ethanol, dried and dissolved in H_20. To create appropriate fragment sizes for inverse PCR, genomic DNA was digested with *HinP1I*, *MspI* or *Sau3AI* and its isoschizomer *BfuCI*. The fragments were circularized by ligation at low DNA concentration. Standard PCR with Plac, Pry and Pwht primers indicated above was carried out, amplification products were separated on a 1.5% agarose gel. Bands were excised, sequenced and mapped to the *Drosophila* genome.

2.2.5.3 Lifespan analysis

For each lifespan series the specific fly line was amplified up to about 50 vials. Then lifespan series were set up according to following scheme:

Monday:	Vials were emptied in the afternoon.
Tuesday (day 0):	10 vials of each food condition were prepared for each genotype; 10 male (< 1 day old) flies were placed in each vial, so that 100 flies per genotype per food condition were examined.
Wednesday (day 1):	Surviving flies in each vial were counted.
Friday, Monday, Wednesday:	Surviving flies were counted and set on fresh food.

2.2.6 Amino acid analysis

For the analysis of the amino acid composition of the fly, 10 male or female flies of lines of interest were set on LSLP and LSHP food for 5 days. For analysis of Schneider S2 cells 6 wells of a 24-well were transfected with the plasmid of interest, splitted 1:4 at day 3 in 6-wells, harvested at day 5 (1,000 x g 3 min) and washed 3 times with PBS.

Whole male flies, female abdomen or cell pellets were pestled in 100 μl of 0.1 N HCl, centrifuged at full speed at 4 °C. Then the supernatant was neutralized with 80 μl of 0.1 N NaOH and again centrifuged at full speed at 4 °C. Samples were freezed at -80 °C. Analysis of the samples was done by Bernhard Michalke (Helmholtz Zentrum).

2.2.7 Triglyceride analysis

For the analysis of triglyceride levels in the fly 12 male flies of lines of interest were set on LSHP food fo 7 days. Then the flies were set in a empty vial for 30 minutes and weighed

afterwards. Flies were homogenized in 100 μl PBS and centrifuged at 4000 rpm for 5 minutes. 5 μl supernatant were mixed with 170 μl Infinity Triglycerides Liquid Stable Reagent (Thermo Scientific) and incubated at 37 °C for 10 min (under agitation, 300 rpm). For the determination of the triglyceride content of the extract the absorbance was measured at 550 nm.

3 Aims of this thesis

- Where is miR-277 expressed in the adult fly and does the expression level change during ageing?
- What are the targets of miR-277 and what is the mode of regulation by miR-277?
- Which role has miR-277 for regulation of longevity on different nutrinional conditions, e.g. dietary restriction?
- Which role has miR-277 for cellular physiology? Has miR-277 influence on the phosphorylation of 4E-BP and thus on the activity of the TOR kinase?
- What is the position of mir-277 in the TOR/IIS-pathway signaling network? Has miR-277 the property to control metabolic flux by regulating BCKAs as signaling molecules?
- Are loading properties into the RISC complex responsible for the regulative effects of miR-277?

4 Results

4.1 Establishing a pulse labeling and fractionation method for parallel quantification of direct and indirect miRNA effects by using an endo-siRNA reporter system

The appearance of a new selfish genetic element in an organism's genome is often accompanied by amplification and mobilization, leading to a highly mutagenic situation until the cells regain control. In *Drosophila*, zebrafish, rat and mouse germ line cells, the piRNA system can efficiently repress selfish genetic elements like transposons in the presence of a pre-existing, maternally contributed pool of piRNAs with corresponding sequence (Blumenstiel and Hartl, 2005; Brennecke et al., 2008); (for review, see Malone and Hannon (2009)). Even though germ cells are the ultimate target for selfish genetic elements, they are not the only cells that can be faced with a newly emerging transposon threat. Thus silencing of transposable elements is important in somatic cells to reduce the possibility of transition into the germ cell lineage (Chalvet et al., 1999; Pelisson et al., 2002). The discovery of endo-siRNAs points to one mechanism which is capable of silencing transposons in the somatic cells of *Drosophila* (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008). Although there was a lot of progress made in elucidation of endo-siRNA biogenesis it still remains unclear what is the mode of regulatory repression by endo-siRNAs.

We were able to generate a reporter system that closely resembles natural endo-siRNA targets. Our reasoning was that some of the features of transposable elements, such as multicopy insertion and the formation of repetitive regions, are shared by transgenes that have integrated into the host cell genome after transfection and selection of stable cell culture lines. Therefore we examined a clonal cell line (63N1) that expresses GFP via an artificial plasmid sequence, stably integrated at high-copy number. We could show that the transgene became subject to endosiRNA- mediated repression, depending on endo-siRNA biogenesis factors (Hartig et al., 2009).

To prove that the GFP transgene is a direct target of the small RNAs, and to rule out that upregulation, i.e. de-repression, of GFP upon depletion of Dcr-2, Loqs an Ago2, is an indirect consequence of endo-siRNA loss leading to increased transcription of the GFP coding gene, we examined transcription and degradation rates of*GFP* mRNA in vivo by pulse labeling newly synthesized RNA (Dolken et al., 2008; Johnson et al., 1991; Kenzelmann et al., 2007).

To verify the procedure (Fig. 4.2A), we determined the ratio of the *hsp70* mRNA, which has a half-life of 15–30 min in S2 cells (Petersen and Lindquist, 1988), to the stable *rp49* mRNA in each RNA fraction. As expected, we found that relative to *rp49*, *hsp70* mRNA was significantly more abundant in the newly synthesized RNA fraction than in unfractionated RNA (Fig. 4.2B).

In addition, we examined the effect of a *dcr-2* RNAi in comparison with a control RNAi against DsRed. As shown in Fig. 4.2C, knock down reduced the *dcr-2* mRNA levels by about 2-fold in total RNA (cDNA synthesis was primed with random hexamers, thus we potentially also detected mRNA degradation fragments), but it was much more pronounced (20-fold) in the flow-through fraction ($> 1h$ old) of our RNA separation procedure. We did not observe any significant change of *dcr-2* mRNA in the newly transcribed fraction. Therefore the 1 h time window for labeling seemed appropriate to separate transcriptional from post-transcriptional events.

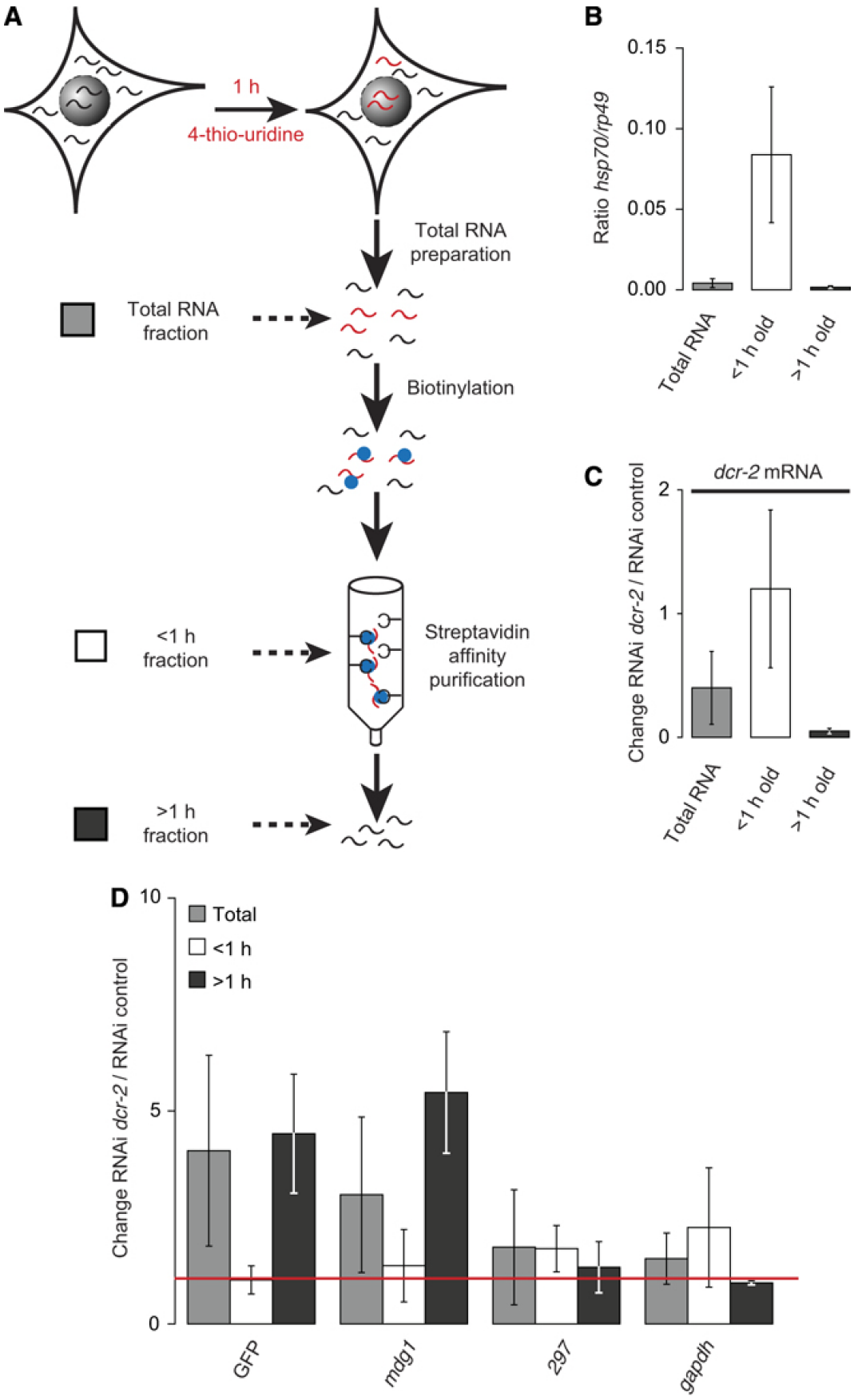
If de-repression of the GFP transgene occurred by a transcriptional mechanism, then inhibition of endo-siRNA biogenesis should cause an increased abundance of target mRNAs in the $< 1h$ RNA fraction. However, if a post-transcriptional mechanism is responsible, the increase should be stronger in the $> 1h$ RNA fraction. We observed a trend towards increased mRNA levels for the GFP transgene and the *mdg1* transposon (an endogenous target of the endo-siRNA pathway) in the total RNA fraction on knock down of *dcr-2*. After fractionation, we could detect significant changes in the $> 1h$ fraction for GFP and *mdg1* (Fig 4.2D, $P < 0{,}05$). We did not detect any significant changes in the newly transcribed RNA fraction, indicating that the increased GFP levels caused by impaired endo-siRNA biogenesis are due to an increased stability of the *GFP* mRNA.

As a positive control for changes in the rates of transcription, we used a UAS–GFP vector with and without co-expression of the GAL4 transcription factor. This resulted in an increase of *GFP* mRNA in all three RNA fractions (Fig. 4.2D and E).

4.2 Detection and analysis of transgene derived endo-siRNAs, endo-siRNA response and endo-siRNA precursor structure

We decided to examine the endo-siRNA response in our GFP reporter system in more detail to identify the sources of the previously observed GFP repression.

In the case that GFP repression is regulated through mRNA targeting by endo-siRNAs, small RNAs with complementary sequence to GFP should be present in the reporter cells. To check this assumption we isolated 18–30-nt long RNAs from the parental S2 cells as well as the stable GFP-expressing clone 63N1 and analysed them by deep sequencing. After removal of adapter sequences and length selection (20–24 nt size window), we obtained 1 098 002 reads for the parental cell library and 2 678 671 reads for the 63N1 cell library. Surprisingly, 359 845 reads from the parental cell library and 1 112 196 reads in the 63N1 library corresponded to miR-184. This exceeds by far the frequency of miR-184 detected in previous studies. Therefore we disregarded these reads during further analysis, leaving 738 157 reads in the parental cell library and 1 566 475 reads in the 63N1 cell library.


A
1 h
4-thio-uridine
Total RNA preparation
Total RNA fraction
Biotinylation
<1 h fraction
Streptavidin affinity purification
>1 h fraction
B
Ratio hsp70/rp49
0.15
0.10
0.05
0.00
Total RNA
<1 h old
>1 h old
C
dcr-2 mRNA
Change RNAi dcr-2 / RNAi control
2
1
0
Total RNA
<1 h old
>1 h old
D
Total
<1 h
>1 h
Change RNAi dcr-2 / RNAi control
10
5
0
GFP
mdg1
297
gapdh

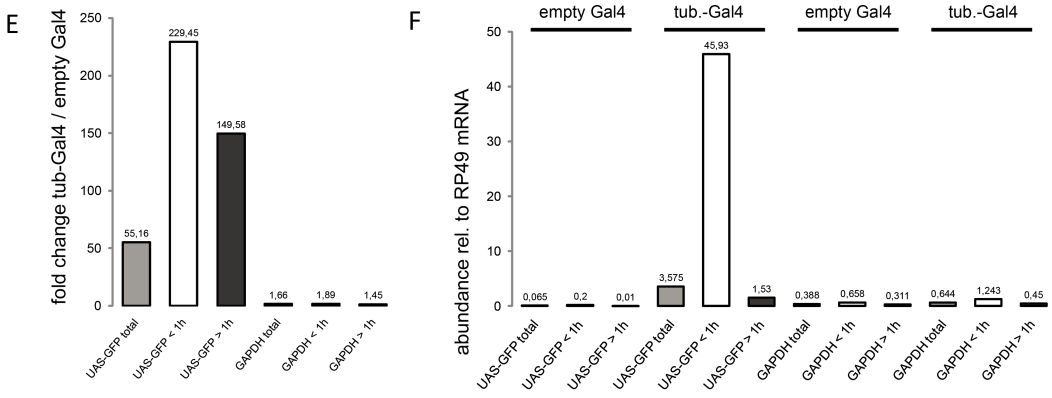


Figure 4.1: **Overview and verfication of the pulse labelling and fractionation technique and expamples for determination of posttranscriptional regulation of endo-siRNAs via degradation and induction of transcription.**
A) Overview of the pulse labeling and purification method adapted from Dolken et al. (2008).
B) To verify the RNA fractionation procedure, the ratio between the short-lived *hsp70* and the long-lived *rp49* mRNAs was determined through qRT–PCR. The separation procedure can extract newly synthesized RNA because the $< 1h$ fraction is enriched for the short-lived message.
C) Further validation was obtained by determining the ratio of *dcr-2* mRNA (normalized to *rp49*) between control and *dcr-2* RNAi treated cells in each fraction. The post-transcriptional effect of 'classical' RNAi affects only the flow-through fraction (i.e. 1 h old).
D) Quantification of *GFP*, *mdg1*, *297* and *gapdh* transcripts by qRT–PCR. The values represent the fold change of each RNA (normalized to *rp49*) between control treated cells (RNAi against DsRed) and *dcr-2* RNAi-treated cells. The horizontal line indicates a value of 1 (no change relative to control). Values represent the mean ± s.d.
E) Changes in the mRNA levels of UAS-GFP and endogenous GAPDH between a promotor-less Gal4-Plasmid and the tubulin-promotor Gal4-Plasmid; an increased mRNA-abundance can be seen in all RNA fractions. Theoretically, the increase in total mRNA should lie between the values for the $< 1h$ and the $> 1h$ fraction, as is the case for GAPDH. The apparent discrepancy for GFP appears to be due to a concomitant increase in mRNA stability (see below), resulting in an exaggerated ratio for the $> 1h$ fraction.
F) For each individual measurement, the mRNA abundance in the total RNA fraction lies between the abundance in the newly transcribed ($< 1h$) and the old ($> 1h$) RNA. However, the contribution of old RNA to the total RNA pool is particularly low for GFP in the uninduced state (see table below). This ratio represents the mRNA half-life relative to *rp49* (< 1: shorter than *rp49*, >1: longer than *rp49*), and this result implies that degradation of the *GFP* mRNA is slower when it is very abundant. One possible explanation could be the saturation of a specific degradation pathway upon the strong induction via the tubulin-promotor Gal4-plasmid.

	old/total
GFP unind.	0.15
GFP ind.	0.43
GAPDH undind.	0.79

After mapping the reads to the sequence of our GFP expression construct, we found 16 424 corresponding reads in the 63N1 cell library and 167 reads in the parental cell

library (see Table 4.1)

The positions of the reads corresponding to our construct showed that not only the GFP-coding region but also many other regions of the construct gave rise to small RNAs (Fig. 4.2). Overall, sense- and antisense-matching reads were equally represented (8 019 sense reads versus 8 405 antisense reads). This is an indication that the small RNAs directed against the construct derive from a double-stranded precursor.

What is triggering the production of double-stranded RNA from the expression construct in the stable cell lines? To assess whether integration is a prerequisite for endo-siRNA generation, we examined a small RNA library generated from transiently with pKF63 (ubi-prom.-GFP) transfected cells. The resulting deep sequencing data were processed, as described before, and a total of 199 418 reads remained after the removal of adapter sequences and length selection. We found 759 reads corresponding to the plasmid sequence. After normalization to small RNAs derived from the CG4068 hairpin (endo-siRNA generating locus, (Okamura et al., 2008b), the amount of reads show a roughly fivefold lower abundance in the transiently transfected cells relative to the 63N1 cells (Table 4.1). This suggests that integration into the host genome - though it may lead to a more efficient response - is not an absolute prerequisite for the generation of endo-siRNAs.

Table 4.1 summarizes the read counts corresponding to the plasmid backbone as well as the endo-siRNA generating hairpin locus CG4068 for normalization.

A detailed analysis of the small RNA population can give insights into the nature of its precursor molecules. We combined all of the transgene-containing deep sequencing libraries discussed in Hartig et al., 2009 (63N1, pKF63 transf., 63-6 mock, 63-6 b-elim., tagged Ago2-IP, (Hartig et al., 2009)) into one dataset and analysed the endo-siRNA population derived from the *mini-white* gene present in all constructs. This marker gene

	Parental	**63N1**	**pKF63**
Total reads (processed)	738 157	1 566 475	199 418
CG4068	397	707	159
pCASPER-2	78[a]	6 930	151
pKF63	167[a]	16 424	759
norm. CG4068	1	1	1
norm. pCASPER-2	0.2[a]	9.8	0.9
norm. pKF63	0.4[a]	23.2	4.8
Neo^R	1	180	–

Table 4.1: Deep Sequencing reads
[a] Size selection of the small RNA from parental, 63N1 and pKF63 transfected cells before adapter ligation was done on the same gel; a small amount of cross contamination may have occurred. The selection plasmid conferring resistance to G418 (Neo^R) was cotransfected only in 63N1 cells. Thus, analysis of the reads matching the neomycin resistance gene can give an independent indication of the extent of cross contamination (last row). The loading order was: parental / 63N1 / pKF63.

is not derived from cDNA and thus still contains introns. Comparison of small RNA occurrence with the exon–intron structure showed that the exonic parts of the gene have a higher propensity to generate small RNAs than the intronic parts (Fig. 4.3, top panel); whereas 38 620 reads were found from the entire *mini-white* gene (3 360 nt), 34 504 reads mapped to the spliced *white* mRNA (2 212 nt). As a 34% reduction of the sequence length reduced small RNA occurrence by only 10.1%, the intron sequences must be less likely to generate endo-siRNAs.

If mRNA can provide the sense strand, then how is the antisense strand generated? We tested whether an RNA dependent RNA-polymerase (RdRP) activity generates the dsRNA precursor by examining endo-siRNAs covering the exon–exon junctions of the *white* gene: while an RdRP-like mechanism will produce double-stranded RNA along the entire transcript, antisense transcripts of the *mini-white* marker gene should not be spliced correspondingly and thus exon–exon junctions in the spliced sense strand cannot anneal with a contiguous antisense transcript. As a consequence, endo-siRNAs should only be produced from within an exon (i.e. not across an exon–exon junction). We, therefore, examined the small RNA density along the spliced white mRNA and compared this data with the location of the exon–exon junctions (Fig. 4.3, bottom panel). In five out of five cases, the exon–exon junctions are preceded by a 15–20 nt region that is essentially devoid of endo-siRNA reads. This is consistent with a lack of endo-siRNAs that cross the exon–exon junction and, consequently, the idea that antisense transcription is providing the second strand to form the duplex precursor for endo-siRNAs.

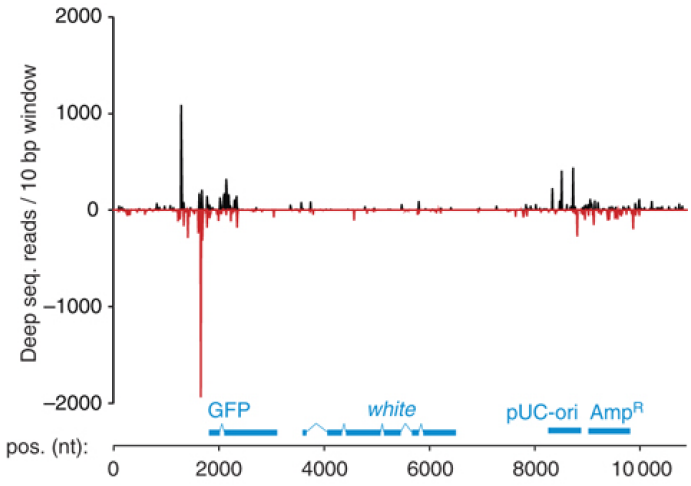


Figure 4.2: **Deep sequencing of transgene-derived endo-siRNAs. The endo-siRNA population lacks the sequences that cross exon–exon junctions.** S2 cell-derived small RNA reads were mapped to the sequence of the transfected GFP expression plasmid (horizontal axis; values above axis: quantification of sense reads, below: quantification of antisense reads). Shown are small RNAs sequenced from 63N1 cells.

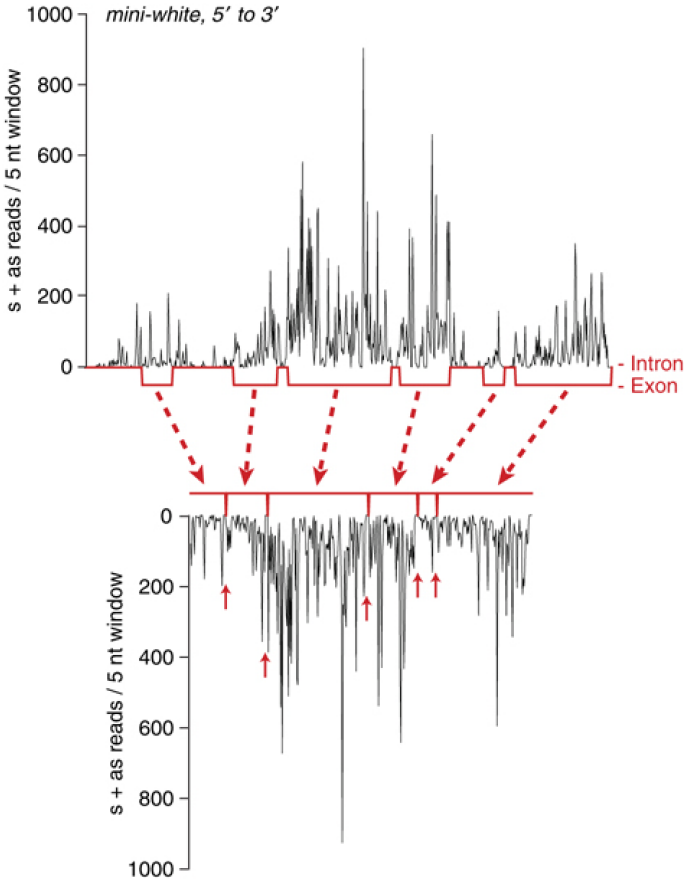


Figure 4.3: **Endo-siRNA population lacks the sequences that cross exon–exon junctions** Small RNA reads were pooled from five different libraries (63N1, pKF63 transf., 63-6 mock, 63-6 beta-el., IP transfected Ago2) and the density of both sense and antisense matching sequences is plotted in comparison with the *white* exon–intron structure. Top panel: the intronic regions give rise to fewer endo-siRNAs than the exonic regions. Bottom panel: when *white* cDNA is used for mapping (individual exons are indicated by dashed arrows), a 15–20 nt window devoid of matching endo-siRNAs precedes each exon–exon junction (solid arrows). The 5 nt bins in the bottom panel are shifted relative to the bins in the top panel because the *mini-white* gene sequence 50 of the transcript start is not an exact multiple of 5. This leads to slight variations in peak heights between corresponding positions in the two diagrams.

4.3 Expression pattern of miR-277

Previous work on miR-277 in *Drosophila* revealed, that the micro RNA is expressed throughout all developmental stages but has its highest expression in the adult fly. To determine the expression pattern of miR-277 in adult *Drosophila* I made use of the UAS-GAL4 system. The GAL4 gene, encoding the yeast transcription activator protein GAL4, is placed under the control of a native gene promoter. Thus GAL4 is only expressed in cells where the driver gene is usually active. The UAS controls expression of a target gene. GAL4 binds specifically to the UAS and activates target gene transcription where UAS has been introduced.

I generated transgenic fly lines expressing GAL4 under the control of a '4kb-miR-277' - promotor sequence. To this end a 4kb sequence downstream of miR-277 was subcloned into the pPTGal-vector and tested via cotransfection with a UAS-GFP construct in *Drosophila* Schneider S2 cells. GFP fluorescence was verified by microscopy and demonstrated the functionality of the generated plasmid. Next the plasmid was sent for embryo injection and several homozygous fly lines were recovered.

The flies were crossed to a UAS-RFP transgenic fly line. Examination of the progeny by fluorescence microscopy revealed that miR-277 is predominantly expressed in the thorax of adult male and female flies, likely in the flight muscles. In female flies there is also some expression in the abdomen visible, probably also in the fat body, but clearly not in the ovaries (see Fig. 4.4).

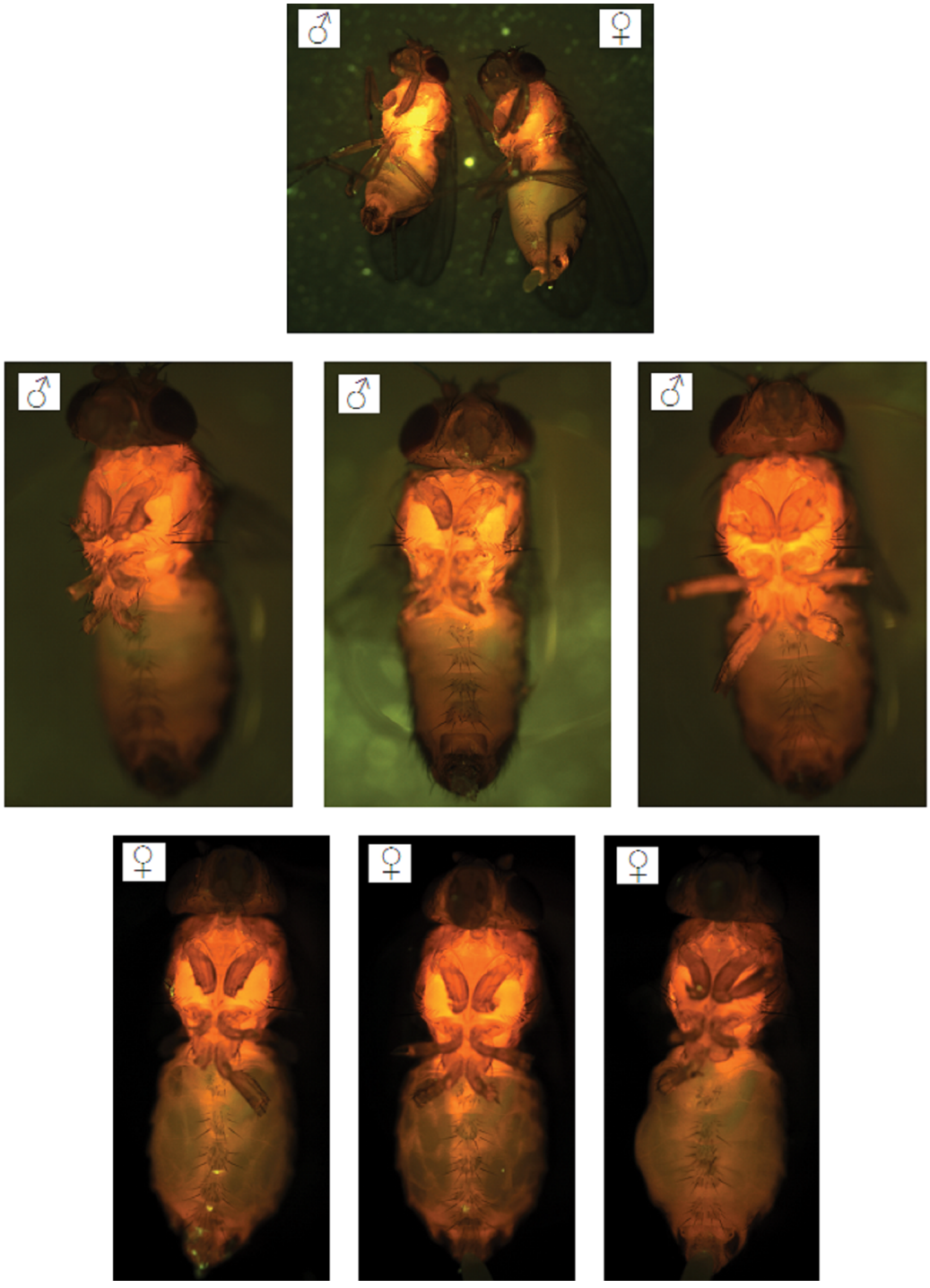


Figure 4.4: **MiR-277 is expressed throughout the thorax of adult male and female *Drosophila melanogaster*.** Stereo fluorescence microscopy of adult progeny of UAS-RFP transgenic flies crossed with 4Kb-miR-277-promotor GAL4 driver transgenic fly line. Male and female flies from three independent 4Kb-miR-277-promotor GAL4 transgenic driver lines are shown.

4.4 Micro RNA profiles of young and old flies are distinct and miR-277 decreases with age

As I was able to show that miR-277 is strongly expressed in the adult fly my next question was if the expression level of miR-277 especially and 80 other microRNAs change during adult life. This can demonstrate that the fly has the ability to regulate microRNA expression probably according to changing demands during life.

We generated expression profiles using a panel of the 80 most abundant microRNAs with RNA extracted on day 1 of lifespan analysis and on the day when 50% of the flies were dead (see also 4.5; chronologic lifespan was recorded from parallel cohorts). After reverse transcription the cDNA was examined via RT-qPCR (real time quantitative PCR). Roughly 40 microRNAs were detectable in all samples. A clustering analysis of the ct-values showed that generally microRNA profiles of young and old flies were distinct and that miR-277 decreases during adult life (Fig. 4.6 and Fig. 4.5). Further changes include an upregulation of miR-14 and miR-8 as well as downregulation of miR-276a and b. We validated these results by Northern Blotting (Romy Böttcher).

One hypothesis is that miR-277 downregulates certain processes in the fly that are important especially at later points of life, which become derepressed upon downregulation of miR-277 itself.

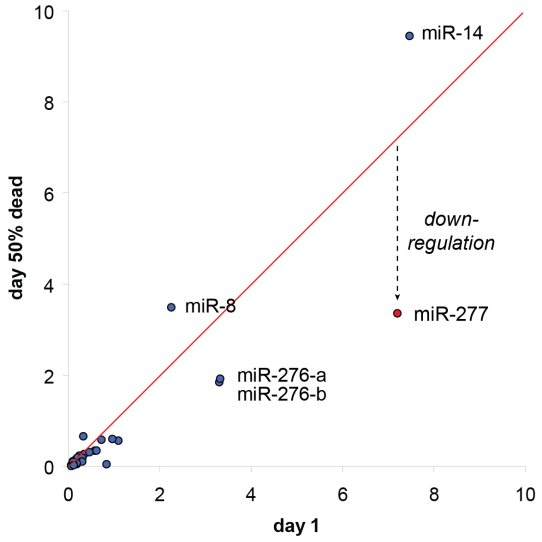


Figure 4.5: **MiR-277 is downregulated with age in *Drosophila*.** Shown are the expression values of the 40 most abundant microRNAs in young and aged wildtype control flies (*yw*) on high sugar high protein food (HSHP) as result of real time quantitative PCR.

Expression values	*yw*(day50%dead)	*yw*(day1)	84(day50%dead)	84(day1)
miR-277	3.9	7.0	7.1	7.8
miR-14	9.7	7.6	7.6	6.3
miR-8	2.4	2.3	3.1	1.7

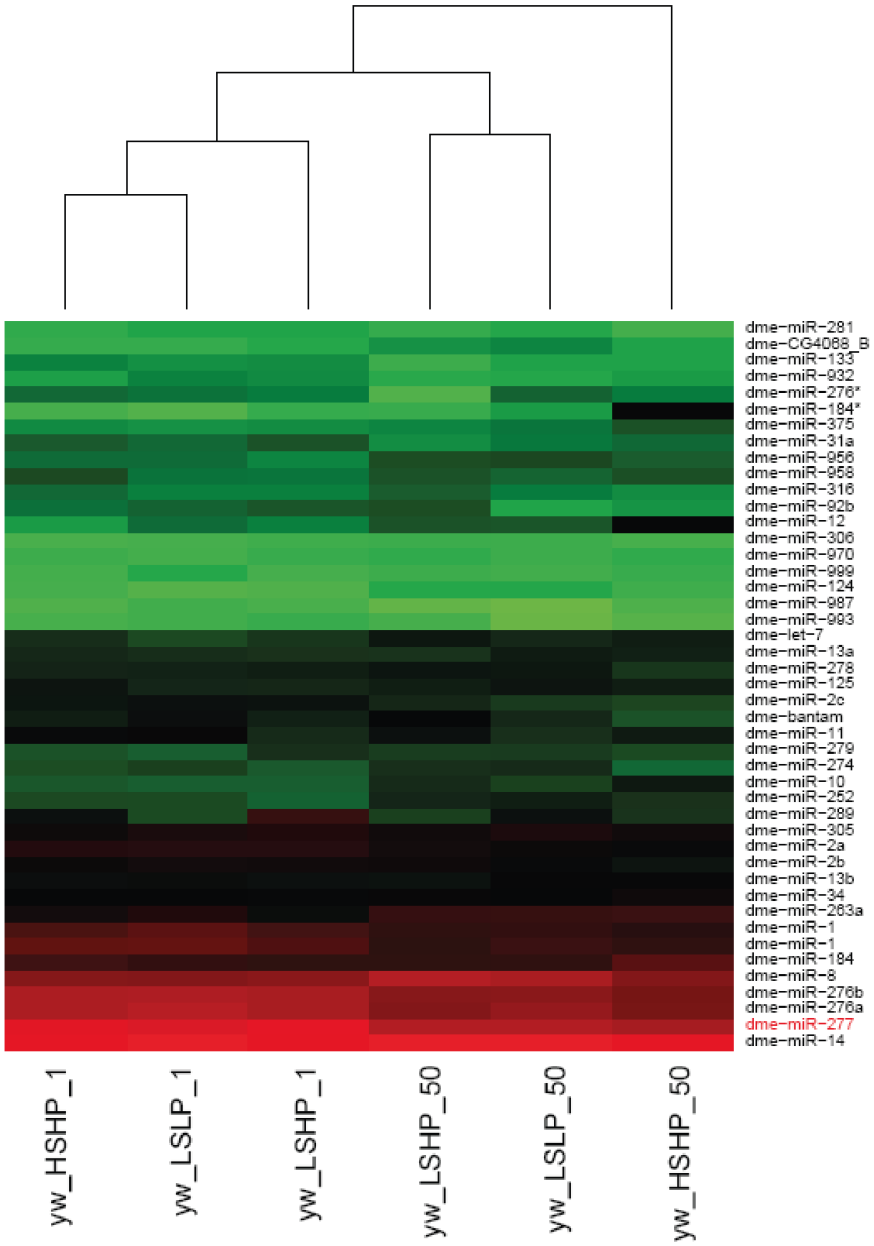


Figure 4.6: **Micro RNA profiles of young and old flies are distinct. MiR-277 is down-regulated with age.** Shown is a clustering analysis of the 40 most abundant microRNAs in young (1) and aged (50) wildtype control flies (*yw*) on three different food regimes (LSLP, LSHP, HSHP) as a result of real time quantitative PCR.

4.5 Analysis of the role of miR-277 for lifespan

4.5.1 Constitutive expression of miR-277 blunts the response to dietary restriction for sugar and protein and decreases lifespan on food with excess protein

My next step was to examine what happens if I impair the observed down-regulation of miR-277 in the fly. Are there visible effects on the lifespan? Additionally I tested how these flies react to different nutritional conditions compared to wildtype flies.

Therefore we generated transgenic fly lines that express miR-277 under the control of a constitutive promoter. For the lifespan analysis series and following experiments 0 - 1 day (after eclosion) old male flies of the constitutively miR-277 expressing fly line (pKF84=ubi-pr.mini-miR-277) and the wildtype fly line (w^{1118}) were set on low sugar low protein food (LSLP), low sugar high protein food (LSHP) and high sugar high protein food (HSHP).

In Fig. 4.7C there are shown the lifespan curves for wildtype and miR-277 overexpressing flies on HSHP.

It is known that dietary restriction (DR), in this case food conditions with LSLP, extends lifespan in many organisms (reviewed in Fontana et al. (2010)). I could confirm this fact for the wildtype flies (w^{1118}) (Fig. 4.7A; Table 4.2). In contrast, flies with constitutive expression of miR-277 (ubi-miR-277) showed no strong lifespan extension on LSLP food. (Fig. 4.7A; Table 4.2).

Next I tested the effect on the lifespan of the flies if I provide them LSHP food, i.e. an unbalanced ratio of sugar an protein. As expected wildtype flies showed a shortened lifespan under these conditions (Fig. 4.7B, Table 4.2). More importantly there was a stronger decrease of lifespan visible for the miR-277 overexpressing flies (Fig. 4.7B; Table 4.2).

Based on these results I could assume that if miR-277 downregulation is impaired, flies are much more sensitive to nutritional condition, like sugar and protein content of the applied food and therefore miR-277 probably plays a role for regulation of the metabolism of nutrients in the fly.

4.5.2 Fat body specific expression of miR-277 decreases lifespan on food with excess of protein

It is known that the metabolic processes of *Drosophila* partially take place in the fat body, the equivalent of the mammalian white adipose tissue and liver. Géminard et al. showed for example that the fat body and the brain of the fly are coupled in ingestion and processing of nutritional signals (Géminard et al., 2009).

To find out in which tissue the regulation by miR-277 takes place that leads to the

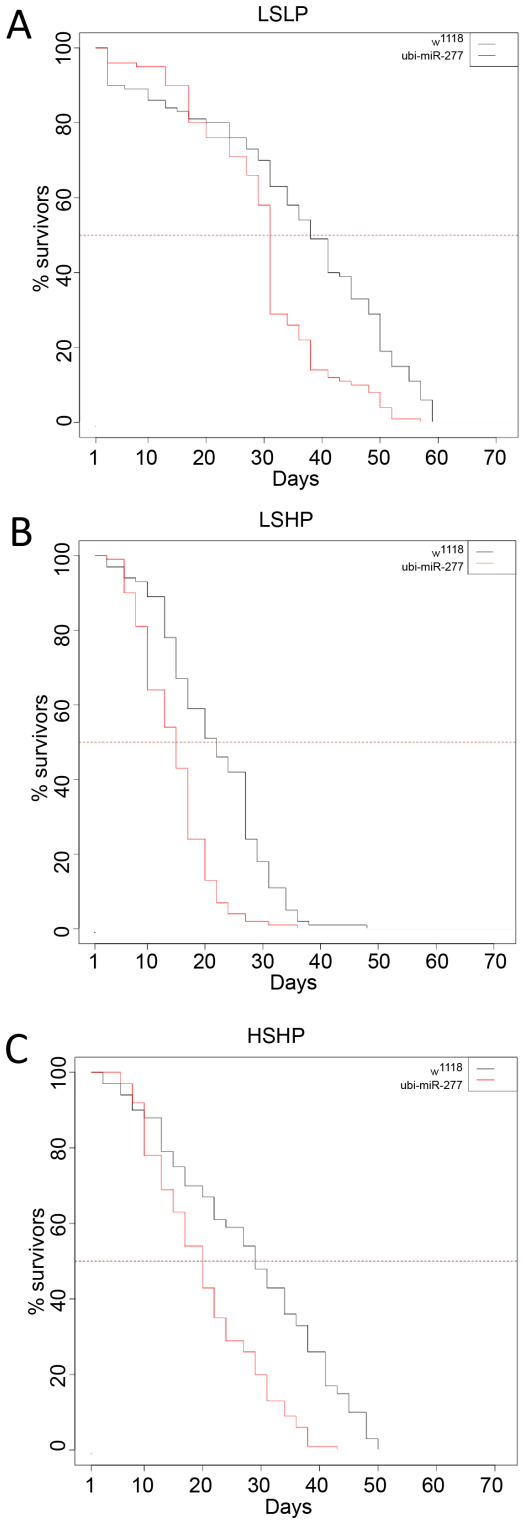

A
LSLP
w1118
ubi-miR-277
% survivors
0
20
40
60
80
100
1
10
20
30
40
50
60
70
Days
B
LSHP
w1118
ubi-miR-277
% survivors
0
20
40
60
80
100
1
10
20
30
40
50
60
70
Days
C
HSHP
w1118
ubi-miR-277
% survivors
0
20
40
60
80
100
1
10
20
30
40
50
60
70
Days

Figure 4.7: **A) Constitutively miR-277 expressing flies show only a slight lifespan extension upon dietary restriction for sugar and protein compared to wildtype control flies (w^{1118}).** Shown is the lifespan analysis of male flies of the constitutively miR-277 expressing line and the wildtype control line on low sugar low protein food (LSLP).
B) Constitutively miR-277 expressing flies show a decrease of lifespan on food with dietary restriction for sugar but excess protein compared to wildtype control flies (w^{1118}). Shown is the lifespan analysis of male flies of the constitutively miR-277 expressing line and the wildtype control line on low sugar high protein food (LSHP).
C) Constitutively miR-277 expressing flies and wildtype control flies (w^{1118}) show a lifespan comparable to conditions on standard lab food. Shown is the lifespan analysis of male flies of the constitutively miR-277 expressing line and the wildtype control line on high sugar high protein food (HSHP).

lifespan shortening phenotype I again took advantage of the UAS-GAL4 system and generated a fly line that was able to express miR-277 under the control of the GAL4 factor. Therefore I subcloned a miR-277 sequence into the pUASp-vector and tested the plasmid via cotransfection with a construct expressing GAL4 under the control of the *Drosophila* tubulin promotor in *Drosophila* Schneider S2 cells. Next the plasmid was sent for embryo injection and several homozygous fly lines were recovered. The flies were crossed to a fat body GAL4-driver line and a nervous system GAL4-driver line and the male progeny was used for lifespan analysis.

I could see a lifespan shortening on food with excess of protein (LSHP and HSHP) if miR-277 is overexpressed in the fat body compared to the control (UAS-miR-277 without any source of GAL4). In the case of overexpression of miR-277 in the nervous system there was no lifespan shortening visible on food with excess of protein. Surprisingly miR-277 overexpression in the nervous system even led to an extension of lifespan compared to the control (Fig. 4.8A and B; Table 4.2); this effect could be due to background effects. These observations suggest that the miR-277 regulation resulting in lifespan shortening may take place in the fat body amongst others. However the effect is weaker than the ubiquitous overexpression of miR-277, apparently other tissues may be involved.

4.5.3 Expression of an artificial miR-277 mRNA-target extends lifespan on food with dietary restriction for sugar but not for protein

As wildtype flies showed a shortened lifespan on food with high protein content and flies with overexpression of miR-277 showed an even stronger lifespan reduction, it would be interesting to examine what happens if the microRNA is caught by an artificial target in a wildtype genetic background. The expected effect would be a rescue of the lifespan shortening.

To test this I used male GFP reporter flies with two perfect matches for miR-277 (pKF67=ubi-GFP 2x pm miR-277), GFP reporter flies with four buldged matches for miR-277 (pKF68=ubi-GFP 4x bm miR-277) and as a control GFP reporter flies without

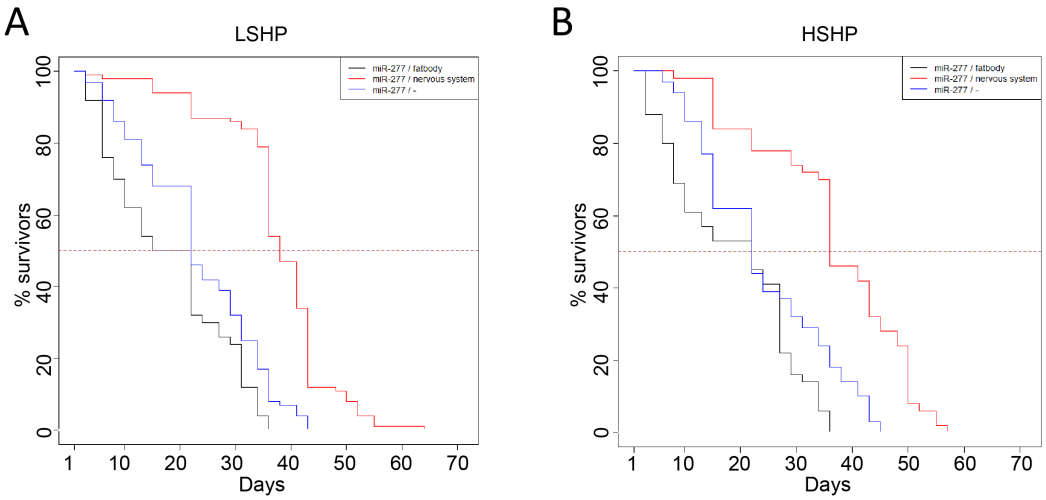


Figure 4.8: **A) Overexpression of miR-277 in the fat body shortens lifespan on food with dietary restriction for sugar but excess protein; whereas overexpression of miR-277 in the nervous system leads to lifespan extension under this nutritional conditions.** Male progeny of transgenic UAS-miR-277 flies crossed to fat body GAL4- or nervous system GAL4-driver line or to wildtype control flies (w^{1118}) were analyzed on low sugar high protein food (LSHP).
B) Overexpression of miR-277 in the fat body shortens lifespan on food with excess sugar and protein; whereas overexpression of miR-277 in the nervous system leads to lifespan extension under this nutritional conditions. Male progeny of transgenic UAS-miR-277 flies crossed to fat body GAL4- or nervous system GAL4-driver line or to wildtype control flies (w^{1118}) were analyzed on high sugar high protein food (HSHP).

any binding site for miR-277 (pKF63= ubi-GFP). Indeed the '67' fly line showed a kind of 'sponge' effect and rescue of the lifespan shortening on LSHP food compared the '68' and '63' fly line. (Fig. 4.9A; Table 4.2) (Constructs for transgenic fly lines see Förstemann et al. (2007)).

Therefore I could conclude that miR-277 affects lifespan in a nutrition dependent manner in *Drosophila*.

4.5.4 Lifespan shortening on LSHP food is not due to elevated BCAA levels specifically

My next question was what the targets of miR-277 are and how their regulation influences the metabolism of the fly.

As I could show that the enzymes of the degradation pathway of BCAAs are direct targets of miR-277 (see 4.6.1 and 4.6.2), my intention for further lifespan analysis series was to mimic the effect of constitutive expression of miR-277 by adding the BCAAs leucine, isoleucine and valine or as a control an isocaloric mix of three other essential amino acids (OEAAs: histidine, threonine, phenylalanine) to LSLP and analyse the lifespan of male wildtype flies (w^{1118}) on this condition. An overexpression of miR-277

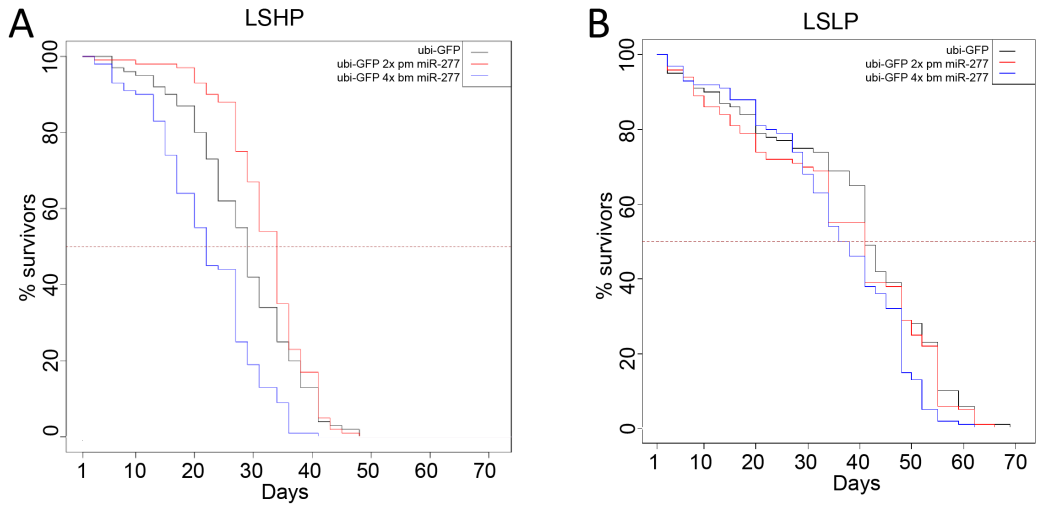


Figure 4.9: **A) GFP reporter flies with two perfect binding sites for miR-277 show an increase of lifespan on food with dietary restriction for sugar and excess of protein; whereas GFP reporter flies with four buldged binding sites for miR-277 show lifespan shortening under this nutritional conditions.** Shown is the lifespan analysis of male GFP reporter flies with two perfect binding sites for miR-277 (ubi-GFP 2x pm miR-277), with four buldged binding sites for miR-277 (ubi-GFP 4x bm miR-277) and with no binding sites (ubi-GFP) on low sugar high protein food (LSHP).
B) GFP reporter flies with two perfect binding sites and four buldged binding sites for miR-277 show lifespan extension on food with dietary restriction for sugar and protein. Shown is the lifespan analysis of male GFP reporter flies with two perfect binding sites for miR-277 (ubi-GFP 2x pm miR-277), with four buldged binding sites for miR-277 (ubi-GFP 4x bm miR-277) and with no binding sites (ubi-GFP) on low sugar low protein food (LSLP).

should lead to inhibition of the degradation enzymes and consequently to elevated levels of BCAAs. I observed a slight lifespan shortening but this effect also appeared on the control food (Fig. 4.10; Table 4.2). Thus the lifespan shortening effect is not due to elevated BCAAs levels specifically and the result likely reflects just the increased caloric content of the supplemented LSLP food.

Nicklin et al. showed 2009 that there is a bidirectional transporter in mice that regulates the simultaneous efflux of glutamine out of cells and transport of leucine and other essential amino acids into cells (Nicklin et al., 2009). The rapid efflux of glutamine in the presence of essential amino acids is the rate-limiting step that activates mTOR , the mammalian TOR-kinase. MTOR regulates protein translation, cell growth and autophagy. Based on these results my next intention was to add, in addition to the BCAAs, glutamine to LSLP food and in this way improve an uptake of BCAAs. I could observe no significant differences for the lifespan analysis curves if I applied LSLP + BCAAS + glutamine (as a control: LSLP + BCAAS + lysine) or LSLP + OEAAs (histidine, threonine, phenylalanine) + glutamine to male wildtype flies (w^{1118}). All mentioned food conditions led to lifespan shortening compared to LSLP food (Fig. 4.11).

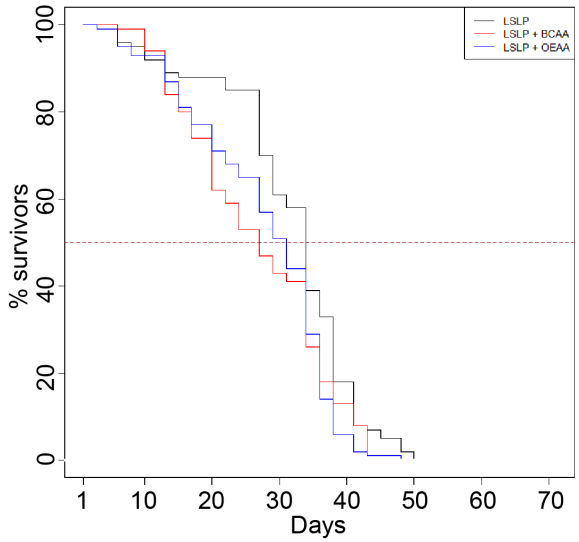


Figure 4.10: **Addition of excess amino acids to food with dietary restriction for sugar and protein shortens lifespan.** Shown is the lifespan analysis of male wildtype flies (w^{1118}) on low sugar low protein food (LSLP) with addition of the three branched-chain amino acids leucine, isoleucine and valine or three another essential amino acids (histidine, phenylalanine, threonine).

Grandison et al. published that methionine acts in combination with one or more other essential amino acids to shorten lifespan with full feeding (Grandison et al., 2009). They could show that adding back all essential amino acids to restricted food except methionine restored lifespan to the level corresponding to DR. Thus there is an imbalance in the ratio of amino acids in yeast and some consequence of this imbalance decreases lifespan with full feeding. I could observe no significant differences for the lifespan analysis curves if I applied following food conditions to male wildtype flies (w^{1118}): LSLP + BCAA + methionine; LSLP + BCAA w/o methionine; LSLP + OEAA (histidine, threonine, phenylalanine) + methionine; LSLP + OEAA (histidine, threonine, phenylalanine) w/o methionine. All mentioned food conditions led to lifespan shortening compared to LSLP food (Fig. 4.12).

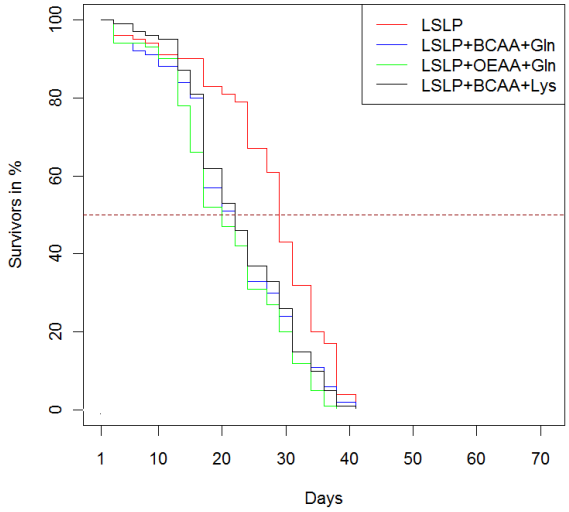


Figure 4.11: **Addition of excess amino acids (and glutamine or lysine) to food with dietary restriction for sugar and protein shortens lifespan.** Shown is the lifespan analysis of male wildtype flies (w^{1118}) on low sugar low protein food (LSLP) with addition of the three branched-chain amino acids leucine, isoleucine and valine or three another essential amino acids (histidine, phenylalanine, threonine) and glutamine or lysine.

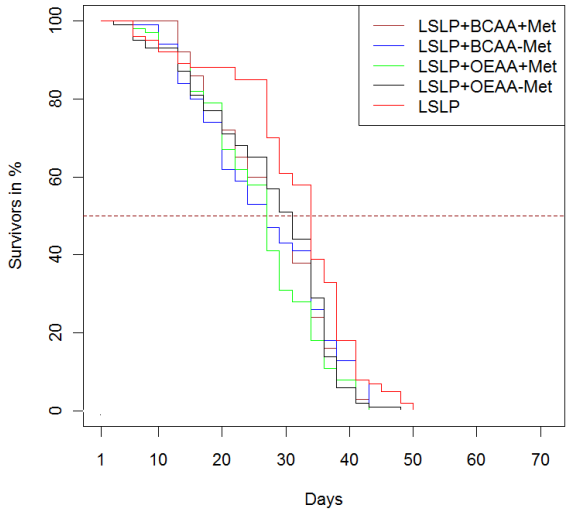


Figure 4.12: **Addition of excess amino acids (and methionine) to food with dietary restriction for sugar and protein shortens lifespan.** Shown is the lifespan analysis of male wildtype flies (w^{1118}) on low sugar low protein food (LSLP) with addition of the three branched-chain amino acids leucine, isoleucine and valine or three another essential amino acids (histidine, phenylalanine, threonine) and methionine.

fly line	food	mean	std. error
'*yw*' (w^{1118} backcrossed in *yw*)	LSLP	33.83	1.74
'*yw*' (w^{1118} backcrossed in *yw*)	LSHP	19.08	0.91
'*yw*' (w^{1118} backcrossed in *yw*)	HSHP	25.5	1.35
ubi-miR-277 (w^{1118} background crossed in *yw*)	LSLP	27.31	1.36
ubi-miR-277 (w^{1118} background crossed in *yw*)	LSHP	12.24	0.65
ubi-miR-277 (w^{1118} background crossed in *yw*)	HSHP	17.9	0.96
yw	LSLP	22.8	1.26
yw	LSHP	13.85	0.77
yw	HSHP	20.05	0.87
ubi-miR-277 (*yw* background)	LSLP	12.3	0.95
ubi-miR-277 (*yw* background)	LSHP	5.8	0.49
ubi-miR-277 (*yw* background)	HSHP	14.3	0.83
ubi-GFP (*yw* background)	LSLP	39.07	1.72
ubi-GFP (*yw* background)	LSHP	28.32	0.95
ubi-GFP 2x pm miR-277 (*yw* background)	LSLP	36.49	1.75
ubi-GFP 2x pm miR-277 (*yw* background)	LSHP	32.28	0.73
ubi-GFP 4x bm miR-277 (*yw* background)	LSLP	35.63	1.43
ubi-GFP 4x bm miR-277 (*yw* background)	LSHP	22.2	0.88
RNAi CG5599 / fat body (w^{1118} background)	LSLP	32.48	2.7
RNAi CG5599 / fat body (w^{1118} background)	LSHP	30.76	2.03
RNAi CG5599 / fat body (w^{1118} background)	HSHP	33.1	2.1
RNAi CG8199 / fat body (w^{1118} background)	LSLP	46.95	1.69
RNAi CG8199 / fat body (w^{1118} background)	LSHP	38.75	1.45
RNAi CG8199 / fat body (w^{1118} background)	HSHP	41.82	1.65
- / fat body (w^{1118} background)	LSLP	41.81	1.54
- / fat body (w^{1118} background)	LSHP	18.27	1.14
- / fat body (w^{1118} background)	HSHP	26.91	1.39
miR-277 / fat body (w^{1118} background)	LSHP	18.1	1.55
miR-277 / fat body (w^{1118} background)	HSHP	18.76	1.61
miR-277 / nervous system (w^{1118} background)	LSHP	34.54	1.11
miR-277 / nervous system (w^{1118} background)	HSHP	32.71	2.25
miR-277 / - (w^{1118} background)	LSHP	27.94	1.34
miR-277 / - (w^{1118} background)	HSHP	36.78	1.81
w^{1118}	LSLP	28.46	1.09
w^{1118}	LSLP+BCAA	24.08	1.07
w^{1118}	LSLP+OEAA	28.46	1.09

Table 4.2: Lifespan analysis series: mean values and standard errors. Depicted in grey are those fly lines whose lifespan analysis curves are not shown.

4.5.5 RNAi of miR-277 targets does not lead to lifespan shortening on LSHP food

Another attempt to mimic the effect of constitutive expression of miR-277 was to directly ablate the transcripts of known miR-277 targets, CG8199 and CG5599, via RNAi. I again employed the UAS-GAL4 system to deplete CG8199 and CG5599 expression in the fat body. Therefore I crossed a fat body GAL4-driver line with UAS-hairpin-CG8199 and UAS-hairpin-CG5599 lines and used the male progeny for lifespan analysis. Upon this knock down I would also expect a lifespan shortening effect as observed upon constitutive expression of miR-277.

However there was no apparent lifespan shortening on LSHP and HSHP food visible. Surprisingly depletion of CG8199 even led to extension of life span; this may be due to background effects. (Fig. 4.13A-C; Table: 4.2) Ubiquitous knock down of CG5599 and CG8199 expression by a tubulin-GAL4 driver line was lethal.

4.6 Transcriptome analysis of miR-277 targets

4.6.1 BCAA degradation enzymes are downregulated upon constitutive expression of miR-277

To further analyze the molecular basis of the lifespan shortening induced by miR-277, I determined the corresponding changes in the transcriptome. It is known that miRNAs regulate their target mRNAs by destabilization and translational inhibition.

Therefore I again used the RNA samples (see 4.4) extracted of whole male wildtype (*yw*) and additionally miR-277 overexpressing flies (pKF84= ubi-pr. mini-miR-277) on day 1 after eclosion and on the day when 50% of the flies were dead, then analyzed them on Affymetrix *Drosophila* 2.0 arrays and categorized the effects of miR-277 overexpression in Gene Ontology (GO) terms according to molecular function (> 1.5 fold change; $> 95\%$ confidence). As microRNAs are predominantly negative regulators I focused on downregulated genes. Flies with a constitutive expression of miR-277 show mainly a significant downregulation of genes annotated with a GO term describing metabolic processes. Three of the TOP 15 GO-terms describe the metabolism of BCAAs (valine metabolic process, leucine metabolic proces and branched chain family amino acid metabolic process). (Fig. 4.14). Four also include BCAA metabolism (cellular ketone metabolic process, oxoacid metabolic process, organic acid metabolic process and carboxylic acid metabolic process). It can be assumed that miR-277 regulates genes involved in the degradation of BCAAs (see Table 4.3); this is also consistent with previous target predictions (Stark et al., 2003).

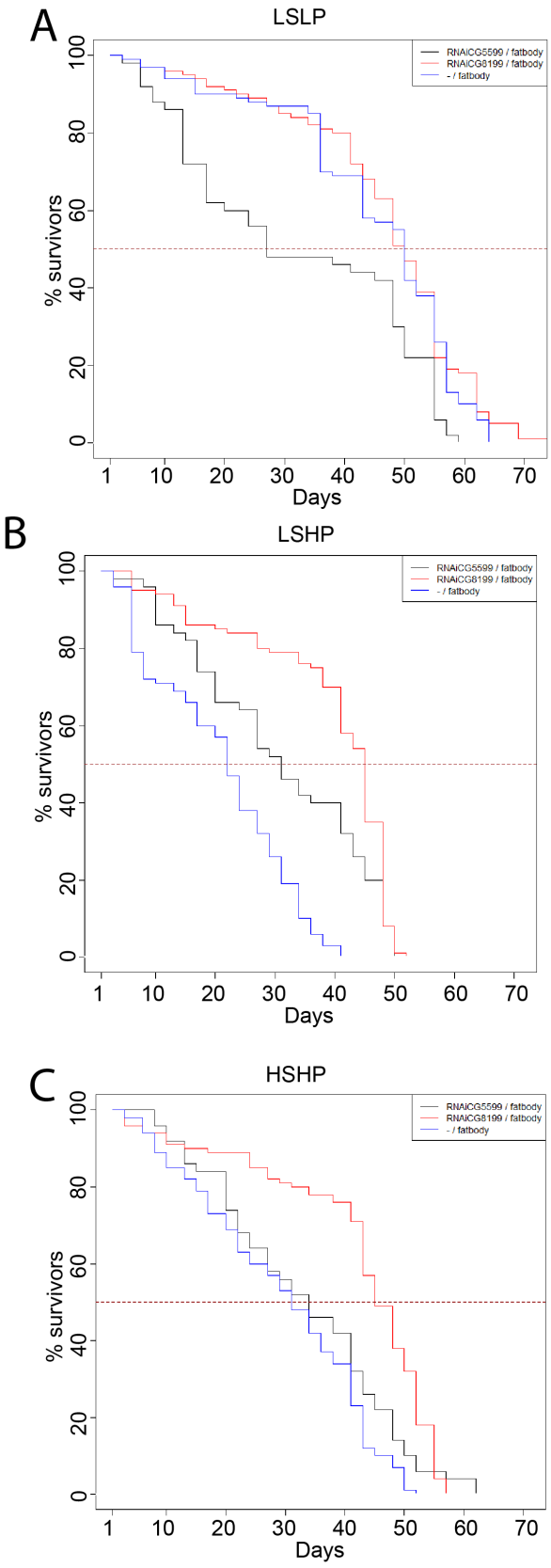
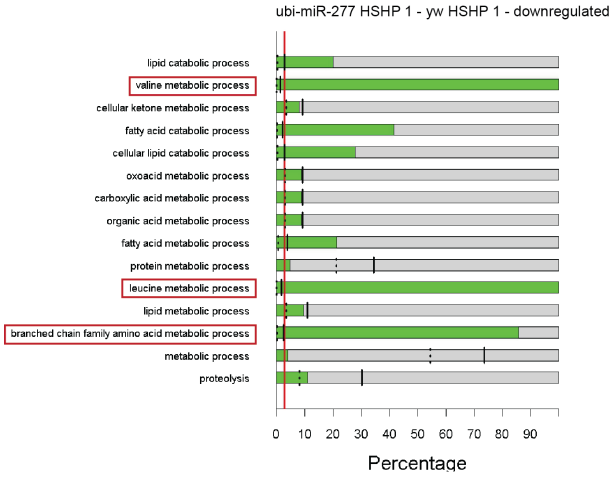
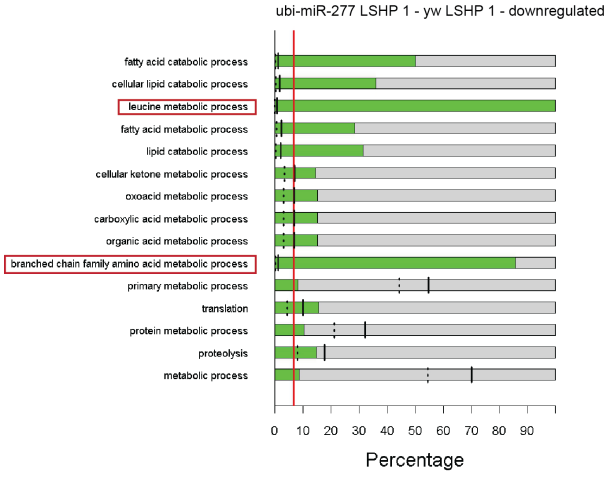

A
LSLP
RNAiCG5599 / fatbody
RNAiCG8199 / fatbody
- / fatbody
% survivors
Days
B
LSHP
RNAiCG5599 / fatbody
RNAiCG8199 / fatbody
- / fatbody
% survivors
Days
C
HSHP
RNAiCG5599 / fatbody
RNAiCG8199 / fatbody
- / fatbody
% survivors
Days

Figure 4.13: **A) Flies with knock down of CG5599 show not as strong lifespan extension upon dietary restriction for sugar and protein compared to control flies and flies with knock down for CG8199.** Shown is the lifespan analysis of male progeny of UAS-RNAi-CG5599 or -CG8199 flies or control flies (w^{1118}) crossed to fat body GAL4-driver line on low sugar low protein food (LSLP).
B) Flies with knock down of CG5599 and CG8199 show no decrease of lifespan on food with dietary restriction for sugar but excess protein compared to control flies. Shown is the lifespan analysis of male progeny of UAS-RNAi-CG5599 or -CG8199 flies or control flies (w^{1118}) crossed to fat body GAL4-driver line on low sugar high protein food (LSHP).
C) Flies with knock down of CG5599 and CG8199 show no decrease of lifespan on food with excess sugar and protein compared to control flies. Shown is the lifespan analysis of male progeny of UAS-RNAi-CG5599 or -CG8199 flies or control flies (w^{1118}) crossed to fat body GAL4-driver line on high sugar high protein food (HSHP).

genes	**fold-change day 1 HSHP**	**fold-change day 1 LSHP**	**fold-change day 1 LSLP**
CG10399	0.38	0.33	0.56
CG3267	0.19	0.20	0.55
CG6543	0.43	0.32	0.69
CG15093	0.43	0.37	0.48
CG6638	0.24	0.20	0.31
CG8199	0.22	0.32	0.43
CG5599	0.25	0.15	0.48
CG1673	0.74	1.1	1.09
CG6984	0.32	0.63	–
CG2118	0.32	0.31	0.41
CG17896	0.47	0.35	0.63
genes	**fold-change day 50%dead HSHP**	**fold-change day 50%dead LSHP**	**fold-change day 50%dead LSLP**
CG10399	0.44	0.51	0.55
CG3267	0.23	0.08	0.05
CG6543	0.18	0.18	0.27
CG15093	0.24	0.26	0.24
CG6638	0.57	0.21	0.23
CG8199	0.24	0.18	0.28
CG5599	0.17	0.17	0.14
CG1673	0.63	0.44	1.51
CG6984	0.27	0.35	–
CG2118	0.43	0.23	0.26
CG17896	0.32	0.27	0.24

Table 4.3: Enzymes of the BCAA degredation pathway are downregulated upon constitutive expression of miR-277. Shown are the results of microarray analysis. Comparison of flies with constitutive expression of miR-277 to wildtype flies at two different timepoints on different food regimes.


ubi-miR-277 HSHP 1 - yw HSHP 1 - downregulated
lipid catabolic process
valine metabolic process
cellular ketone metabolic process
fatty acid catabolic process
cellular lipid catabolic process
oxoacid metabolic process
carboxylic acid metabolic process
organic acid metabolic process
fatty acid metabolic process
protein metabolic process
leucine metabolic process
lipid metabolic process
branched chain family amino acid metabolic process
metabolic process
proteolysis
0 10 20 30 40 50 60 70 80 90
Percentage


ubi-miR-277 LSHP 1 - yw LSHP 1 - downregulated
fatty acid catabolic process
cellular lipid catabolic process
leucine metabolic process
fatty acid metabolic process
lipid catabolic process
cellular ketone metabolic process
oxoacid metabolic process
carboxylic acid metabolic process
organic acid metabolic process
branched chain family amino acid metabolic process
primary metabolic process
translation
protein metabolic process
proteolysis
metabolic process
0 10 20 30 40 50 60 70 80 90
Percentage

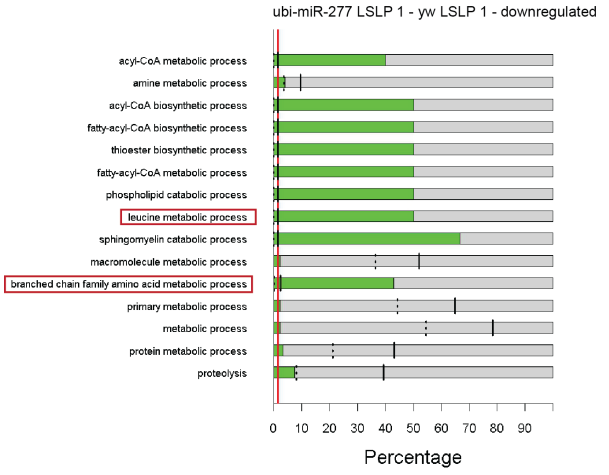

ubi-miR-277 LSLP 1 - yw LSLP 1 - downregulated
acyl-CoA metabolic process
amine metabolic process
acyl-CoA biosynthetic process
fatty-acyl-CoA biosynthetic process
thioester biosynthetic process
fatty-acyl-CoA metabolic process
phospholipid catabolic process
leucine metabolic process
sphingomyelin catabolic process
macromolecule metabolic process
branched chain family amino acid metabolic process
primary metabolic process
metabolic process
protein metabolic process
proteolysis
0 10 20 30 40 50 60 70 80 90
Percentage

Figure 4.14: **Constitutive expression of miR-277 in flies leads to down-regulation of genes that are involved in metabolic processes of BCAAs.** Effects of constitutive expression of miR-277 in adult flies (ubi-miR-277) compared to wildtype control flies (*yw*) on day1 (1). Flies were tested on 3 different food conditions (LSLP= low sugar, low protein; LSHP= low sugar, high protein; HSHP= high sugar, high protein). Down-regulated genes were categorized according to their GO-terms. Each bar represents a Gene-Ontology term. The green portion shows the percentage of changing genes within a term. The red line show the expected amount of changing genes. The dotted black lines display the amount of changing genes related to the whole genome. The continuous black lines display the amount of changing genes related to total changing genes under given conditions. Red boxes mark the GO-terms that describe metabolic processes of BCAAs.

4.6.2 Inhibition of miR-277 in *Drosophila* Schneider S2 cells shows no transcriptional changes

To find out if multiple enzymes of the degradation pathway of BCAAs are directly targeted by miR-277 or whether miR-277 is responsible for an upstream event leading to transcriptional co-regulation of the enzymes I used a 4-thio-uridine pulse-labeling and fractionation technique (also see 4.1). Changes in mRNA stability, i.e. changed degradation rates, are most obvious in the old mRNA fraction, whereas changes in transcription rates are reflected in the newly transcribed mRNA fraction.

I profiled mRNA levels after inhibition of endogenously expressed miR-277 via use of a 2'-O-methyl antisense oligo in *Drosophila* Schneider cells. This should identify targets by their increased abundance. After analyzing the three RNA pools on Affymetrix *Drosophila* 2.0 microarrays I found that only 8 genes showed significant upregulation in the total and unlabeled (old) RNA fraction as well as an increased half life after inhibition of miR-277 compared to the effect of a treatment with a control oligo (> 1.5 fold change; $> 95\%$ confidence). No significant changes were detected in the newly transcribed fraction. Consistent with the results obtained in flies, all of the upregulated genes were predicted miR-277 targets and involved in the degradation of BCAAs (Table 4.4).

I could conclude that the upregulation is due to stabilization of the corresponding mRNAs rather than an increased rate of transcription and this strongly argued that all BCAA degradation enzymes are direct targets of miR-277 in *Drosophila* Schneider S2 cells. Thus regulation of this enzymatic cascade by miR-277 occurs at a posttranscriptional level.

In a cumulative distribution graph of the changes upon miR-277 inhibition we saw a general shift towards increased steady-state levels for predicted miR-277 targets, and this shift was more pronounced in the older RNA fraction, indicating a post-transcriptional mechanism (Fig. 4.16).

One notable exception is the first enzyme of the BCAA degradation cascade, CG1673, a transaminase that converts amongst others leucine to α-keto isocaproic acid (KIC). The abundance of this mRNA changed neither in the transgenic flies with constitutive

miR-277 expression (Table 4.3, day 1), nor in the Schneider cells upon miR-277 inhibition (for an overview of the degradation pathway of BCAAs see Fig. 4.15).

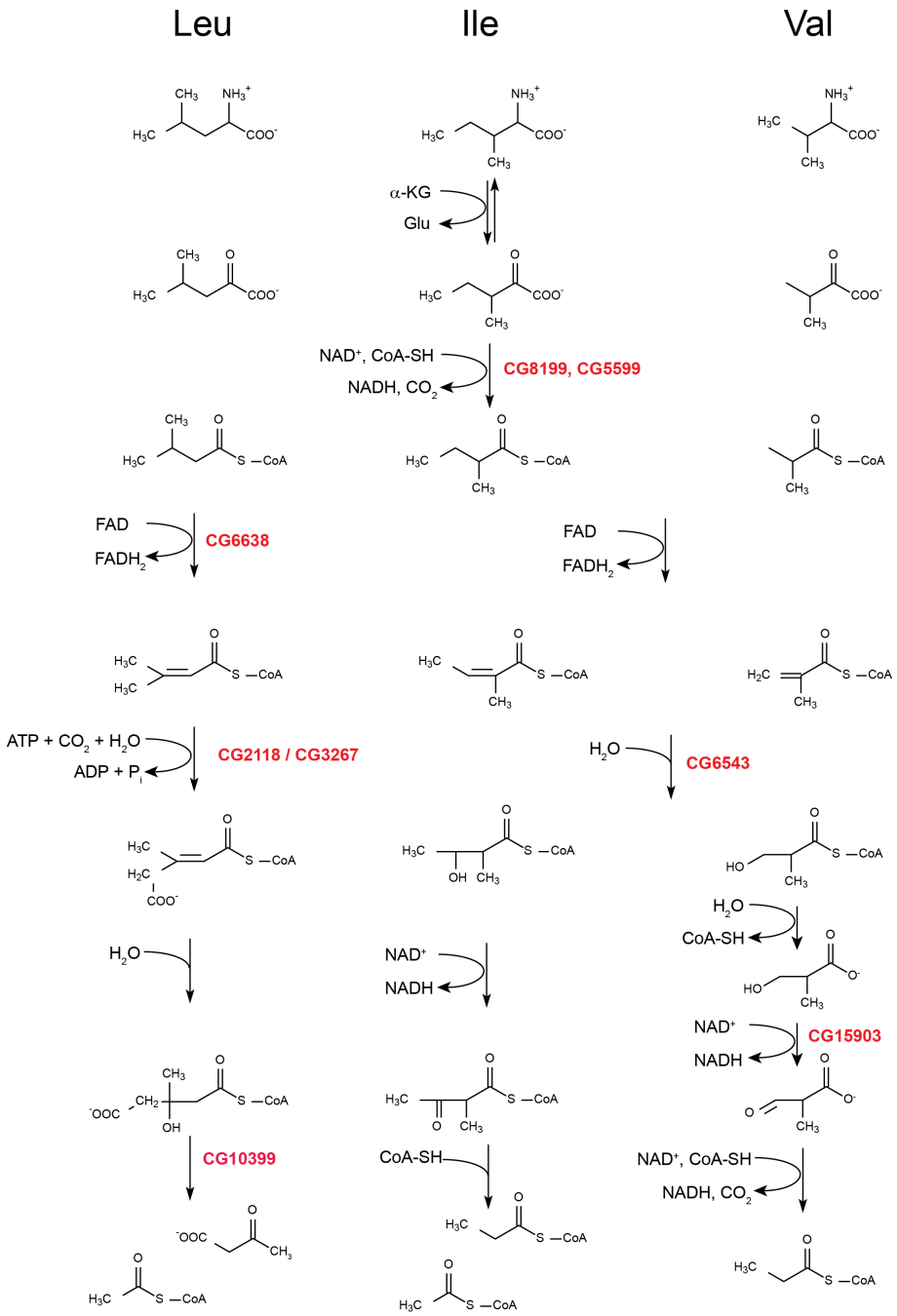


Figure 4.15: **Overview of the degradation of branched-chain amino acids.** The validated targets of miR-277 are marked in red.

Gene	**Significant changes as-miR-277 vs. control:** RNA fractions			**Half-lifes (minutes):** as-oligo	
	labeled	unlabeled	total	control	as-miR-277
CG10399	no	yes	yes	151	293
CG3267	no	yes	yes	320	602
CG6543	no	yes	yes	553	633
Atta	no	yes	no	–	–
CG15093	no	yes	yes	480	878
CG6638	no	yes	yes	529	772
CG8199	no	yes	yes	133	187
CG2118	no	yes	yes	189	295
CG5599	no	yes	yes	173	250

Table 4.4: Direct targets of miR-277

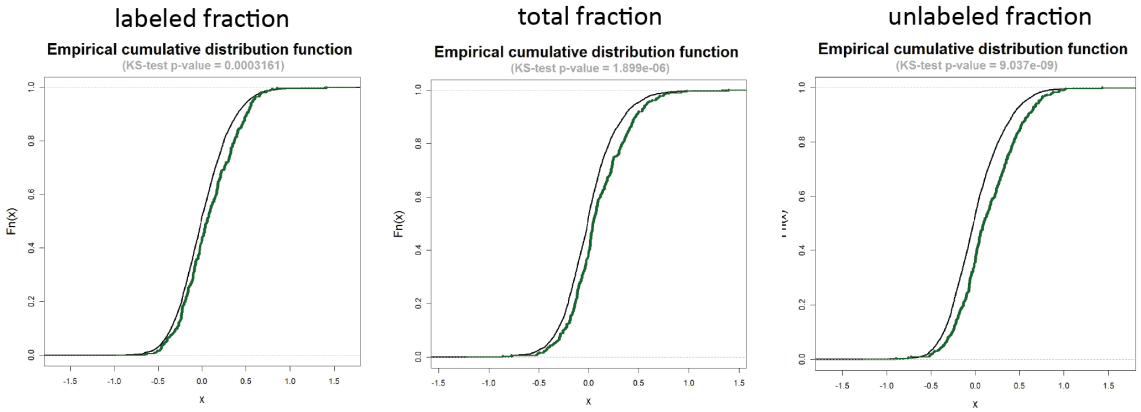


Figure 4.16: **Mir-277 target genes show an increase of expression in total an unlabeled RNA fractions after inhibiton of miR-277; they are therefore regulated post-transcriptionally and accordingly direct targets of miR-277.** Shown are results of Affymetrix *Drosophila* 2.0 array analysis (Björn Schwalb, AG Tresch) for the three different RNA fractions. Black curve: Empirical cumulative distribution of all genes of the array. Green curve: Distribution of all predicted miR-277 targets.

4.7 Profiling of ageing related genes in miR-277 overexpressing flies

Mir-277 overexpression leads to a reduced lifespan, especially on LSHP food. This effect raises the question if flies with a constitutive expression of miR-277 age faster than wildtype flies. To elucidate this I profiled the expression of ageing related genes at two timepoints for miR-277 overexpressing flies compared to wildtype flies. Therefore I extracted RNA of the transgenic fly line (ubi-pr. mini-miR-277) and wildtype flies (w^{1118}) on day 1 after eclosion and on day 15 after eclosion. After reverse transcription cDNA was examined via RT-qPCR (real time quantitative PCR). I could confirm the results of the transcriptome analysis. Overexpression of miR-277 leads to downregulation of the val-

idated targets, i.e. genes of the degradation pathway of BCAAs. Furthermore the ageing related genes are slightly up or downregulated at day 15 but there is not yet a significant difference visible between wildtype flies (w^{1118}) and the transgenic fly line (ubi-miR-277) (Fig. 4.17).

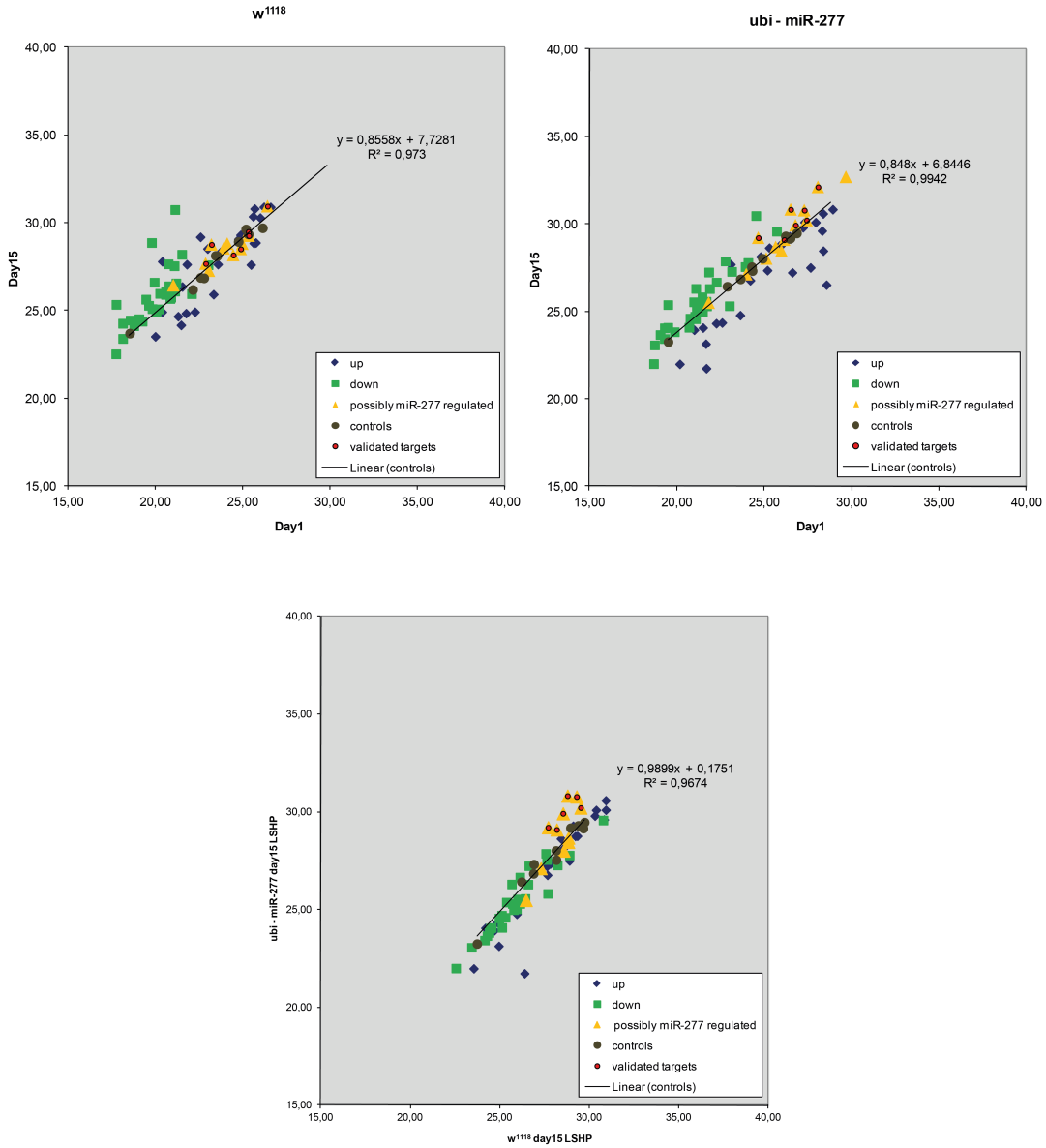


Figure 4.17: **Flies beginn to age at day 15 (panels above).Genes of BCAA degradation are downregulated in miR-277 overexpressing flies (panel below).** Shown are the Ct values of ageing related genes in wildtype flies w^{1118} and miR-277 overexpressing flies (ubi-pr. mini-miR-277) on day 1 and 15 as a result of real time quantitative PCR. Possibly miR-277 regulated genes, i.e. validated miR-277 targets and components of the TOR and IIS pathway, are marked with a yellow triangle.

4.8 Analysis of amino acid composition upon constitutive expression of miR-277 in the fly

As the transcriptome analysis revealed that the enzymes of the degradation pathway of BCAAs are direct targets and post-transcriptionally regulated by miR-277 I wanted to know if I can observe the expected accumulation of the three BCAAs leucine, isoleucine and valine upon modulation of miR-277 levels in the fly and in *Drosophila* Schneider S2 cells.

Neither constitutive expression of miR-277 in male transgenic flies nor overexpression of miR-277 in Schneider cells increased the concentration of BCAAs (Fig. 4.18A). The transgenic miR-277 expression construct is driven by a constitutive ubiquitin-promotor, leading to ubiquitous expression including germ line tissue. Since endogenous miR-277 is not expressed in the germ line, any effects of miR-277 should be most evident in this tissue. Consistent with this notion, a moderate increase in the concentration of valine was detectable in extracts prepared from the abdomen of female miR-277 overexpressing flies, leucine and isoleucine were also slightly increased (Fig. 4.18B).

These results raised the question how miR-277 overexpression represses the enzymes responsible for the degradation of BCAAs without detectably raising the concentration of the amino acids. It is conceivable that transamination can take place, because CG1673 is no direct target of miR-277 (see 4.6), but further degradation by the branched-chain α-keto acid dehydrogenase complex (BCKDH) is diminished. In this case branched chain α-keto acids (BCKAs) like KIC should build up.

Interestingly I could also observe a higher amount of threonine in the abdomen of female flies overexpressing miR-277 (Fig. 4.18B). One possible explanation could be that accumulating isoleucine in miR-277 overexpressing flies leads to a feedback inhibition of the threonine dehydratase and thus threonine is no longer metabolized. Furthermore the BCKDH is also able to degrade the product of the threonine dehydratase, 2-oxobutanoate. In flies overexpressing miR-277 the inhibition of the BCKDH could lead to a accumulation of threonine.

4.9 Measurement of triglycerides levels upon constitutive expression of miR-277 in the fly

Manipulations of the IIS and the TOR signaling in species ranging from yeast to humans dramatically influences metabolic responses, such as lipid and glucose homeostasis (Saltiel and Kahn, 2001; Tatar et al., 2003; Vellai et al., 2003). The findings of Birse et al. indicate that e.g. *Drosophila* on a high-fat diet can become obese, leading to metabolic and cardiac dysfunction. These effects are evolutionary and functionally conserved and mediated by

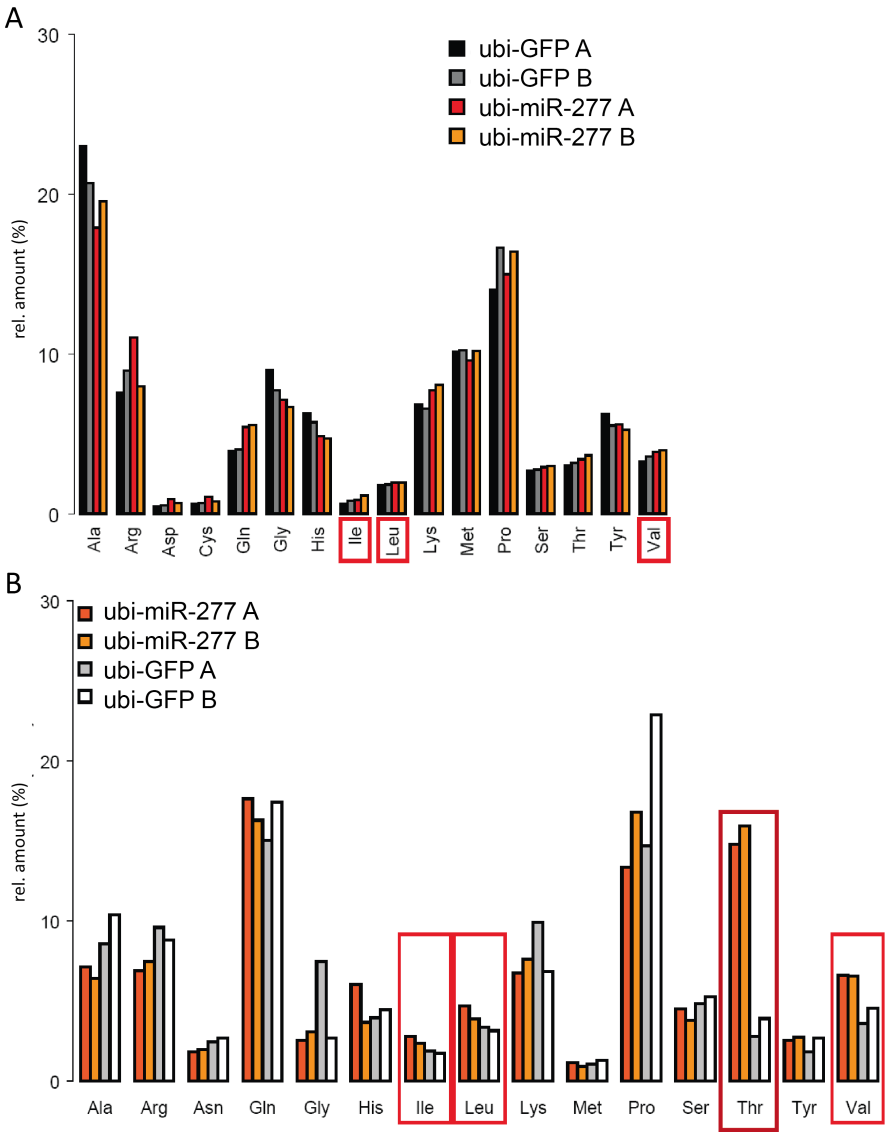


Figure 4.18: **A) The relative amounts of branched-chain amino acids leucine, isoleucine and valine don't change upon constitutive expression of miR-277 in *Drosophila* Schneider S2 cells.** Shown are the relative amounts of amino-acids extracted of *Drosophila* Schneider S2 cells after transfection of the constitutively miR-277 overexpressing construct (ubi-miR-277) compared to cells transfected with a control construct (pKF63). A and B mark the two replicates of each extract.
B) The relative amount of branched-chain amino acids leucine, isoleucine and valine change slightly upon constitutive expression of miR-277 in *Drosophila* female abdomen. The relative amount of threonine is higher. Shown are the relative amounts of amino-acids of the abdomen of adult female flies of the constitutively miR-277 overexpressing fly line (ubi-miR-277) compared to control flies (ubi-GFP). A and B mark the two replicates of each extract.

a nutrient-sensitive circuit, which includes IIS-TOR signaling and the control of lipid metabolism (Birse et al., 2010).

As I could show a role for miR-277 for modulation of the TOR-kinase (4.10), I next wanted to know if miR-277 moreover affects lipid metabolism. Therefore I measured initially the triglyceride levels of wildtype flies compared to flies that constitutively and tissue specifically overexpressed miR-277. After 7 days on LSHP food body weight and triglyceride content were determined. I did not detect changing levels of triglycerides in any sample (Table 4.5).

4.10 Analysis of the role of miR-277 for cell physiology

4.10.1 Constitutive expression of miR-277 in *Drosophila* Schneider S2 cells leads to activation of the TOR-kinase

How can metabolic changes in BCAA degradation rates lead to a shortened lifespan? Based on our analysis, we assume that constitutive expression of miR-277 reduces the capacity for metabolic clearance of BCAAs. It is already known that these amino acids activate the TOR-kinase in *Drosophila*; phosphorylation by TOR inhibits the activity of 4E-BP, which enables the mRNA cap-binding protein eIF4E to bind mRNAs leading to increased translation and shortened lifespan (Giannakou and Partridge, 2007). Therefore the TOR-kinase acts as a signaling module that coordinates growth with nutrient availability.

To test this potential link I transfected *Drosophila* Schneider-2 cells with the construct overexpressing miR-277 (pKF84= ubi-pr.mini-miR-277). This should lead to a reduced BCAA clearance and activation of the TOR pathway. Indeed I could detect an increasing level of phosphorylated 4E-BP in miR-277 overexpressing cells compared to control GFP-transfected cells (pKF63) (Fig. 4.19A). The straightforward interpretation is that constitutive expression of miR-277 shortens life span via inappropriate activation of the TOR-kinase.

Transfection of *Drosophila* Schneider S2 cells with a construct overexpressing miR-277

fly line	**weight per fly (mg)**	**triglyceride level (mg/ml)**
w^{1118}	4.4	0.8129
yw	4.8	0.6017
ubi-miR-277 (w^{1118} background)	5.3	0.6236
ubi-miR-277 (*yw* background)	4.7	0.5282
UAS-miR-277 x fat body Gal4	5.7	0.7703

Table 4.5: Measurements of triglyceride levels

with a mutation in the *-strand (pKM4) also led to an increasing level of phosphorylated 4E-BP.

4.10.2 Treatment of *Drosophila* Schneider S2 cells with leucine and KIC leads to activation of the TOR-kinase

I could show that overexpression of miR-277 leads to increased TOR-kinase activity without detectably raising the concentration of leucine. The derivate of leucine, KIC, is also known to be a strong activator of the TOR-kinase in mammals (Xu et al., 2001). Upon overexpression of miR-277 transamination occurs but further degradation by the BCKDH is diminished, consequently KIC should build up.

To answer the question if the accumulation of KIC also could lead to activation of the TOR-kinase, as published for mammals (Xu et al., 2001), I treated *Drosophila* Schneider-2 cells with an extra dose of leucine (in addition to the amino acids present in the culture medium) and KIC, extracted protein and looked again for the phosphorylation state of 4E-BP. I saw both for leucine and even more potently and more persitently for KIC an increased level of phosphorylated 4E-BP, indicating an activation of the TOR-kinase (Fig. 4.19 B).

Uptake of leucine can be limited by the availability of glutamine (Nicklin et al., 2009), but this is unlikely to have limited leucine-mediated stimulation of TOR since in normal Schneider's medium glutamine is still present in a 2.5-fold molar excess over the supplemented leucine. Thus, at equimolar extracellular concentration, KIC is a more potent activator of TOR than leucine.

Taken together, our results suggest that miR-277 alters the metabolic flux of BCAA degradation such that the BCKAs build up. Since these derivatives are more potent activators of TOR than the original amino acids, miR-277 modulates TOR activity indirectly by adjusting the clearance of BCKAs.

4.10.3 Combination of constitutive expression of miR-277 and mutation of the insulin receptor substrate *chico* is lethal

To coordinate nutrient availability with cellular and organismal growth, the TOR signaling pathway interacts with the IIS signaling system (Giannakou and Partridge, 2007). I next wanted to test a possible genetic interaction between the two pathways by combining constitutive miR-277 expression with a mutation in the insulin receptor substrate *chico* that reduces insulin signal transduction.

While a control cross of $chico^1/cyo$ flies gave offspring in the expected ratio of 2:1 for heterozygous vs. homozygous animals (62 vs. 36 animals; homozygous *cyo* flies are lethal), the combination of the transgenic miR-277 overexpression construct was synthetic

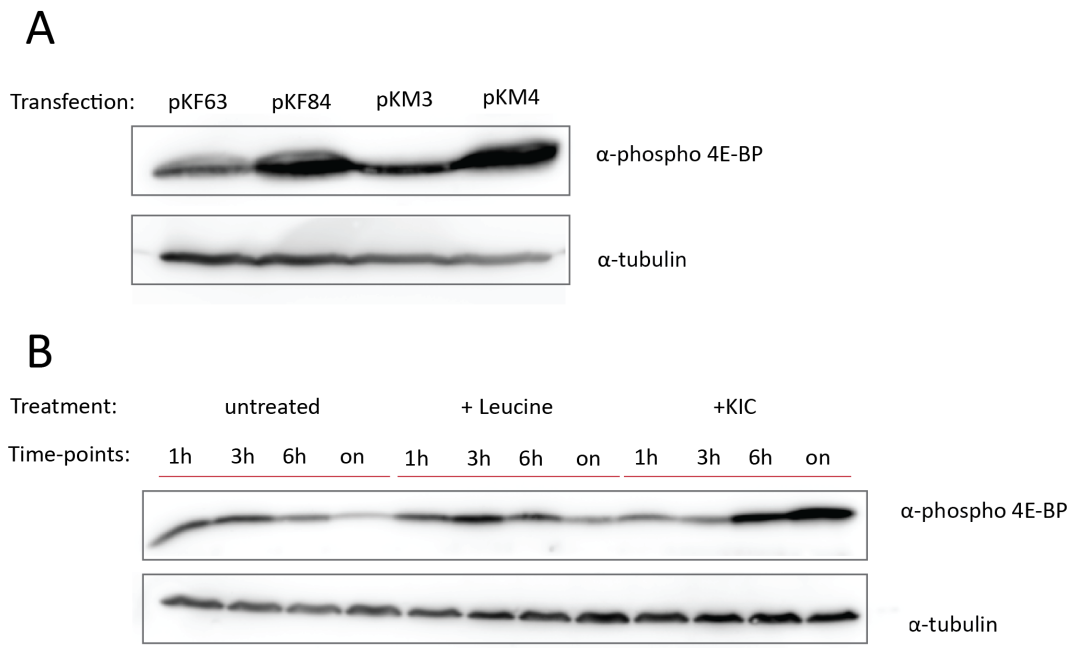


Figure 4.19: **Both constitutive expression of miR-277 and treatment with leucine and KIC leads to activation of the TOR signalling pathway in *Drosophila* Schneider S2 cells.**
A) Effect of transfection of *Drosophila* Schneider S2 cells with constructs resulting in a constitutive expression of miR-277 (pKF84) and miR-277 with mutated *-strand (pKM3, pKM4). Overexpression of GFP serves as a control. The Western Blot was probed with α-phospho 4E-BP antibody to confirm an activation of the TOR signalling pathway. α- tubulin served as a loading control.
B) Effect of treatment of Drosophila Schneider S2 cells with equivalent amounts of leucine and KIC. The Western Blot was probed with α-phospho 4E-BP antibody to confirm an activation of the TOR-signalling pathway. α- tublin served as a loading control.

lethal with a homozygous *chico*^{1} mutation (only heterozygous adult offspring observed, $n = 57$) (Fig. 4.20).

4.11 Generation of mutations of the miR-277 * strand

Drosophila miR-277 is unusual because about 2/3 of the mature miRNA are loaded in Ago2 rather than Ago1 (Förstemann et al., 2007).

To find out if this also plays a role for the lifespan phenotype of miR-277 overexpressing flies and its role for metabolic regulation we generated constructs with mutated *-strands of miR-277 (Katharina Michalik) to get a miR-277 with typical microRNA Ago1-loading properties (pKM3) and a miR-277 with typical siRNA Ago2-loading properties (pKM4) (Fig. 4.21).

The plasmids were sent for embryo injection and several homozygous fly lines were

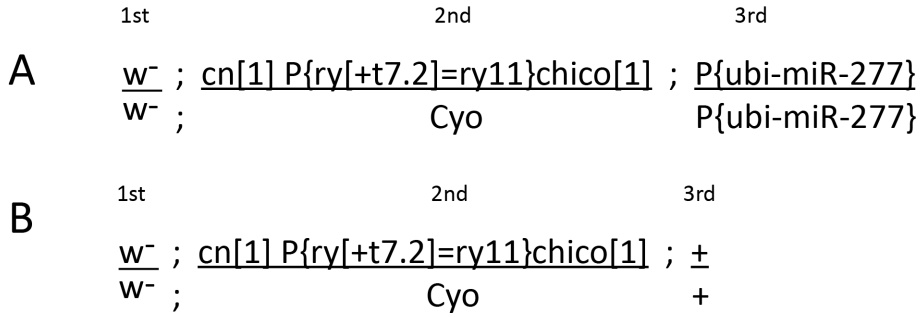


Figure 4.20: Crossing brothers and sisters of above mentioned generated fly lines, has following result shown in the table below.

Markers (expected)	cyo	+
A) chico mutant / constitutive miR-277	57 (50%)	0 (25%)
B) chico mutant / + 25% are lethal cyo/cyo!	62 (66%)	36 (33%)

recovered. Furthermore, the plasmids were tested via Northern Blot analysis for their functionality and via immunoprecipitation to test the achieved loading properties of the mutant pre-miR-277 hairpins.

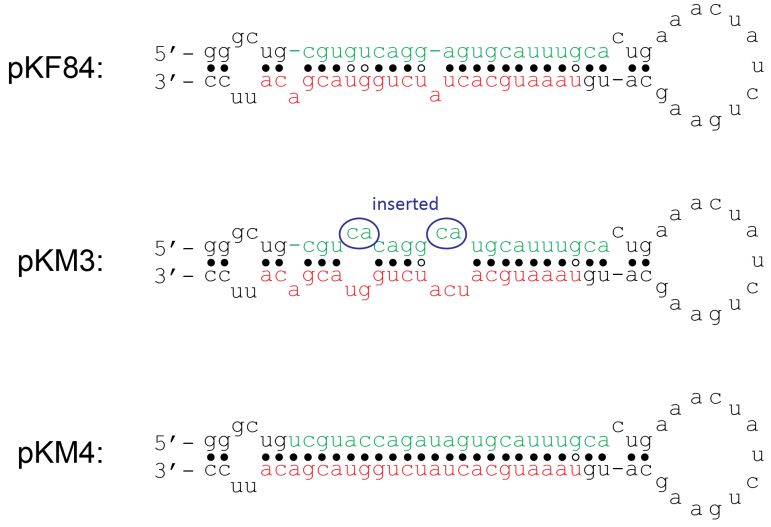


Figure 4.21: **Mutations of the miR-277 *-strand to generate miRNA like miR-277 (pKM3) and siRNA like miR-277 (pKM4)** Shown are the schemes of the miR-277 hairpin and the two mutated constructs. To generate a more miRNA like miR-277 and to shift the loading to an AGO1-RISC, the *-strand exhibits more uncomplementarity. To generate a more siRNA like miR-277 and to shift the loading to an AGO2-RISC the *-strand exhibits no uncomplementary sequences.

4.11.1 'super' - miRNA like miR-277

In the case of the pKM3 construct I could not see any overexpression of miR-277 in the Northern Blot analysis of fly extracts as well as in Northern Blot analysis after immunoprecipitation (Fig. 4.22 and Fig. 4.23).

4.11.2 'super' - siRNA like miR-277

To test the capacitiy of the generated constructs to overexpress miR-277 I extracted RNA from the abdomen of male flies of the constitutively miR-277 expressing fly lines and examined them via Northern Blot analysis. I could show that the KF84-lines as well as the KM4-lines overexpress miR-277. As a control served w^{1118} flies; I could also show that miR-277 is endogenously higher expressed in the head/thorax fraction of the fly, this also matches the expression analysis results of 4.3 (Fig. 4.22).

Next I wanted to find out if we were able to generate a mutated form of pre-miR-277 and if its loading is shifted to Ago1-RISC in the case of pKM3 and to Ago2-RISC in the case of pKM4. Therefore I transfected *Drosophila* Schneider S2 cells (stable cell line with flag-tagged Ago2 generated by Katharina Elmer) with pKF84, pKM3, pKM4 and as a control pKF63. After immunoprecipitations of Ago1 and Ago2 (Flag) miR-277 was detected via Northern Blot analysis. I could see that for pKM4 we were able to shift loading of miR-277 even more towards Ago2-RISC than wildtype pre-miR-277. For Ago1 we could see some microRNA intermediates (Fig. 4.23). It needs to be mentioned that in *Drosophila* Schneider S2 cells there is also endogenous Ago2, maybe even more abundant than the transfected construct.

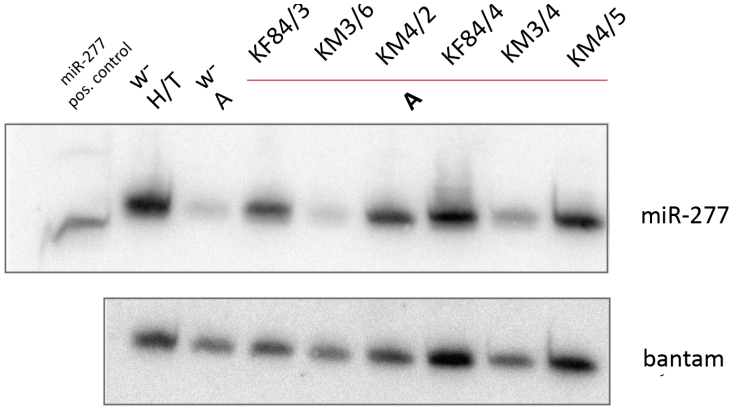


Figure 4.22: **KF84 and KM4 transgenic fly lines overexpress constitutively miR-277 in the adult abdomen.** Northern Blot of RNA extracts of abdomen of flies that constitutively express miR-277 (KF) or miR-277 with a mutated *-strand (KM3, KM4) compared to head/thorax and abdomen extract of wildtype control flies. Shown are two independent transgenic fly line for each P-element. W^{1118}flies serve as control. Also shown is the stronger endogenous expression of miR-277 in the thorax of adult flies.

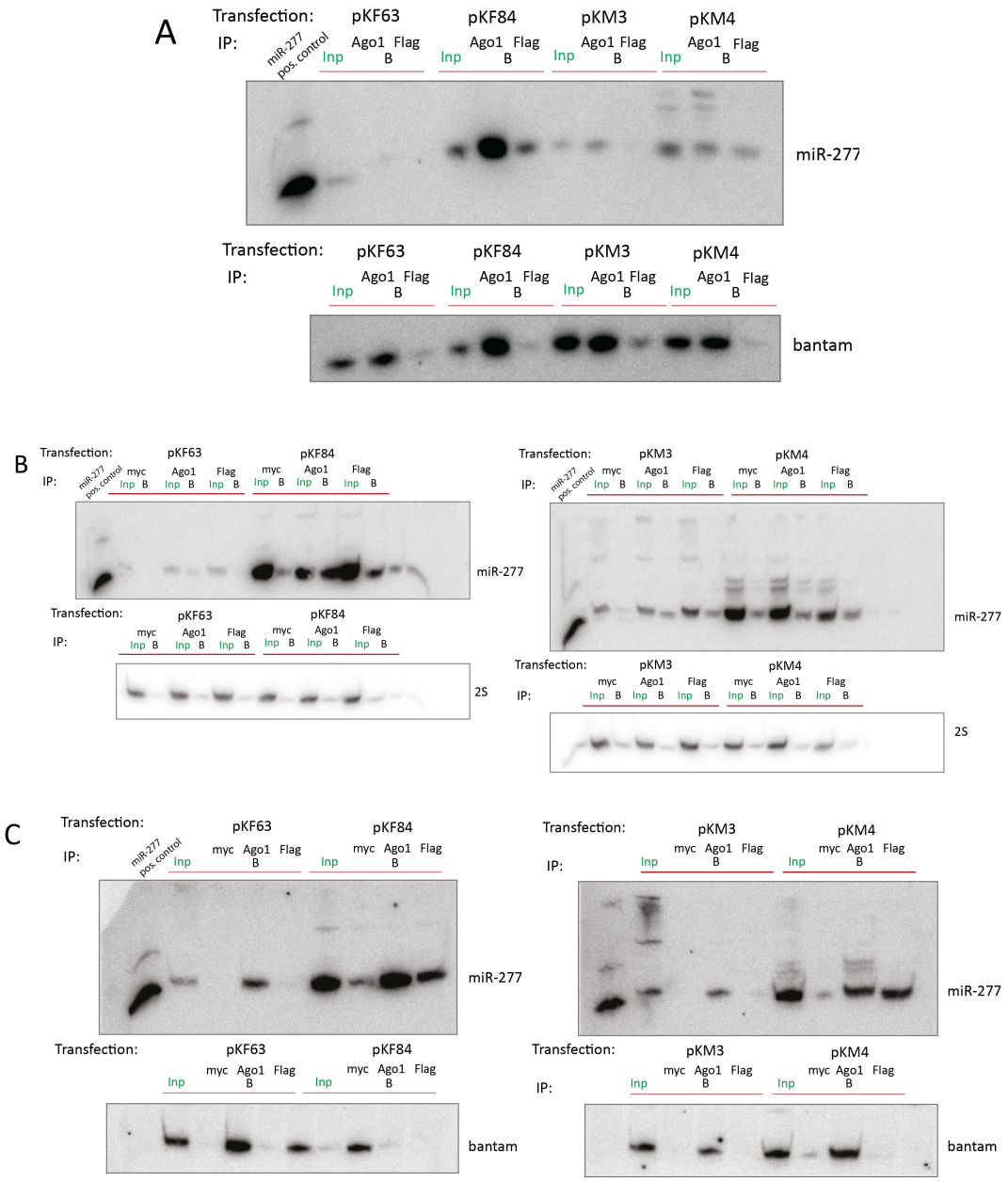


Figure 4.23: **panels above:**
Mir-277 is loaded into both AGO1- and AGO2-RISC and proper loading of mature miR-277 can be shifted to AGO2 through "siRNA like" mutation of the *-strand (pKM4).

A-C) Immunoprecipitations of AGO1 and AGO2 (Flag) and detection of miR-277 via Northern Blot. Shown are three independent experiments (3 biological replicates of transfection and subsequent immunoprecipitation and Northern Blot analysis). PKF63 transfection shows the loading properties of endogenous miR-277. myc-IP serves as a negative control. Inp: Input; B: Bound

panels below
Bantam is loaded into AGO1-RISC.

A and C) Immunoprecipitations of AGO1 and AGO2 (Flag) and detection of loaded bantam via Northern Blot. Shown are two independent experiments (3 biological replicates of transfection and subsequent immunoprecipitation and Northern Blot analysis). PKF63 transfection shows the loading properties of endogenous miR-277. myc-IP serves as a negative control.
B) Immunoprecipitation of AGO1 and AGO2 (Flag) and detection of 2S-rRNA via Northern Blot as a quantitiy control of RNA extraction and applied input.

5 Discussion

5.1 Flies with impaired downregulation of miR-277 with age have a shortened lifespan

Using a qRT-PCR assay I could measure the abundance of 80 microRNAs in young and aged flies. MiR-277, as one of the most abundant microRNAs in *Drosophila*, showed an about 2-4 fold downregulation with age in wildtype flies. The age related downregulation could be prevented in the transgenic line expressing a short form of miR-277 under the control of the constitutive ubiquitin promoter. MiR-277 levels were now constant; the other age related changes of the microRNA profiles were unperturbed.

I also examined the genome wide transcriptional changes in the fly samples by microarray analysis to confirm age related effects - as shown by (Pletcher et al., 2002) - in my lifespan analysis setup. The samples of flies overexpressing miR-277 revealed that the microRNA controls genes involved in the degradation of BCAAs, consistent with previous predictions (Stark et al., 2003). All genes of the degradation pathway, except CG1673 (gene for branched-chain amino acid transaminase, BCAT) are direct targets of miR-277. BCAAs are in the first enzymatic step of their degradation transaminated to BCKAs. As BCAAs and BCKAs are known activators of the TOR kinase we assumed that miR-277 probably plays a role in the TOR signaling network. Therefore miR-277 is not an exception to the conclusion of many target prediction and validation approaches, that microRNAs often target genes with a function in signaling systems.

The fact that miR-277 is downregulated with age in normal flies implies that the BCKA levels should decrease, because their degradation is no longer inhibited. In humans the analysis of serum BCKA levels revealed a significant decrease in old versus young individuals (Pailla et al., 2000). Hence BCKA levels could serve as metabolic markers for human age related pathologies. Recent studies have shown that levels of BCAAs are elevated in prediabetic patients (Newgard et al., 2009; Tai et al., 2010).

However much remains unknown about the patterns of molecular changes in different tissues and how different tissues interact with each other during aging. Regulatory pathways such as the insulin-like pathway, the TOR pathway, the Jun kinase pathway and the Sir2 deacetylase pathway control the rate of aging. Several studies in mammals have shown that the effects of aging are tissue specific. Zhan et al. measured genome-wide expression profiles of ageing in *Drosophila melanogaster* for seven tissues representing nervous, muscular, digestive, renal, reproductive, and storage systems at six adult ages. This study showed that that different tissues age in different patterns in an organism (Zhan et al., 2007). Acquiring the consequences of modulations induced by aging regulatory pathways (e.g. TOR) in different tissues is an important task for future experiments.

Dietary restriction (DR), a reduction in food intake without malnutrition, extends median lifespan and survivorship in many organisms (Fontana et al., 2010). Numerous

studies demonstrate different approaches to put flies on diet (reviewed in Tatar (2007).

In my lifespan analysis setup I applied three different food compositions to male flies: LSLP = low sugar, low protein; LSHP = low sugar, high protein; HSHP = high sugar, high protein (as applied in Mair et al. (2005)). I could show that male flies overexpressing miR-277 (thus with an impaired downregulation of miR-277 with age) show shortened lifespan on food with dietary restriction for sugar but excess of protein compared to wildtype flies.

Stephanie Helfer (AG Förstemann) also tested female miR-277 overexpressing flies on different food conditions. In general female miR-277 overexpressing flies showed a slightly decreased fertility compared to wildtype flies. Importantly they had a longer lifespan than male siblings on all food regimes. Because the lifespan shortening effect on LSHP food was more visible for male flies, I decided to set up my further lifespan analysis series with male individuals.

Resources for reproduction can not be allocated for maintenance and repair of somatic cells, leading to lifespan shortening. Partridge et al. showed that lifespan can be prolonged in female flies by reduction of reproduction upon dietary restriction (Partridge et al., 2005). Thus DR slows ageing processes by downregulating genes important for metabolism, reproduction and cell growth (Pletcher et al., 2002).

Recently it has been shown that manipulations of signaling pathways can also be gender specific. For mice the manipulation of the TOR pathway displays a gender specific effect clearly distinguishable from DR (Evans et al., 2011; Harrison et al., 2009; Selman et al., 2009). Reduced TOR signaling is thought to be the putative mechanism mediating lifespan extension by DR (reviewed in Stanfel et al. (2009)). Rapamycin treatment in mice exerts prolongevity effects. But, unlike DR, rapamycin is more efficacious in female than in male mice. This gender specific pattern of TOR inhibition in aged individuals remains a problem to be solved. The effect of rapamycin in *Drosophila* is controversal (Bjedov et al., 2010; Harrison et al., 2010). As we also observed a more visible effect of different nutritional regimes in male as in female miR-277 overexpressing flies it would be important if we also see gender-specific effects upon modulation of the important signaling pathways as TOR and IIS.

To prove that the observed life span shortening is really due to the overexpression of miR-277 we tested first three transgenic fly lines with different P-element insertion sites; all of them showed a reduced life span compared to the control. Next I compared two transgenic lines with different genetic backgrounds (yw and w^{1118}) to rule out that this could have an effect on the (amino acid) metabolism and accordingly the life span of the flies. The y gene for example is important for the melanin biosynthetic process from tyrosine. Overexpression of miR-277 leads in both genetic backgrounds to life span shortening.

Flies with a deletion (including Fmr1) in the 3rd chromosome that also lack miR-277 are viable but sick. *Fmr1* is the fragile X mental retardation gene. The full mutation of *fmr1* in humans causes the Fragile-X syndrome, a genetic syndrome that is the most commonly known single-gene cause of autism and the most common inherited cause of intellectual disability. This is also the reason why these flies are not appropriate for lifespan analysis experiments because there are severe effects to expect that are rather due to the deletion of fmr1 than to the lack of miR-277. It would be interesting for the future to find out which life span phenotype miR-277 knock out flies show on different food regimes. We got a first hint by analyzing the lifespan of flies expressing an artificial target of miR-277 on different food regimes. Indeed we could observe an elongation of lifespan, arguing that the effect of lifespan shortening is specifically due to the constitutive expression of miR-277.

Kadener et al worked on the role of microRNAs for the circadian clock of *Drosophila*. Their approach identified miRNAs, among them miR-277, expressed in fly head circadian tissue. Three core clock mRNAs - *clock (clk), vrille (vri)*, and *clockworkorange (cwo)* are under miRNA mediated control (Kadener et al., 2009). I set up life span analysis series of miR-277 overexpressing and wildtype flies in constant dark and in a 12 hours light/dark cycle to examine if the lifespan shortening effect of miR-277 overexpression is dependent of the circadian rhythm of the fly. There was no connection between lifespan on different nutritional regimes and phases of dark and light visible. This suggests that miR-277 and its regulation of metabolism are not dependent of genes of the circadian clock in *Drosophila*.

5.2 Parallel quantification of direct and indirect effects of microRNAs: miR-277 regulates post-transcriptionally enzymes of the degradation cascade of BCAAs

It has already been published, that miR-277 in *Drosophila* has a predicted role for the degradation pathway of BCAAs (Stark et al., 2003). MicroRNAs can silence gene expression by inhibiting translation or favoring degradation of the message (Okamura et al., 2004). I employed a transcriptome-wide analysis of changes in mRNA degradation and synthesis rate, called dynamic transcriptome analysis (DTA) (Dolken et al., 2008; Hartig et al., 2009), to distinguish between direct effects on mRNA stability and indirect effects on transcription rates after inhibition of miR-277. Inhibition of the microRNA will increase the steady-state levels of its target mRNAs. But secondary effects i.e. secondary changes that occur as a consequence of loss of miRNA function have to be taken into consideration.

This mRNA pulse-labeling technique allowed me to conclude that *Drosophila* miR-277

regulates post-transcriptionally enzymes that degrade BCAAs. There were no significant changes of transcription rates detectable. Hence all changing genes are direct targets of miR-277. Upon inhibition of other microRNAs (e.g. miR-14) transcriptional alterations were seen. Thus DTA is useful to identify targets of miRNAs and examine their physiological relevance. In the first enzymatic step of the degradation cascade, BCAAs are transaminated to BCKAs, e.g. leucine is converted to KIC. Importantly the BCAT did not change upon miR-277 inhibition.

5.3 Mechanism of TOR activation by leucine and KIC

The degradation of BCKAs is inhibited by overexpression of miR-277. BCKAs levels should increase. It is known that BCAAs and their corresponding BCKAs (e.g. leucine and KIC) activate the TOR kinase (Fig. 5.1), leading to phosphorylation of 4E-BP. In mammals starvation and exercise stimulate protein breakdown, thereby increasing the levels of BCAAs in adipose and muscle cells and accordingly catabolism of BCAAs (Fig.5.3).

I could demonstrate that indeed constitutive expression of miR-277 inhibits 4E-BP via increased phosphorylation, presumably due to activation of the TOR pathway via increased BCKA levels.

It is not yet fully understood how BCAAs and BCKAs activate TOR. Nicklin et al. showed that the cellular uptake of L-glutamine and its subsequent rapid efflux in the presence and import of essential amino acids (like for example leucine) is the rate limiting step that activates mTOR (Nicklin et al., 2009). The direct sensor of leucine concentration is unknown. KIC can also be transaminated back to leucine. Is this re-transamination required for the activation of TOR? She et al. generated mice in which the gene encoding the BCAT was disrupted. These animals showed a more persistent activation of TOR upon refeeding, consistent with the notion that leucine can stimulate TOR without a need for metabolic conversion to KIC (She et al., 2007). I could show that in *Drosophila* Schneider cells KIC stimulates the TOR pathway in the presence of a high concentration of leucine.

It could be possible that there are two independent modes of TOR activation by leucine and KIC and that both signals can be differentially interpreted by the organism.

Amino acids like leucine are important precursors of TCA (tricarboxylic acid) cycle compontents (Fig. 5.2), assuring the energy supply of the organism but have also a second function as substrate for protein translation (Fig.5.1). Therefore a long-term upregulation of leucine concentrations (e.g. by miR-277) may - additional to activation of the TOR pathway and adverse effects on the energy maintenance of the organism - also lead to inappropriate translational events. It could be possible that KIC as a

derivate of leucine could serve as a signaling factor for long-term purposes such as the down-regulation I observed in the aging study.

5.4 Serves KIC as a qualitatively different signal of TOR activation?

Under normal conditions the BCKAs are present at much lower levels than the corresponding BCAAs (see Table 5.1).

The K_m of the BCAT for KIC is 140 μM in rat heart. This value is much higher than the physiologic concentrations of KIC (see Table 5.1). Thus, the re-transamination of KIC to leucine only occurs upon a very substantial increase of KIC concentration. An important question is, what can lead to increased levels of BCAAs and BCKAs in an organism? There are two possible scenarios (see Fig. 5.4). The first possibility is the dietary uptake of BCAAs. Work in mammals shows that the uptake of BCAAs results in a roughly corresponding increase of BCAA and BCKA levels, the leucine to KIC ratio remains the same (Schauder, 1985; Wiltafsky et al., 2010). Also starvation and exercise can lead to increasing BCAA levels by stimulating protein breakdown in adipose tissue and muscle cells (Fig. 5.3).

The second possibility is a changed activity of the BCKDH enzyme. This should lead to a more pronounced increase of BCKAs (over BCAAs). The human autosomal metabolic disorder Maple syrup urine disease (MSUD) is caused by a deficiency of the BCDKH complex. The disorder is characterized by a buildup of BCAAs but also of BCKAs to essentially equal levels. As miR-277 negatively regulates the degradation of BCAAs, hence the BCKDH complex, a constitutive expression of miR-277 could lead to the same physiologic effect in the fly as observed upon MSUD. Interestingly we could

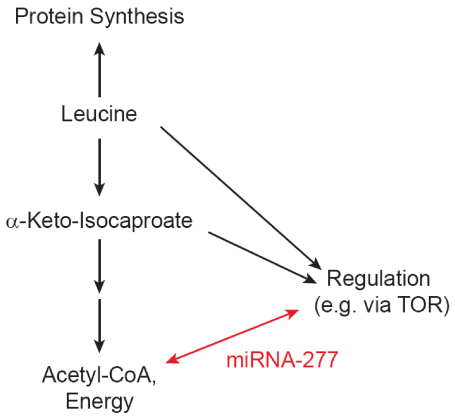


Figure 5.1: Speculated role of miR-277 for energy metabolism of the fly by regulating BCAA levels and accordingly TOR signaling.

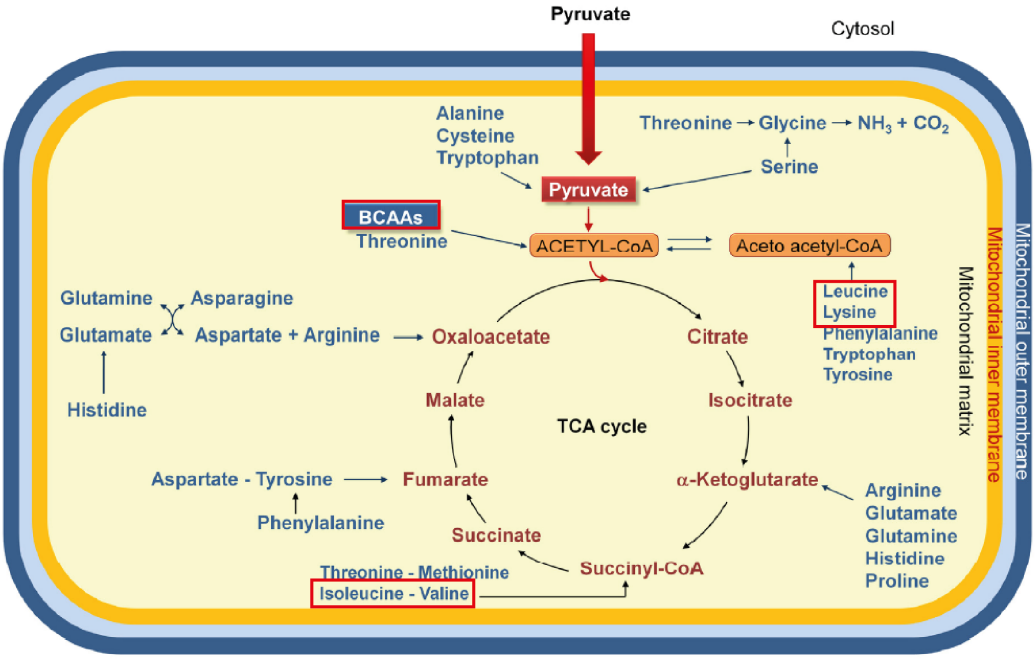


Figure 5.2: **More detailed view of amino acid degradation.** Amino acids are degraded to compounds that can be metabolized to CO_2 and H_2O, or used in gluconeogenesis. Amino acids can be divided into two groups, on the basis of their catabolic pathways: 1) Gluconeogenic amino acids, which are catabolized to pyruvate, α-ketoglutarate, succinyl-CoA, fumarate, oxalacetate and are glucose precursors; 2) ketogenic amino acids, which are catabolized to acetyl-CoA or acetoacetate, and may be transformed into fatty acids or ketone bodies (adapted from Valerio et al. (2011)). BCAAs are framed red.

	BCAAs	**BCKAs**
human blood	300-500 μM	40-60 μM
human plasma	149 μM (Ile/Leu), 235 μM (Val)	33 μM (KIC), 18.9 μM (KMV), 12.7 μM (KIV)
rat kidney	–	8 μM
rat skeletal muscle	–	12 μM

Table 5.1: Measurements of BCAA and BCKA levels. (Livesey and Lund, 1980, 1988; Newgard et al., 2009; Pailla et al., 2000)

not detect increasing levels of BCAAs neither in male flies overexpressing miR-277 nor in *Drosophila* Schneider cells, transfected with a miR-277 overexpressing construct. The transgenic miR-277 expression construct is driven by a constitutive ubiquitin-promotor, leading to ubiquitous expression including germ line tissue. Since endogenous miR-277 is not expressed in the germ line, any effects of miR-277 should be most evident in this tissue. Consistent with this notion, a moderate increase in the concentration of valine was detectable in extracts prepared from the abdomen of female miR-277 overexpressing flies, leucine and isoleucine were also slightly increased. It is absolutely necessary for future

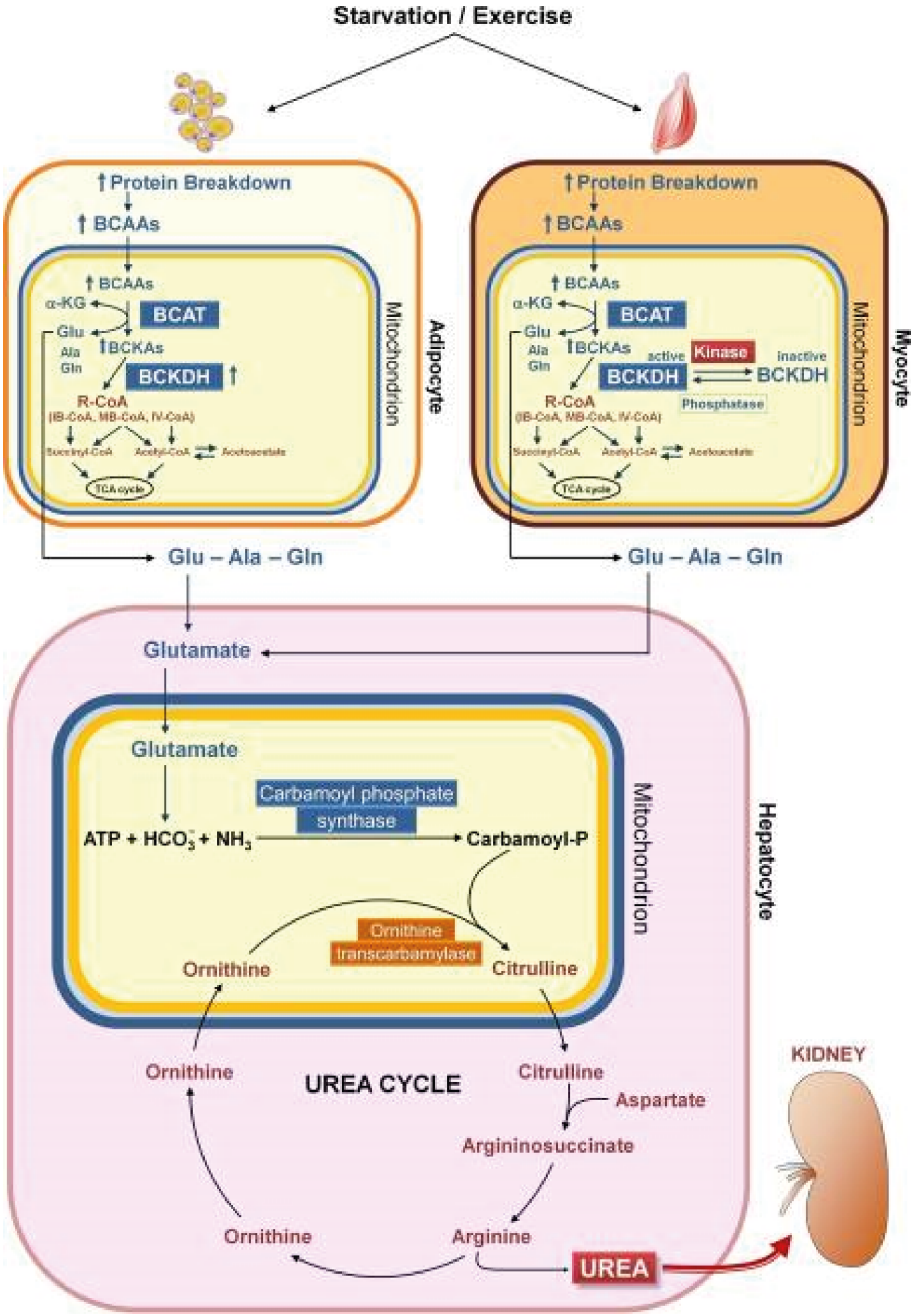


Figure 5.3: **Catabolism of BCAAs in mammals** The BCAAs are transaminated in muscle mitochondria by branched-chain aminotransferase (BCAT), and branched-chain alpha-keto acids (BCKAs) inhibit branched-chain alpha-keto acid dehydrogenase kinase, resulting in elevation of the active state of the rate limiting enzyme branched-chain alpha-keto acid dehydrogenase complex (BCKDH) (adapted from Valerio et al. (2011)).

work to quantify KIC levels in *Drosophila* upon constitutive expression of miR-277.

The elevated BCAA and BCKA level could be an explanation for the shortened lifespan of miR-277 overexpressing flies. I tried to mimic the effect of constitutive expression of miR-277 in two ways: First by directly ablating the transcripts of subunits of the BCKDH complex (CG5599 and CG8199). Possibly the ratio of BCAAs to BCKAs is modulated by the control of the BCKDH. BCAAs and BCKAs are potent activators of the TOR pathway. Maybe a short-term increase of the amino acid levels upon dietary intake and therefore the activation of TOR can be distinguished from a long-term adaption by modulation of the BCKDH (e.g. by miR-277). After depletion of CG8199 and CG5599 expression in the fat body, there was no apparent lifespan shortening visible on LSHP and HSHP food. Maybe there are some unknown feedback mechanisms that are able to rescue the knock down of the BCKDH function in the fat body.

In a second lifespan analysis setup I added BCAAs to LSLP food and chronicled the lifespan of wildtype flies. There was only a minimal lifespan shortening visible. This effect also appeared on the control food (LSLP with additional three other amino acids). This likely reflects the increased caloric content of the supplemented LSLP food. Importantly in this case, the BCAA to BCKA ratio is not affected, because there is no modulation of the activity of the BCKDH. My next approach will be to add only KIC to LSLP food. My hypothesis is that this shift of the leucine to KIC ratio towards higher KIC levels would lead to a comparable lifespan shortening as observed upon miR-277 overexpression.

Grandison et al. published that an imbalance of amino acids is important for regulation of lifespan of *Drosophila* especially upon DR. Methionine acts in combination with one or more other essential amino acids to shorten lifespan with full feeding (Grandison et al., 2009). They could show that adding back all essential amino acids to restricted food except methionine restored lifespan to the level corresponding to DR. Thus there is an imbalance in the ratio of amino acids in yeast and some consequence of this imbalance decreases lifespan with full feeding.

I could show that BCAAs are not the one or not the only one among the essential amino acids,that interact with methionine. There were no significant differences for the lifespan analysis curves if I applied additional methionine and BCAAs (or OEAAs) to LSLP.

The major mechanism regulating BCAA oxidation in human muscle and liver is a reversible phosphorylation, that inactivates the BCKDH complex (Herman et al., 2010; Paxton and Harris, 1984). Recent results indicate a novel mechanism for regulation of BCAA oxidation in adipose tissue, i.e. changes in expression of BCAA enzymes (She et al., 2007).

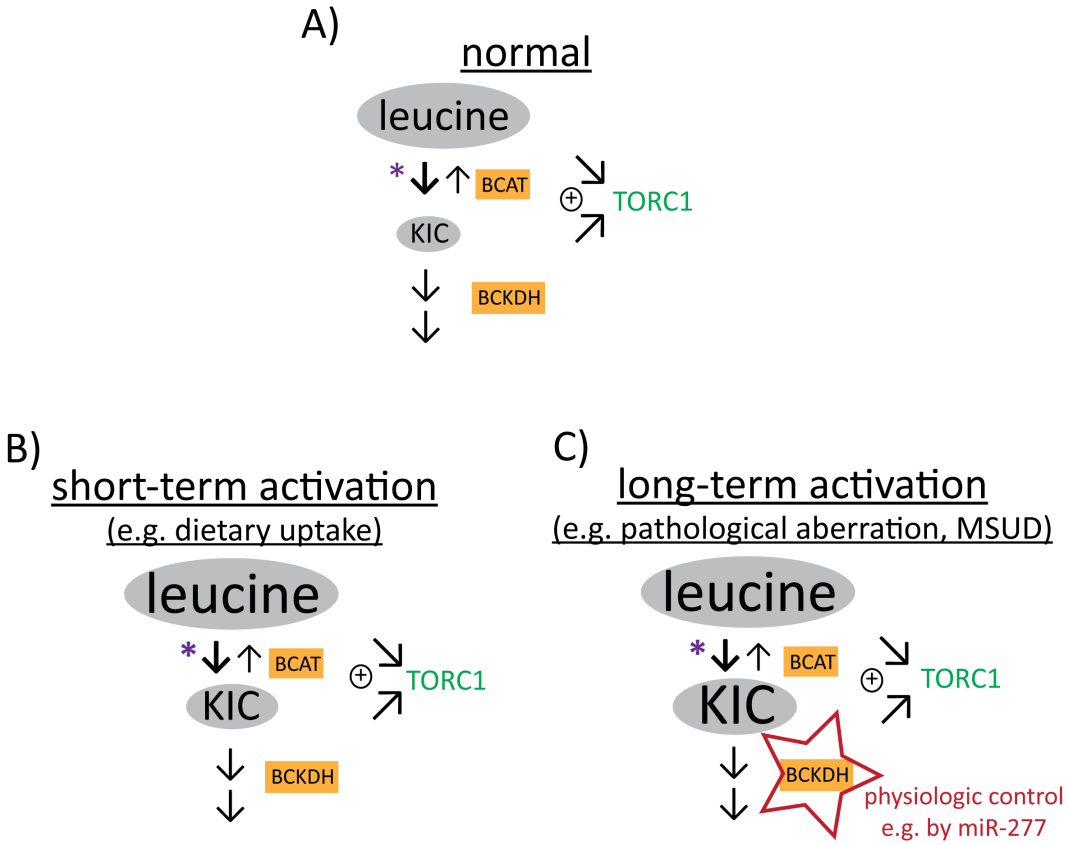


Figure 5.4: **A)** Under normal circumstances the BCKAs are present at much lower concentration than the corresponding BCAAS.
B) Dietary uptake of BCAAs resulting in a roughly corresponding increase in BCAA and BCKA levels.
C) Changes in activity in activity of the BCKDH enzyme should lead to a more pronounced increase of BCKAs over BCAAs.
* The K_m of BCAT for KIC was determined 10 times higher than the estimated physiologic concentration of KIC. Thus, reverse transamination of BCKAs to BCAAs will only occur efficiently upon a very substantial increase of BCKA concentration.

5.5 The expression of miR-277 in thoracic muscles and the fat body serves as source of circulating BCKAs emanating from these tissues

I determined the expression pattern of miR-277 by using the conditional UAS-Gal4 expression system in *Drosophila* (Brand and Perrimon, 1993). MiR-277 is expressed in thoracic (flight) muscles, the fat body, but not the gonads, arguing that the physiological role of miR-277 is strongest in muscles and fat body. The next step is to generate a new transgenic fly line which expresses Gal4 under the control of a longer (than the present 4kb sequence) miR-277 specific promoter sequence. This would show if we were able to identify the whole sequence of the miR-277 genomic region necessary for a complete

picture of miR-277 pattern.

I was able to demonstrate that miR-277 probably reduces BCKDH activity, leading to increasing BCKA levels and thus activation of the TOR kinase. Kapahi et al. could demonstrate that TOR signaling in the fly is tissue specific. A nervous-system-specific reduction of TOR activity had no effect on life span, whereas reduction of TOR signaling in the muscle and fat, or muscle, fat and gut did (Kapahi et al., 2010). Also IIS activity in the adult *Drosophila* fat body has been shown to regulate adult survival; thus it is likely that both TOR and IIS function in the adult fat body to regulate life span. To find out which tissue is responsible for the lifespan shortening effect of miR-277 I manipulated regulation by miR-277 directly in the fat body (the equivalent of mammalian white adipose tissue and liver) and in the nervous system and chronicled the lifespan of these flies. Overexpression of miR-277 in the fat body, leading to an activation of the TOR kinase in this tissue, leads to the expected lifespan shortening on food with an excess protein content. Overexpression of miR-277 in the nervous system led to an extension of life span. The second effect is probably due to genetic background effects.

An important approach for the next life span analysis series would be to examine miR-277 overexpression in further tissues of the fly, especially the muscles, and if this leads to the lifespan relevant increase of BCAAs and BCKAs.

An important question is if this effect of miR-277 is restricted to a certain tissue (e.g. muscles) or rather to certain cells. Can the modulation of the BCKDH complex in one tissue lead to activation of the TOR pathway in another tissue? It is known that in rats BCKAs can cross the blood-brain barrier by a carrier system (Amaral et al., 2010). It is also important to mention that MSUD patients show very high levels of BCAAs and BCKAs in their plasma, therefore it can be concluded that BCKAs can exit the cell of their origin (Tanaka et al., 1988). Table 5.1 shows the results of Linvesey et al.. The skeletal muscles of rats have a higher level of BCKAs as the liver tissue, which is another argument for the transfer of BCKAs between tissues (Livesey and Lund, 1980, 1988).

Muscle tissue plays a very important role for metabolic homeostasis and a decrease in muscle function is partly responsible for age-related pathologies. D'Antona et al. showed that supplementation of BCAAs promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice (D'Antona et al., 2010). It is also known that in humans a higher branched-chain amino acid intake is associated with a lower prevalence of being overweight (Qin et al., 2011).

Geminard et al confirmed with their results that a diffusible factor produced in one tissue can affect a signaling pathway in a distant tissue (Géminard et al., 2009) in *Drosophila*. They could show a connection between the availability of nutrients, that is sensed in the fat body and conveyed to the brain by a humoral signal that transits through the hemolypmph and activates Dilp (Drosophila insulin like proteins) secretion.

I can argue that probably miR-277 regulates BCKA levels in the tissues of its expression, i.e. muscles and the fat body. Probably the BCKAs are then released and serve as signaling factors (in addition to their role as metabolic intermediates), leading to inhibition of 4E-BP via increased phosphorylation by the TOR-kinase. Since BCKAs can exit the cell, they may function as a hormone like signal to activate TOR at distant locations.

5.6 *Drosophila miR-277* is part of a signaling network

D. melanogaster probably uses miR-277 to control the levels of BCKAs.

The TOR pathway interacts with the insulin/insulin-like growth factor-like signaling (IIS) pathway in *Drosophila* at the level of PKB, which phosphorylates TSC2, a negative regulator of TOR, in addition to dFOXO, thereby activating the TOR pathway (Giannakou and Partridge, 2007). Reduced IIS can extend life span in Drosophila (Piper et al., 2008). *Chico* encodes the insulin receptor substrate and it has been shown that $chico^1$ homozygous flies have a dwarf phenotype and are long-lived (Clancy et al., 2002; Tatar et al., 2001). I could show a genetic interaction of constitutive miR-277 expression and decreased insulin signaling in $chico^1$ mutants (Fig. 5.5).

The interaction has to occur at a stage prior to the eclosion of adults, because the combination is lethal. Most likely a developmental function was perturbed.

It would be interesting to examine further the genetic interaction of miR-277 with the insulin pathway by combining overexpression of miR-277 with mutations of other pathway components. For example genetic deletion of three of the *Drosophila* insulin-like peptides (dilps) extends life span (Grönke et al., 2010). It is not yet known whether the fly FOXO is required for extension of life span as in *C.elegans*. Probably it would also be of interest what effects show alterations of the TOR pathway that lead to extended life span in the fly like feeding of rapamycin (Bjedov et al., 2010) or genetic modulation of pathway components in combination with overexpression of miR-277.

The muscle-specific overexpression of a constitutive active form of 4E-BP can delay muscle ageing, regulate feeding behavior, modulate insulin release from the brain and as a result extends lifespan (Demontis and Perrimon, 2010).

By contrast *Drosophila* 4E-BP mutants are viable with little or no growth phenotypes (Miron et al., 2001). These mutants are hypersensitive to nutrient starvation and oxidative stress and have defective fat metabolism (Teleman et al., 2005; Tettweiler et al., 2005). Thus *in vivo*, 4E-BP function may only be critical when nutrients are limiting and levels of insulin/TOR signaling are low. Under these conditions, 4E-BP activity is high due to both FOXO-mediated transcription and removal of the TOR-mediated inhibitory phosphorylation. 4E-BP can subsequently function as a metabolic brake to ensure levels of mRNA translation are kept low. Translation is a large consumer of cellular energy of

an organism.

As miR-277 leads to phosporylation of 4E-BP, this could also be the explanation why the fly downregulates miR-277 with age and why flies with overexpression of miR-277 are short-lived.

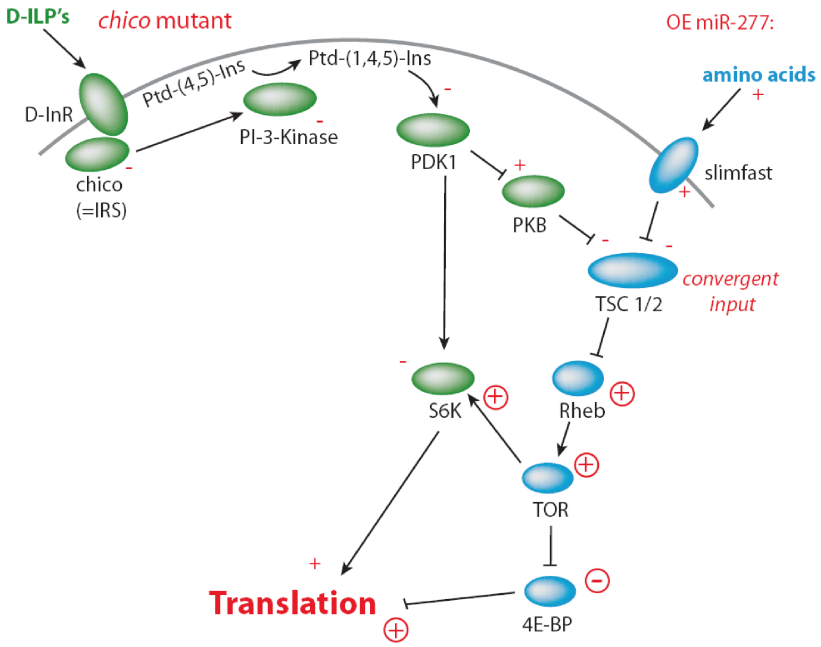


Figure 5.5: **The *Drosophila* IIS and TOR signaling pathways.** High levels of miR-277 are synthetic lethal with reduced activity of insulin-receptor signal transduction. (adapted and modified from Giannakou and Partridge (2007)).

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Stephanie Maria Esslinger

Persönliche Daten

Geburtsdatum	17.10.1981
Geburtsort	Nijmegen (NL)
Nationalität	deutsch
Familienstand	ledig

Ausbildung

seit 09/2007 **Promotionsstudium Chemie**
Ludwig-Maximilians-Universität München
Prof. Dr. Klaus Förstemann, Genzentrum München
Titel: 'Analysis of miR-277 in *Drosophila melanogaster* and its role for metabolism and lifespan'

10/2001 - 06/2007 **Diplomstudium Biologie**
Universität Regensburg
Diplomarbeit am Institut für Zoologie in Kooperation mit dem Institut für molekulare Pathologie Universitätsklinikum Regensburg
Titel: 'Analysis of the fussel gene in *Drosophila melanogaster*'
Diplom Gesamtnote: 1,6

08/1992 - 06/2001 **Allgemeine Hochschulreife**
Maristengymnasium Fürstenzell

Berufliche Tätigkeiten

2003 - 2006 **Studentische Hilfskraft**
Institut für Botanik, Universität Regensburg
Mitarbeit im Projekt BayFlora-Kryptogamen

Veröffentlichungen

2011 ***Drosophila* miR-277 tunes TOR activity via control of leucine metabolism**
Esslinger S., Förstemann K., in Arbeit

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