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**Abstract**

Insulin signaling is one of the master keys for performing proper regulation of energy metabolism. Serious diseases such as cardiovascular disease, type 2 diabetes and obesity occur due to mis-regulation of insulin pathway. Therefore, it is necessary to recognize the factors that function in the production of insulin and it's signaling. *Drosophila melanogaster* is the model organism used for this study in order to explore the function of Protein kinase D *(PKD)*, which is a serine-threonine kinase. *D. melanogaster* has shown lower lipid content when *PKD* expression is reduced. The knockdown of *PKD* on transcription level in the *D. melanogaster* flies did not affect the transcription levels of both the insulin-like peptides *(ILPs)* and Adipokinetic hormone *Akh*. Starvation has affected the expression of different *Ilps* in the fly brain and high protein rich diets have affected the expression of *PKD* and *Ilp3* in the fly brain.

**Key words:** Protein kinase D, Obesity-linked gene, Homeostasis

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**Abbreviations**

AKHs adipokinetic hormone

CNS central nervous system

DAG diacylglycerol

DMSO dimethylsulfoxide

GWAS genome wide association study

ILPs insulin like peptides

IPC insulin producing cell

GPCR G protein coupled receptors

PI pars Intracerebralis

PKD protein kinase D

Strn-Mlck stretchin-mlck

WHO world health organization

**Introduction:**

There are more than 1 billion adults overweight globally, estimated by world health organization (WHO) in 2008. More than 300 million of people are obese and should be treated clinically. Some of the major chronic diseases like cardiovascular disease, specific types of cancer, hypertension and type-2 diabetes are also linked to overweight and obesity. Since 1980, the rates of obesity have increased three times in the world due to the consumption of saturated fats, high level of sugar and insufficient physical activity [1].

The genome of *Drosophila melanogaster* has a single protein kinase D (*PKD)* homologue. Protein kinase D (*PKD)* is a multifunctional protein that is broadly expressed at the time of development. Some portion of the *PKD* protein localizes to the Golgi complex, which functions in the secretory transport. There is about 65% overall degree of similarity between human and *D. melanogaster PKD* at the protein level. There are two domains of *PKD* in *D. melanogaster*, a regulatory domain consisting of two cysteine-rich regions (C1, C2) necessary for membrane localization and diacylglycerol (DAG) binding that is needed for the phosphorylation of the target protein, and a pleckstrin homology domain (PH) performing multiple functions. Maier et al 2007 identified that *PKD* in overexpression leads to incomplete wing vein development, tissue loss, and retina degeneration [2].

In mammals, the protein kinase family comprises three isoforms of *PKD*. These isoforms of *PKD* were identified in 1994 (genes named *PRKD1, PRKD2, PRKD3*). In human, they are called *PKCμ*, *PKD2* and *PKCV*. *PKCμ* was also called protein kinase C (*PKC*). *PKD* is a serine/threonine kinase and in mammals, these *PKDs* perform multiple biological activities such as apoptosis, secretory transport from the trans-Golgi complex and cell proliferation. Mammalian cells apply a particular pathway to regulate nuclear imports of *PKDs*, which may not function in the fly. In humans, *PKC* is necessary for the nuclear transport of *PKD* that responds to G-protein coupled receptors (GPCR) activation [3].

The function of *PKD* in insulin secretion was initially observed from cell studies. Firstly, in INS-1 cells (rat insulinoma-derived pancreatic B cell line) one of the isoform of *PKD* (*PKD1*) shows inhibition of insulin secretion. Moreover, the supplementation of glucose enhanced *PKD1* (*PRKD1*) initiation, which functions in fusion of vesicle membranes at the trans-Golgi complex. Secondly, in *Caenorhabditis elegans* deprived of the *dfk-2* gene (*PKD* homologue) there was an enhanced life span by 40% compared to the wild type of nematodes [23]. Meanwhile, knocking down of *daf-16* in *dfk-2* mutant nematodes rescues the extended lifespan. These data from cell studies and from the study of *C. elegans* as mentioned above support further proof that *PRKD1* (one of the isoform of *PKD*) is involved in insulin signaling regulation. Furthermore, ablation of IPCs in *D. melanogaster* also enhanced longevity [2, 23,25]. The results from mammals and *C. elegans* further confirm that *PKD* could be included in the regulation of the insulin signaling mechanism [4, 2].

***Drosophila insulin like peptides (Dilps)*:**

The genome of *Drosophila melanogaster* contains eight insulin-like peptide *(Ilps)* genes. These *Ilps* are expressed in particular in two clusters of seven cells called insulin-producing cells, IPCs present in the PI and generate three of the eight *Ilps* called *Ilp2*, *Ilp3,* and *Ilp5*. All three *Ilps* show a parallel and associated structure compared to human insulin [5,6]. Larval salivary glands, ovaries, midgut, larval and adult brain are the main tissues that express *drosophila insulin-like peptides (dilps)* [5,7]. Detection of *dilp*1 transcript occurred in larval IPCs whereas *dilp*7 occurred in the abdominal neuromeres and thoracic-abdominal ganglia. *Dilp*6 was detected in the fat body adipose cells, heart and salivary glands. *Dilp*8 has been detected in the imaginal discs and *dilp*4 in the mesoderm and anterior midgut. *Ilps*2, 3 and 5 are all co-expressed in the median neurosecretory cells (MNCs) in a protocerebrum region called *Pars intracerebralis (PI)* of adults and larva [5,8]. In larval MNCs the expression of *Ilps*2, 3 and 5 are independently regulated, *Ilp*3 and *Ilp*5 expressions are controlled through

nutrient availability, due to starvation the amount of detectable transcripts is reduced and the peptide gets accumulated in the axonal termini and IPCs. The *Ilp*2 has been detected in the salivary glands and imaginal discs and expressed in the glial cells in the CNS of first larval stages where they perform the function of neuroblast reactivation.

***Drosophila melanogaster* as a model organism:**

*Drosophila melanogaster* is a key model organism in genetics, studied by T. H. Morgan more than 100 years ago. He used it to study heredity. The criteria for choosing *Drosophila melanogaster* are. a) It is a small fly having a short life cycle and producing a huge number of offspring. b) Environmental and genetic manipulations can change its life span, c) its maintenance is easy and inexpensive compared to other model organisms like rats and mice etc. d) female and male show visible differences and virgins are easy to separate.

More than half of the *D. melanogaster* genes have homologs in humans. A lot of molecular pathways explained in *D. melanogaster* are conserved in mammalian systems. Recently, *D. melanogaster* has been also used in research on developmental genetics and molecular development. Due to the small size of *D. melanogaster*, it can easily be handled and one can easily study its behavioral activities related to the human condition like male-male aggression and courtship. The main goal of using *D. melanogaster* is to explore fundamental biological processes that may be directly applicable to human beings [9,10].

The size of *D. melanogaster* genome is 180-Megabase (MB), of which 120 MB is euchromatin. Sixty MB of the *D. melanogaster* genome is composed of transposons, simple sequence satellites and two big blocks of Ribosomal RNA genes. In comparison to vertebrate (mammals) adipose tissue and liver, *D. melanogaster* has an orthologous organ called fat body and oenocytes that stores the majority of the lipids and carbohydrates, contains triglycerides and glycogen is accomplished mostly by the fat body. *D. melanogaster* fat body cells consist lipid droplets that use many of the similar enzymes to regulate the synthesis of

glycogen and breakdown similar as in mammals. Oenocytes perform the role of lipometabolism. In vertebrates, lipids are mobilized as free fatty acids, but in *D. melanogaster,* they are mobilized as diacylglycerol (DAG) from the fat body. Two types of hormones are involved in inducing mobilization of lipids, octopamine and adipokinetic hormone (AKHs). AKHs are a huge family composed of 8-to 10-amino acids peptides secreted into hemolymph via neurosecretory cells of the *Corpora cardiaca* [5,11].

***UAS/GAL4* System in *Drosophila melanogaster:***

The *GAL4* system was recognized in the yeast *Saccharomyces cerevisiae*. *GAL4* encodes a transcription regulator that binds to an upstream activating sequence (*UAS*) consisting of four related 17 bp DNA motif that are analogous to an element in multicellular eukaryotes, called enhancer [12]. The *GAL4/UAS* system in *D. melanogaster* has definite benefits: 1. One can examine the function of a single transgene in various developmental stages in different tissues. One can also analyze several genes at a particular time in a specific tissues or cells. 2. The expression level of a particular transgene can also be amplified via the *GAL4* system [13]. In *D. melanogaster,* the *GAL4/UAS* system was constructed on separation of *GAL4* and *UAS* parts into two diverse parental lines, driver and responder lines. In driver line, the native promoter was used to control the *GAL4* gene. Therefore, wherever the native promoter is active then *GAL4* function in those cells/tissues and no transcription of the target gene will occur in parental responder line because of the absence of *GAL4* expression [14]. This absence of expression assists scientists to construct a responder lines for lethal or toxic genes. When crossing over the driver and the responder lines, offspring's express both the target and the *GAL4* gene beneath the control of the *UAS* element that induced to expression of the specific target gene in certain cells or tissues which express *GAL4*.

*GAL4/UAS* system was constructed to study gain of function phenotypes; *GAL4/UAS* method combination with the RNA-mediated interference (RNAi) method supplies a strong tool for

investigating a loss of function phenotypes. To sustain a targeted gene knock down, a construct is designed that expresses an RNA molecule, which forms double-stranded RNA (dsRNA) that mediates gene-specific RNAi. The anti-sense RNA molecule is generated by a designed construct that is complementary to the mRNA of the specific target gene. This RNA molecule then folds into a stem-loop construct that is identified and chopped into short fragments < 30n nucleotides via an enzyme Dicer called small interfering RNA (SiRNA). RNA Induced Silencing Complex (RISC) then unwinds the SiRNA molecules and one strand of the SiRNA is selected and remains bound to the argonauts proteins, this strand is called the guide strand. The second strand of the SiRNA degrades. The SiRNA binds to the specific mRNA and this target is precise because it is determined by base pairing between the SiRNA and the target mRNA. The SiRNA frequently have perfect complementarity to the target sites. Once bound argonauts cleaves the mRNA. This break down in mRNA occurred due to degradation through exonucleases in cell [15, 16].

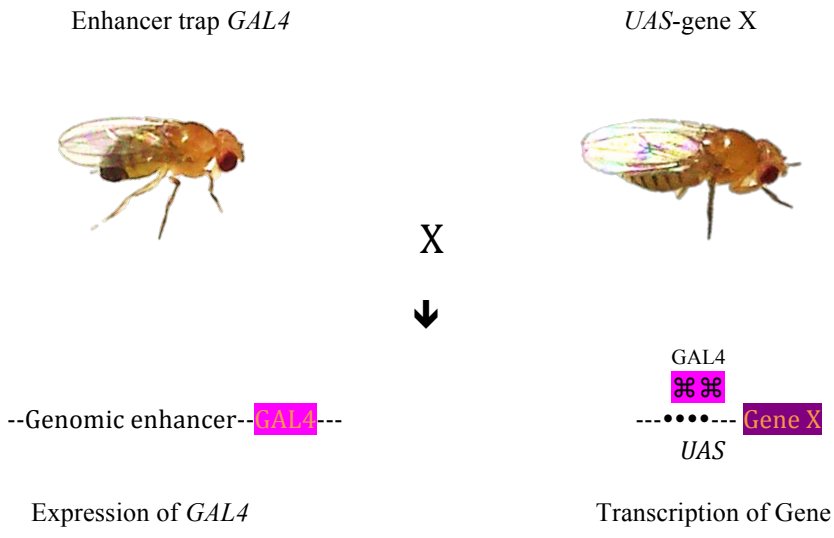


**Figure 1**. **Targeted gene expression in *D. melanogaster* applying the *GAL4/UAS* system.** The *GAL4* gene was randomly integrated into the genome of parental driver line that influences the *GAL4* gene expression into

various genomic enhancers. The desired gene (gene x) was sub-cloned behind the *UAS* element into the parental responder line. In the parental responder line, the gene x was silenced because of the absence of *GAL4* gene expression. In order to stimulate gene x expression in a tissue or cell particular method, the responder line (*UAS* gene x) was crossed with the parental driver line called enhancer trap *GAL4*. In the next generation offspring of this cross-expressed gene x as a consequence of a *UAS* stimulation via binding with *GAL4* (picture edited from St Johnston D. 2002) [17].

**Aim of the Project:**

Our aim in the project was to explore the in vivo role of the *PKD* (a serine-threonine kinase) in the *IPCs*. It was hypothesized that change in the *PKD* indicates abnormal production or secretion of insulin-like peptides.

To confirm our hypothesis the protein kinase D gene was knocked out in the *D. melanogaster* whole fly by crossing the specific *PKD-Daughterless GAL4* driver line to the *UAS* responder line that generates RNAi against the target gene. We would be able to knock out the specific *PKD* gene in the whole fly of *D. melanogaster*. To investigate whether the behavioral and metabolic assay performed the function of *PKD* and *Ilps,* this function was studied by knocking down the *PKD* using RNAi. *Drosophila melanogaster* activity-monitoring system (DAMS) has been developed to study the behavior of the flies. In the over expression and starvation assay, the survival time of the flies was studied. A metabolic assay called lipid assay and starvation was used, and applying the RNAi caused the knocking down of *PKD*.

**Material and Methods:**

***Drosophila melanogaster* Stock:**

Flies were collected from the stock available in the Department of Neuroscience, Functional Pharmacology, Uppsala University and the flies were kept for 12:12 hour's period's light: dark cycles at 25°C. All flies, unless otherwise stated, were fed on the standard fly food called jazz mix (Fischer Thermo Scientific). The fly strains, which were used: *CSORC* (wild type), *UAS-PKD*$^{RNAi}$, yellow white (*Yw*), *Elav-GAL4*, *Da-Elav-GAL4*, *PKD* over-expression (*PKD*$^{OE}$), yellow white over-expression (*Yw*$^{OE}$), were collected from Bloomington *Drosophila* stock center (BDSC; University of Indiana, Bloomington, USA).

**Genetic Crosses of Flies:**

For experimental crosses, virgin females of daughterless *da-Elav-GAL4* and *Elav-GAL4* driver line (trip line) were collected from the stock and crossed with the UAS reporter line called *UAS-PKD*$^{RNAi}$. The *UAS-PKD*$^{RNAi}$ line produces RNAi against *PKD*, which results and knocks down the *PKD* gene in the whole fly. The *Elav-GAL4/PKD*$^{RNAi}$ and *Da-Elav-GAL4/PKD*$^{RNAi}$ line were obtained as a result of a cross. The *PKD* gene was knock down in the whole body of *Drosophila melanogaster* with the *da-Elav-GAL4* driver line. The *Da-Elav-GAL4/PKD*$^{RNAi}$ line was only used for the knock down experiment and in the rest of the experiment *Elav-GAL4/PKD*$^{RNAi}$ was used (lipid and starvation assay).

For the control crosses, two different strains were used. Virgin females from *Yw* line were collected and crossed with the same UAS reporter line *UAS-PKD*$^{RNAi}$ and *Elav-GAL4*. As a result of the above cross *Yw/PKD*$^{RNAi}$ and *Elav-GAL4/Yw* line flies were obtained.

In the overexpression line, *PKD*$^{OE}$ line was crossed with the *Elav-GAL4* driver line and *Yw*. As a result of this cross *Elav-GAL4/PKD*$^{OE}$ and *Yw/PKD*$^{OE}$ line flies were obtained and these two genotypes were also used in the lipid extraction assay with the following three genotypes (*Elav-GAL4/PKD*$^{RNAi}$, *Yw/PKD*$^{RNAi}$ and *Elav-GAL4/Yw*). All these flies were feed on the

standard fly food called jazz mix (Fischer Thermo Scientific). They were kept in narrow vials in an incubator at 25°C. The crosses were then transferred into new bottles. After 5-6 days the flies' larvae crawled up to the walls of the bottles. The bottles containing flies were shifted from the 25°C incubator to another incubator having 29°C temperature due to the purpose that the *GAL4/UAS* system activation works well at 29°C temperature and expresses well instead of 25°C.

**Starvation Assay:**

The starvation assay was performed on transgenic fly lines after collecting males from the F1 and F2 generations and the flies were shifted into another bottles having food. They were aged for 5-7 days in a 29°C incubator. Afterward, 20 flies were put in a vial having 6 ml agarose (10 g/l) that supplied humidity and water to the flies but no food was provided. The vial was incubated in 25°C incubator. After every 12 hours the number of dead flies was counted and a survival curve was raised. Ten replicates of 200 flies for 3 different genotypes were selected for the experiment.

*D. melanogaster CSORC* flies were also starved for the qPCR experiment. All flies were fed on the standard fly food called jazz mix (Fischer Thermo Scientific). The *CSORC* flies were aged for 5-7 days in a 29°C incubator. Afterward, these flies were divided into three groups. The first group of flies was starved for 24 hour and the second group of flies was starved for 48 hour. Flies in these groups were put into a separate vial and incubated in a 25°C incubator. Flies of the control group were not starved. Afterward, heads of the flies were removed, mRNA was extracted from flies' heads, cDNA was synthesized and qPCR was performed.

**Lipid Assay:**

Fresh males from the new generations (F1 and F2) of transgenic fly lines were selected and aged for 5-7 days at 29°C. To confirm the lipid content, 30 male flies were enclosed into a glass vial and they were dried for 1h at 65°C incubator. The dried flies were collected on

filter paper from the vials and each of the replicate was weighted separately. The dried flies were transferred into the respective vials and 10 ml of diethyl ether (Sigma-Aldrich, Germany) were added into each of the glass vials. The glass vials were incubated for 24 hours in a hood at room temperature in order to obtain the lipid content. Diethyl ether was discarded and flies were dried in vials in a 65°C incubator for 1 hour and weighted again to achieve the lean dry weight. The total lipid contents of the flies were determined by calculating the difference between the dry weight and lean dry weight. For each genotype a maximum of 450 flies in 15 replicates, and at least 240 flies in 8 replicates were used for the experiment.

**Different Concentration of Diets:**

When *Drosophila melanogaster CSORC* flies were 2 days old then the males were collected from the F1 and F2 generations. Flies were fed for five days on various diets in a 25°C incubator. A standard diet consisting of (10:10 g $dl^{-1}$ sugar: protein) was called the control. The rest of the diets were called the experimental diets having different concentrations, a restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), high caloric diet (10:40 g $dl^{-1}$ sugar: protein), high sugar diet (40:10 g $dl^{-1}$ sugar: protein), and a nutrient dense diet (40:40 g $dl^{-1}$ sugar: protein). After 5 days on various diets flies were frozen. Flies' heads were removed by banging against table or vortexing for 15 seconds. The mRNA was isolated from flies' heads and cDNA was synthesized, then a quantitative RT-PCR was carried out on cDNA of flies' heads. Fifty flies heads were used in each replicate and 5 replicates were applied in each condition.

***Drosophila* Activity monitoring system (DAMS) Assay:**

Three different strains *Yw*/*UAS-PKD*$^{RNAi}$, *Elav-GAL4/UAS-PKD*$^{RNAi}$, *Elav-GAL4/Yw* were used in order to record the activity and behavior of the flies. For each strain, 30 male flies were collected and each one fly was put into a narrow glass vial. There was food on one side

of the glass vials and a cotton plug on the other side and the fly was put in the middle of the glass vials. All of these 30 vials were then put into a small chamber having small holes according to the size of the glass vials and then the chamber was transferred inside the incubator having 29°C temperature. Afterward, the chamber was connected with a computer program, when the flies move inside the small vials that the activity should be recorded. The experiment was run for 48 hours. On the first day, there was no activity-recorded because the environment was new for the flies and only the flies have to get use with the system for the first 24 hours. The activity and behavior were recorded on the 2$^{nd}$ day. As mentioned above, the experiment was performed for the 3 different strains only for the purpose to confirm the behavioral assay between the control flies (*Yw/UAS/PKD$^{RNAi}$, Elav-GAL4/Yw*) and the knock down (*Elav-GAL4/PKD$^{RNAi}$*).

**Isolation of RNA:**

During the time of collection, flies were anesthetized with carbon dioxide on a small chamber under the microscope. Only male flies were collected and transferred into 1 ml Eppendorf tubes chilled on ice and stored for at least 24 hours in -80°C freezer. Flies' heads were removed by banging the tubes with a table or by vortexing for 15 seconds. Flies' heads were counted over a Petri dish placed on the dry ice and shifted into a new Eppendorf tube with a small brush. But in wild-type flies *CSORC* of *Drosophila melanogaster* 10 whole flies were obtained and RNA was isolated from them. (Similar RNA Isolation procedure was used for the flies' heads and for the whole flies body).

Fifty flies' heads from *Drosophila melanogaster* were obtained in a 1.5 ml Eppendorf tube, 700 µl Qiazol lysis reagent (miRNeasy Micro Kit Qiagen) was added to the tubes. Flies' heads were homogenized with mortar and pestle. Qiazol lysis reagent maintains the integrity of RNA, while disrupting cells and dissolving cell components. The samples were incubated for 5 minutes at room temperature. Afterward, 140 µl chloroform was added into the RNA

sample that denatures the proteins. The RNA samples were incubated again for 2-3 minutes and spun down (24 X 1.5/2.0 ml rotor of Thermo Scientific Sorvall$^{TM}$) at 8000 X g for 15 minutes at 4°C. Afterward 300 µl aqueous phases were collected from the RNA samples and transferred to the collection tubes. The RNA samples were precipitated by adding 525 µl of 100% ethanol to the aqueous phase. All of the RNA samples were passed into the RNeasy Mini Elute spin columns and 700 µl buffer RWT was added to the tubes and the samples were centrifuged (24 X 1.5/2.0 ml rotor of Thermo Scientific Sorvall$^{TM}$) for 15 seconds at 8000 X g at 4°C. The flow-through was discarded from the tubes and 500 µl buffer RPE was added to the tube columns. The tubes columns were centrifuged as above and the flow-trough was discarded. The RNA samples were washed with 500 µl of 80% ethanol and a clean RNA pellet was collected. After centrifugation the flow-through was discarded. Tube columns were transferred into new 2 ml collection tubes and were centrifuged for 5 minutes to dry the column membrane. RNA tube column was shifted into new 1.5 ml collection tube and the RNA was eluted with a 14 µl of RNA free water. After, centrifugation for 1 minute RNA was collected in the collection tube and the column was discarded.

**RNA Concentration:**

The RNA concentration was measured by nanodrop ND 1000 spectrophotometer (Saveen Werner) after spinning down the samples.

**Complementary DNA (cDNA synthesis):**

Total RNA of 2 µg was used to synthesize cDNA. The reaction mix consisted of 1 µl (5U/µl) reverse transcriptase enzyme (AB applied biosystem, Qiagen), 10 µl reverse transcriptase buffer (AB applied biosystem, Qiagen) and remaining 9 µl included RNA according to calculation plus water to make a total volume of 20 µl. Afterward, the reactions were performed in a PCR cycler, with initial denaturation at 95°C for 3 minutes, and cDNA synthesis at 37°C for 1 hour. In order to confirm the synthesis of cDNA samples this was

then amplified by PCR program with a condition, initial denaturation at 95°C for 3 min, 30 cycles of denaturing at 95°C for 30 sec, annealing temp at 52.8°C for 30 sec and extension at 72°C for 30 sec. Rp49 was used as a primer and Taq DNA polymerase enzyme (5U/ µl) along with DMSO in the reaction. Afterward, the cDNA was analyzed on a 1.5% agarose gel using a 100 bp ladder as a size marker. 10 ng/µl of cDNA was used in qPCR.

**Primer Design:**

All primers were designed using Beacon primer design 8 (Premier Biosoft, Palo, CA, USA), SYBR Green setting was selected. First of all mRNA sequences for the genes of interest *PKD Str-mlck*, *Akh, Rp49* and *Ilps* 2, 3, 5 and 6 were obtained from a fly base. The mRNA sequences were uploaded to the beacon designer and run for online blast search. Afterward, pick primers option was choosed and the best primers were selected and the primers were sent for the order to the company (Fischer Thermo Scientific). For each gene, two sets of primers forward and reverse were designed, shown in Table 1.

**Table 1. List of primers used.**

| | | |
|---|---|---|
| 1 | RP49 F. CACACCAAATCTTACAAAATGTGTGA | RP49 R. AATCCGGCCTTGCACATG |
| 2 | Pkd F. CTCCAAGACCATCACACT | Pkd R. ATCCTGTCCAACGAAGTA |
| 3 | Str-mlck F. TCACTGGCGGAGAACTGTTC | Str-mlck R. CGGGTGCTTTCGTATCCAGT |
| 4 | Dilp2 F. AGCCTTTGTCCTTCATCT | Dilp2 R. CATACTCAGCACCTCGTT |
| 5 | Dilp3 F. AAACTGCCCGAAACTCTC | Dilp3 R. AGCATCTGAACCGAACTAT |
| 6 | Dilp5 F. CTTGATGGACATGCAGAG | Dilp5 R. GAAAAGGAACACGATTTG |
| 7 | Dilp6 F. GTCCAAAGTCCTGCTAGTCCT | Dilp6 R. TCTGTTCGTATTCCGTGGGTG |
| 8 | Akh F.CTGGTCCTGGAACCTTTT | Akh R. GAGCTGTGCCTGAGATTG |

**Polymerase chain reaction (PCR):**

The method of PCR is particularly used to amplify the target DNA fragment for the purpose to enhance the desired DNA to a measurable level. Kary Mullis developed PCR in 1984 [18]. This has a great influence on all molecular studies such as those in the diagnosis of different diseases. One can detect the lowest amount of pathogens; can detect pathogens of mixed populations in infections and also conclude weight of pathogens. This method is particularly used in molecular biology, medical and forensic application as well as research in the laboratory. The three major steps of PCR can be summarized as follows.

**Denaturation:**

The two strands of DNA (dsDNA) denature when heated to 90-97°C. The reaction sample is first heated that separates the DNA into two pieces of single-stranded DNA.

**Annealing:**

The primers binds to their complementary single stranded DNA template molecules when the reaction mixture temperature lowers down to about 50-60°C.

**Elongation:**

The replication process starts, once the temperature of single-stranded DNA primers solution is increased from 50°C to 72°C for about 1-3 minutes, when dNTPs, $Mg^{2+}$, eventually DMSO, PCR buffer and heat stable Taq polymerase enzyme are present.

With every repetition of this process of PCR, the amount of the desired DNA is doubled and approximately after 30 cycles, the reaction will generate about 1 million copies of the desired gene fragment [18, 19].

**Electrophoresis:**

Agarose is the utmost standard method for the separation of medium and large sized nucleic acids. Agarose concentration is stated to as the percentage of agarose to a volume of buffer (w/v). DNA moves faster at lower agarose concentration. In general, a low concentration of

agarose is used to separate large fragments of DNA and a high concentration of agarose is used for the isolation of small fragments of DNA.

We used 1.5% agarose gel dissolved in buffer having the same concentration as the one used in the gel tank and for the gel. The preparation of the gel used was as follows: 6 grams of agarose was dissolved in 400 ml of a solution having tris base, acetic acid and EDTA (pH 8.0) called TAE buffer. Five microliter of ethidium bromide was added to 100 ml of gel solution and the gel solution was poured into the gel tank and comb was inserted to make holes. The DNA or RNA samples were analyzed. The loading buffer contains 6 X dyes normally used as 0.25% bromophenol, which assists inspection of the DNA/RNA sample during gel loading and electrophoresis. Afterward, 10 µl of cDNA/RNA samples were run on a 1.5% agarose gel for 20 minutes and 130 volt. The observation of DNA/RNA inside the agarose gel is studied under the ultraviolet light. For a record purpose photos were taken from the gel in a dark room [20].

**Real time PCR (qPCR):**

Relative mRNA expression levels of a housekeeping gene (Rp49) and of the genes of concern *PKD*, *Akh, str-mlck* and *Ilps* (2,3,5,6) were determined through quantitative RT-PCR (qPCR). QPCR was performed in a total volume of 20 µl reactions, IQ$^{TM}$SYBR® Green supermix (Bio-Rad) was used in qPCR and the gene of interest was amplified by instrument IQ5 (Bio-Rad). All experiments of the qPCR were performed in duplicates. Template concentration of 10ng/µl cDNA and the concentration of each of the primer was 2 pmol/µl in 96 well plate. Water was used as a negative control for each qPCR. Beacon designer was used for primer design using SYBR Green as a setting. The following qPCR condition was used, initial denaturation at 95°C for 3 min, 50 cycles of denaturing at 95°C for 30 sec, annealing temp at 52.8–63°C for 30 sec and extension at 72°C for 30 sec. Data of the qPCR was analyzed using My IQ 5 software (BioRad) [21]. LinReg PCR program was applied to

calculate the efficiencies of the primers [22]. Afterward, the GeNorm protocol described by Vandesompele et al [23] was applied in order to obtain normalization values from the expression levels of the housekeeping gene. Then Grubbs test was applied to exclude outliers. Gene expressions between different groups were analyzed through ANOVA. The names and sequences of the primers were mentioned above in table 1.

**Statistical analysis:**

The starvation assay results were analyzed through log rank test, a nonparametric test that evaluates the survival curve of two samples via online. The data from the quantitative real-time PCR were analyzed by one-way anova using bonferroni compared selected pairs of columns option. All graphs were obtained in graph pad prism 5 or Microsoft excel 2007.

**Results:**

***PKD* knockdown in the whole fly of *Drosophila melanogaster* with *Daughterless Elav-GAL4* driver:**

In order to perform any assay, the crosses were performed between the three genotypes as mentioned in Materials and Methods. First of all crosses were established between the *UASPKD*$^{RNAi}$ lines with the uniqitious driver *Elav-GAL4*. Then the crosses were performed once again between *UASPKD*$^{RNAi}$ line and the *daughterless-Elav-GAL4* (*Da-Elav-GAL4*), to confirm the results of the experiment. These two RNAi lines were a *TRIP* line (one line). (Refered to as *PKD*$^{RNAi}$). RNase III similar enzyme in *Drosophila melanogaster* generates smaller fragments of 22 nucleotides, like the size generated throughout RNAi mechanism. This particular enzyme called Dicer degrades dsRNA into equally sized small interfering RNA SiRNA. In flies, mammals, plants and worms nucleases are evolutionary conserved. The SiRNA detaches into two single strands and is incorporated into the RISC complex. Afterward, SiRNA combines to the target mRNA and argonauts catalysis cleavage of the mRNA, so inhibiting the process of translation. *Da-Elav-GAL4* lines were crossed with *UAS-PKD*$^{RNAi}$ and *yw*. *Da-Elav-GAL4* heterozygous knockdown males had 0.41 fold (p=0.0136) (Figure 2A) of normal *PKD* expression level. *PKD* expression in *yw-PKD*$^{RNAi}$ control was set as 100% represented as 1 on the graph. The *PKD*$^{RNAi}$ line was crossed with both the experimental line (*da-Elav-GAL4/PKD*$^{RNAi}$) and with the control *yw* (*yw-PKD*$^{RNAi}$). Therefore the *yw-PKD*$^{RNAi}$ control was set as 100% indicated as 1 on all the graphs. Unexpectedly *Str-Mlck* gene had also shown a knock down instead of up-regulation. Therefore, this line was predicted to have a possible off-target effect that disrupts the expression of *stretchin-Mlck (Str-Mlck)*, another serine/threonine kinase. We concluded from these results that both *Strn-Mlck* and *PKD* were knocked down at the same time (Figure 2A, 2B).

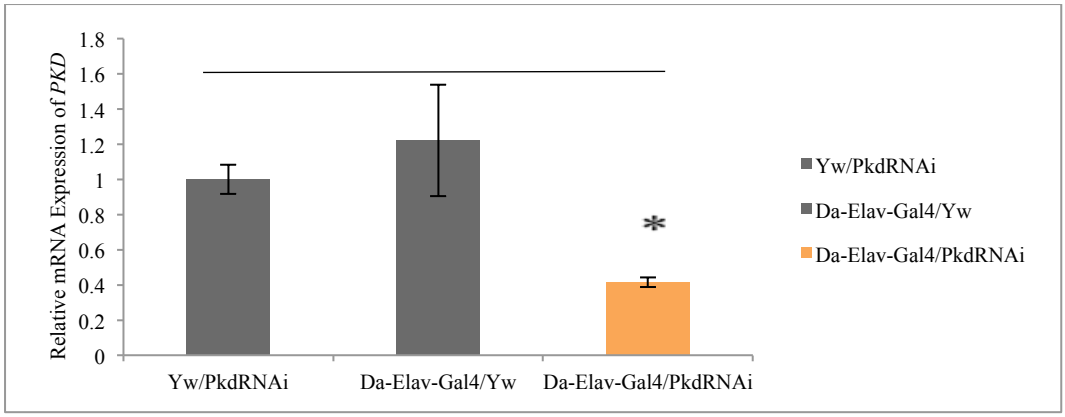


**Figure 2A)** Protein kinase D (*PKD*) knockdown reduces *PKD* transciption in *da-Elav-GAL4/PKD*$^{RNAi}$ in the whole fly of *Drosophila melanogaster* which was compared to the two control lines *da-Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$. Five replicates were used for each of these three genotypes and each replicate consisted of 8 flies. All values are given as mean ± SE. A single asterisk (*) denotes the significant difference of *PKD* knockdown *da-Elav-GAL4/PKD*$^{RNAi}$ compared to the control. P<0.05 was used as a significant difference. One-way anova with bonferroni post hoc test was applied. The orange bar in the graph shows the *PKD* knock down and the black bars represent controls. The *yw* line was crossed with the *PKD*$^{RNAi}$, therefore *Yw/PKD*$^{RNAi}$ control was set as 100% indicated as 1 on the graph instead of selecting the second control.

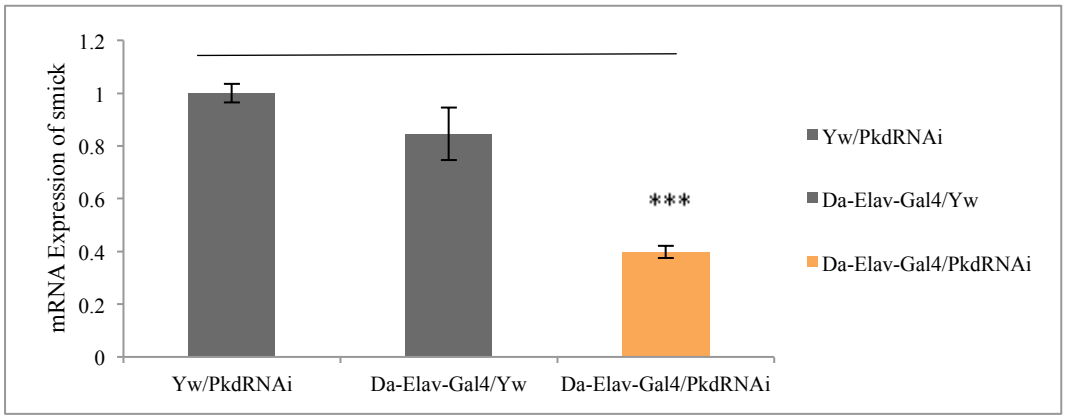


**Figure 2B)** Protein kinase D (*PKD*) knockdown reduces *Smick* in *da-Elav-GAL4/PKD*$^{RNAi}$ in the whole fly of *Drosophila melanogaster*. *Smick* function was confirmed in *PKD* knock down flies compared to the two control lines *da-Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$. Five replicates were applied per condition and each replicate consisted of 8 flies. All values are given as mean ± SE. A triple asterisks (***) indicates the significant difference of *PKD* knock down to control (p<0.0001). P<0.05 was used as a significant level. One-way anova with bonferroni post hoc test was applied. The orange bar in the graph shows the *PKD* knock down and the

black bars show the controls in the graph. The *Yw/PKD*$^{RNAi}$ was obtained as a result of the cross of *yw* and *PKD*$^{RNAi}$, therefore *Yw/PKD*$^{RNAi}$ control was set as 100% indicated as 1 on the graph.

**_PKD_ expression in the IPCs and its affect on _ILPS_ transcript levels:**

The function of *PKD* was investigated in the insulin producing cells IPCs by determining the relative mRNA expression level. After the knocking down of *PKD*, the transcript levels of various *Ilps* were also investigated in the whole body of *Drosophila melanogaster*. All of the *Ilps* including *Akh* were not raised up to the significant level compared to the control. The knocking down of *PKD* did not significantly affect the transcript level of *Ilp3* ($p<0.0568$) one-way anova post hoc test, (Figure 3A), *Ilp5* ($p<0.8503$) one-way anova post hoc test, (Figure 3B), *Ilp2* ($p<0.0975$) one-way anova post hoc test, (Figure 3C), and *Akh* ($p<0.4852$) one-way anova post hoc test, (Figure 3D). The transcript level for *Ilp3* was raised but the increase was not significant compared to both control *Yw/PKD*$^{RNAi}$ and *da-Elav-GAL4/Yw*. *Ilp6*, transcript level in the *PKD* knock down did not increase significantly (Figure 3E). The *yw* line was crossed with the *PKD*$^{RNAi}$ line, therefore the *Yw/PKD*$^{RNAi}$ control was set as 100% indicated as 1 on the graph (Figure 3A-3E) while the second control was not crossed with the *PKD*$^{RNAi}$, therefore this control was not set as 1 on the graph.

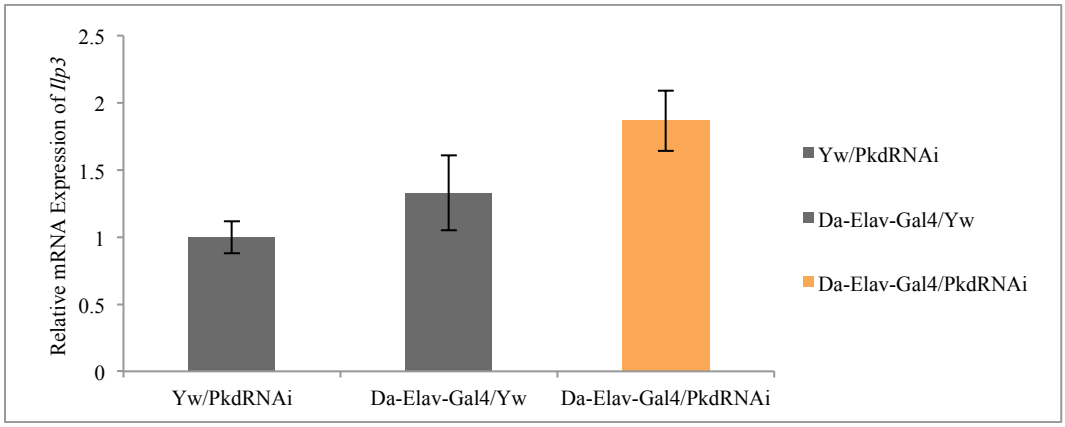

**Figure 3A)** The expression of *Ilp3* was not significantly affected by Protein kinase D (*PKD*) knockdown (*da-Elav-GAL4/PKD*$^{RNAi}$) in the whole fly of *Drosophila melanogaster* compared to the controls (*da-Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$). The *yw* line was crossed with the *PKD*$^{RNAi}$, so the *Yw/PKD*$^{RNAi}$ control was set as 100% represented as 1 in the figure, instead of choosing the second control on the graph. Five replicates were used per condition and each replicate consisted of 8 flies. All values are given as mean ± SE. No significant difference for *Ilp3* ($p<0.0568$) was found compared to control. $P<0.05$ was used as a significant difference. One-way anova with bonferroni post hoc test was applied. The black bars show the controls and the orange bar shows the *PKD* knock down in the graph.

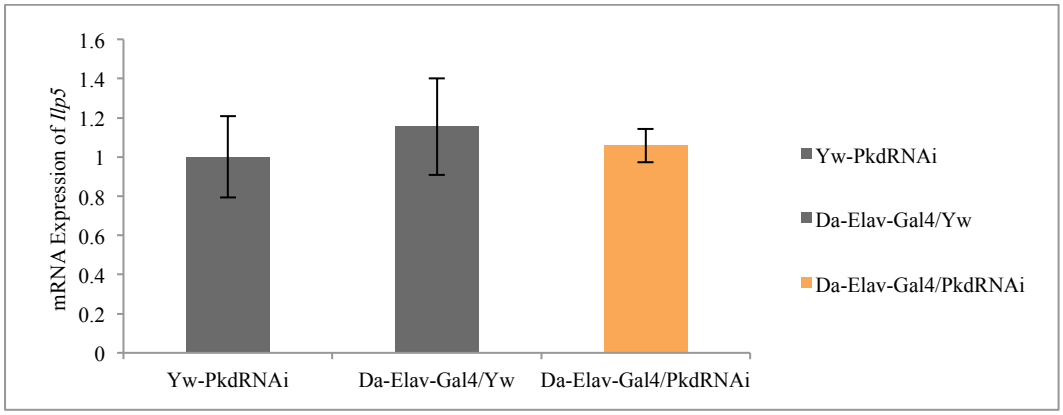


**Figure 3B)** The transcription of *Ilp5* was not affected by the *PKD* knockdown (*da-Elav-GAL4/PKD*$^{RNAi}$) in the whole fly of *Drosophila melanogaster* compared to the controls (*da-Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$). The *yw* was crossed with *PKD*$^{RNAi}$ line as a result *Yw/PKD*$^{RNAi}$ was obtained. Therefore the *Yw/PKD*$^{RNAi}$ control was set as 100% represented as 1 on the graph. Five replicates were used for each of these three genotypes and each replicate consisted of 8 flies. All values are given as mean ± SE. No significant difference was found for *Ilp5* ($p<0.8503$) compared to control. The $p<0.05$ was applied as a significant difference. One-way anova with bonferroni post hoc test was used. The black bars show the controls and the orange bar shows the *PKD* knock down in the graph.

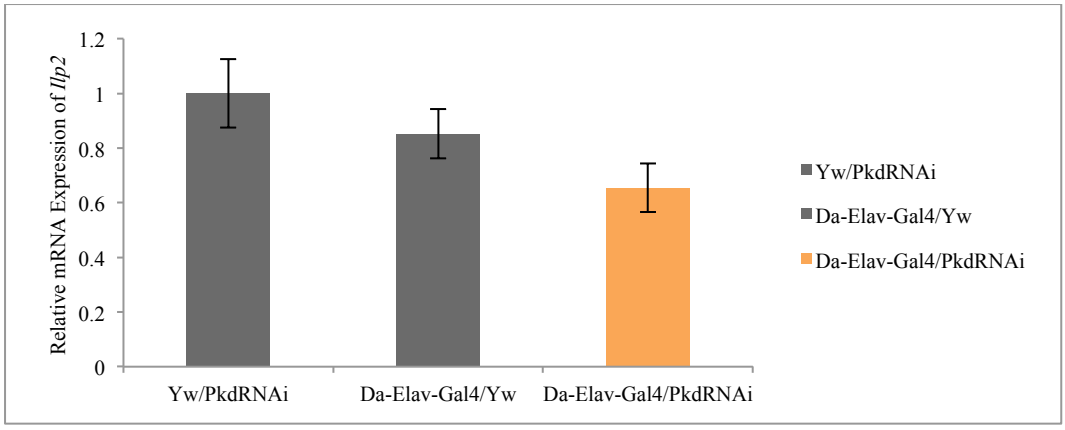


**Figure 3C)** The transcription of *Ilp2* was not significantly affected by the *PKD* knock down (*da-Elav-GAL4/PKD*$^{RNAi}$) in the whole fly of *Drosophila melanogaster* compared to the controls (*da-Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$). The *Yw/PKD*$^{RNAi}$ control was set as 100% indicated as 1 in the figure because the *Yw/PKD*$^{RNAi}$ was obtained as a result of the cross of *yw* with the *PKD*$^{RNAi}$. Five replicates were used for each genotype and every replicate consisted of 8 flies. All values are given as mean ± SE. No significant difference was found for *Ilp2* ($p<0.0975$) compared to control. The $p<0.05$ was used as a significant difference. One-way anova with bonferroni post hoc test was applied. The orange bar represents the *PKD* knock down and the the black bars show the controls in the graph.

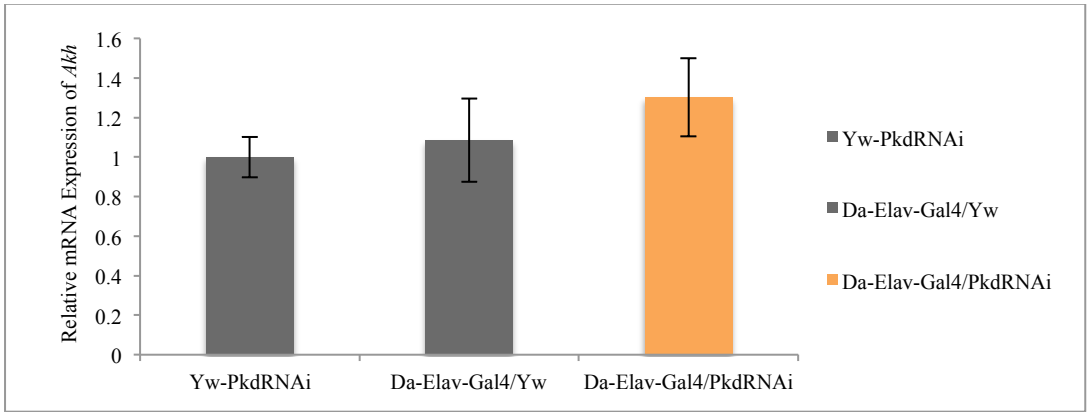


**Figure 3D)** Transcription of *Akh* was not significantly affected by *PKD* knockdown (*da-Elav-GAL4/PKD*$^{RNAi}$) in the whole fly of *Drosophila melanogaster* compared to controls (*da-Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$). The *Yw/PKD*$^{RNAi}$ control was set as 100% represented as 1 in the figure because the *PKD*$^{RNAi}$ was crossed with the *yw* as a result *Yw/PKD*$^{RNAi}$ control was obtained and the *PKD*$^{RNAi}$ was not crossed with the second control. Five replicates were used for each condition and each replicate comprised of 8 flies. All values are given as mean ± SE. No significant change for *Akh* ($p<0.4852$) was found compared to control. $P<0.05$ was applied as a

significant difference. One-way anova with bonferroni post hoc test was used. The black bars show the controls and the orange bar shows the *PKD* knock down in the graph.

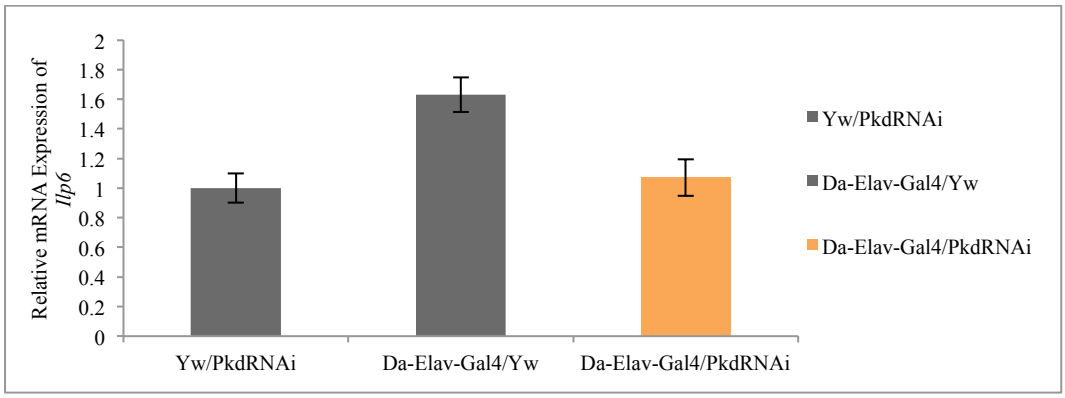


**Figure 3E)** The transcription of *Ilp6* was not significantly affected by *PKD* knockdown (*da-Elav-GAL4/PKD*$^{RNAi}$) in the whole body of *Drosophila melanogaster*. The *Ilp6* transcription was confirmed in *PKD* knockdown flies compared to the controls. The *PKD*$^{RNAi}$ was crossed with *yw* as a result *Yw/PKD*$^{RNAi}$ control was obtained while in the second control *PKD*$^{RNAi}$ was not crossed. Therefore *Yw/PKD*$^{RNAi}$ control was set as 100% represented as 1 in the figure. Five replicates were applied for each genotype and each replicate consisted of 8 flies. All values are given as mean ± SE. When compared to control the *Ilp6* did not cross the significant difference. $P<0.05$ was used as a significant difference. One-way anova with bonferroni post hoc test was applied. The orange bar shows the *PKD* knock down in the graph and the black bars show the controls in the graph.

**Effect of starvation on the survival time of transgenic fly lines:**

Three various genotypes were obtained from the crosses called *Elav-GAL4/Yw, Yw/PKD*$^{RNAi}$ and *Elav-GAL4/PKD*$^{RNAi}$. The first two genotypes were called the control and the 3$^{rd}$ one was called the *PKD* knock down, as known as *Elav-GAL4/PKD*$^{RNAi}$. These three genotypes were used for the starvation experiment. We have the assumption that the *PKD* knock down *Elav-GAL4/PKD*$^{RNAi}$ has lowered *PKD* expression and in general shortened lifetime compared to the control. To confirm our assumption these three genotype were enclosed in different vials for starvation. These flies were carefully observed after every 12 hours and the number of dead flies was noted until all of the flies had died.

The survival time and expression of *PKD* was investigated in the transgenic flies through starvation assay. The starvation assay reveals different activities of the flies such as the movement of the flies in a vial, survival time of flies and storage of lipids in flies' body. The *PKD* knock down flies died rapidly compared to the control (*Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$). The starvation assay shows *PKD* knock down were more starvation vulnerable and died earlier than the control *Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$. The control flies were resistant to starvation and died later. The p value was <0.001 for both controls, log Rank test, (Figure 4). The standard deviation for the proportion of survivors was determined. The survival time of *Elav-GAL4/Yw* was 72 hours (SEM± 0,07), *Yw/PKD*$^{RNAi}$ survival time was 60 hours (SEM± 0,03) and *Elav-GAL4/PKD*$^{RNAi}$ survival time was 36 hours (SEM± 0,03), after this time half of the flies had died in the three genotypes. Therefore, the shortest time of 48 hours was recorded for the *PKD* knock down flies when all of the flies have died (Figure 4).

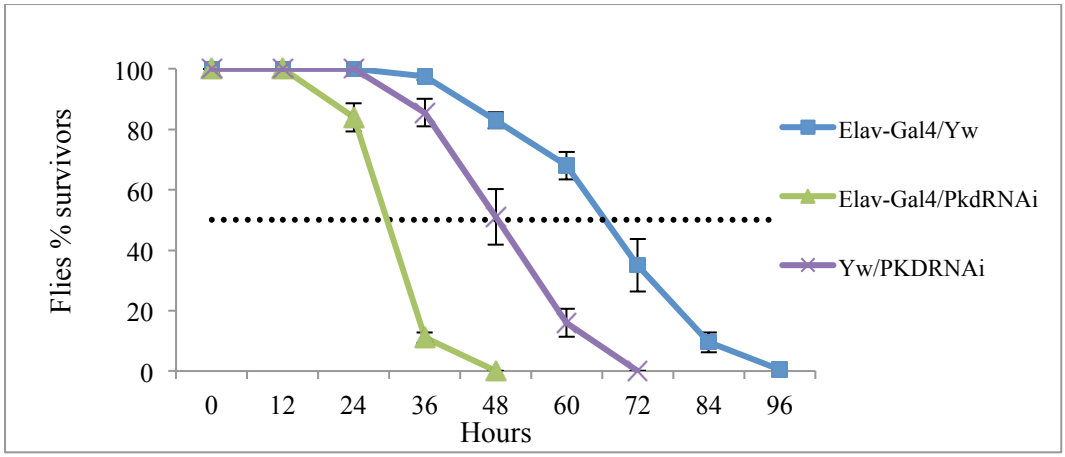


**Figure 4) Survival time of transgenic flies lines was affected by starvation:** Survival time was measured in the transgenic fly lines. The *PKD* knock down *Elav-GAL4/PKD*$^{RNAi}$ had shown a lower survival time compared to the control *Yw/PKD*$^{RNAi}$ and *Elav-GAL4/Yw*. Twenty male flies from each genotype were put into vials having 6 ml agarose (10 g/l) that provided humidity and water to the flies but no food, and incubated in a 25°C incubator. The number of dead flies was counted after every 12 hours until all of the flies died and a survival curve was obtained. Ten replicates were used for each genotype and each replicate consisted of 20 flies. The *PKD* knock down flies have shown minimum starvation resistance compared to the control. All values are given as mean ± SE. P value of <0.001 for both controls was obtained, log-rank test). Y-axis shows the survival time of the transgenic flies and X-axis shows the time in hours. The green line in the graph shows the *PKD* knock down and blue and purple line shows the controls in the graph.

**Effect of lipid on the transcription of transgenic fly lines:**

Two over-expression controls (*Elav-GAL4/PKD*$^{OE}$, *Yw/PKD*$^{OE}$) were added with the three genotypes used in the starvation experiment. Therefore a total of five different genotypes were used for the lipid extraction assay. The total lipid content of the above-mentioned strains were studied, to investigate the possible function of *PKD*, and to find out whether it affects the survival times of the flies and its correlation with the above mentioned starvation experiment.

The *PKD* knock down had shown lower lipid contents compared to controls *Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$ (Figure 5). After 0 hours of starvation, *PKD* knockdown flies had on average 39.04 µg (SEM ± 4.02 µg) of lipids compared to the control flies, *Yw/PKD*$^{RNAi}$ 56.9

µg (SEM ± 5.1 µg) and *Elav-GAL4/Yw* 53.75 µg (SEM ± 4.08 µg). Two new groups of the *PKD* knockdown (*Elav-GAL4/PKD*$^{RNAi}$) flies were starved for 6 and 12 hours. After 6 hours starvation the *PKD* knockdown had on average 27 ± 2.05 µg; and 12 hours starvation had on average 17 ± 2.95 µg of lipids. There was no loss of lipids content observed from 6 to 12 hours of starvation in the control *Elav-GAL4/Yw* (6 hours 41.33 ± 2.17 µg of lipids: 12 hours 50 ± 4.12 µg of lipids) and *Yw/PKD*$^{RNAi}$ (6 hours 34.84 ± 3.95 µg of lipids: 12 hours 40.27 ± 2.64 µg of lipids). Overall the lipid content of *PKD* knockdown (*Elav-GAL4/PKD*$^{RNAi}$) was significantly lowered than both the control *Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$ for the two different time periods of starvation.

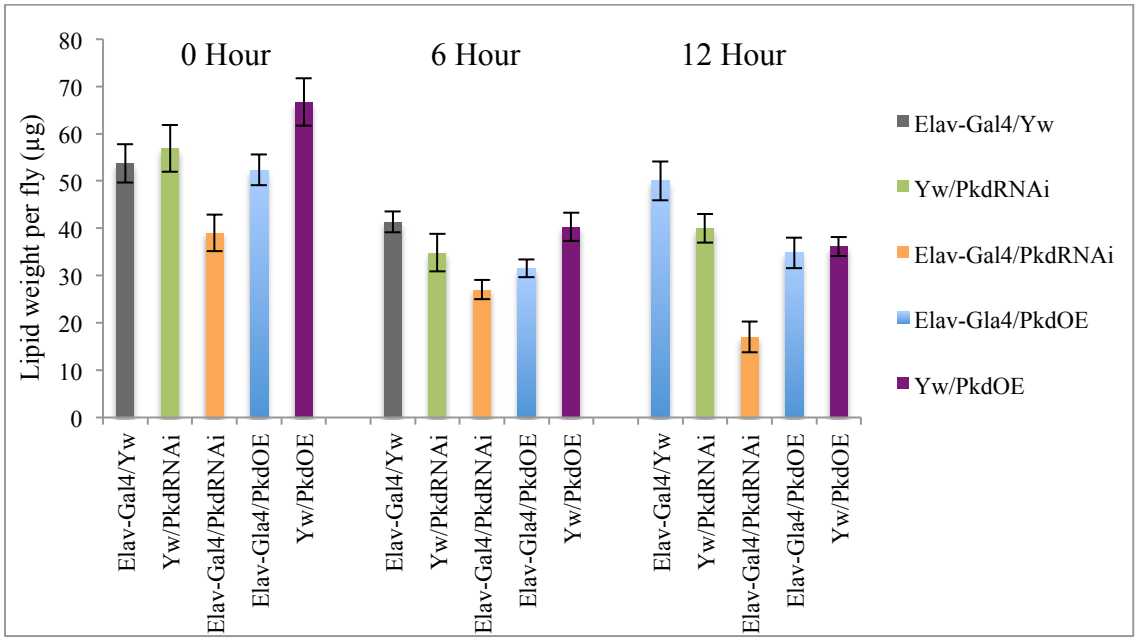


**Figure 5) Reduction in lipid content in the transgenic fly lines affects the expression of *PKD*.** Total lipid content was measured in flies with reduced *PKD* expression in the *PKD* knockdown and in the controls, at three different time points, 0 hours, 6 hours and 12 hours of incubations. Thirty male flies of each genotype were enclosed into glass vials and investigated for the lipid content (explained in Materials and Methods). The difference between the dry weight and lean dry weight was calculated and the actual weight of lipid was obtained. In the two starvation intervals, the *PKD* knockdown flies had shown lower lipids content than the control *Elav/Yw* and *Yw/PKD*$^{RNAi}$, (0h: $p<0,05$, one-way anova bonferroni post hoc test; 6h: $p<0,05$, one-way

anova bonferroni post hoc test; 12h: $p<0,01$, one-way anova bonferroni post hoc test). For each genotype, 30 flies per condition were used and a minimum of 10 replicates was applied. All values are given as mean ± SEM. Y-axis in the graph shows the amount of lipid in microgram and the x-axis shows the different genotypes. The first and the last two bars in the graph shows the controls and the middle bar (orange) represent the *PKD* knock down in the graph in the three interval (0 hours, 6 hours, 12 hours)

***PKD* and *ILPs* transcripts level on nutrient rich diets.**

The *PKD* mRNA level was also measured in wild type *Drosophila melanogaster* flies called *CSORC* under different environmental conditions in the heads of the flies. When the flies were two days old males were collected and kept for five days on various diets having the following concentrations: Restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), standard diet (10:10 g $dl^{-1}$ sugar: protein), high calorie diet (10:40 g $dl^{-1}$ sugar: protein), high sugar diet (40:10 g $dl^{-1}$ sugar: protein), and a nutrient rich diet (40:40 g $dl^{-1}$ sugar: protein). After 7 days of aging the flies from various diets were collected and frozen, then the flies were decapitated and mRNA was isolated from the flies' heads. The cDNA was synthesized and confirmed with the gel electrophoresis. Afterward, the cDNA was run on qPCR for various genes and the expression levels of mRNA for various genes were analyzed. The *PKD* expression level was decreased only on the nutrient rich diet, 40:40 g $dl^{-1}$ sugar: protein, compared to the standard diet, 10:10 g/dl. Expression of *PKD* was decreased up to 60% (SEM ± 4.1 $P<0.0045$ one-way anova) of the standard diet (Figure 6A). The rest of the diets did not cross the statistical significance level associated to standard diet ($p<0.05$ for all diets were used). The mRNA level was raised up to 80% for *Ilp*3 when flies were kept on nutrient rich diet (40:40 g/dL protein: sugar) ($p<0.041$, Figure 6B). There were no significant changes measured in *Ilp*5 and *Ilp*2 mRNA expression level on various diets, compared to the standard diet (*Ilp*5, $p<0.3531$, *Ilp*2, $p<0.1249$ one-way anova) (Figure 6C, 6D). The expression level of mRNA for *Akh* was different on all diets. The qPCR data indicated higher expression for the *Akh* on high-calorie diet (10:40 g $dl^{-1}$ sugar: protein). The *Akh* had shown less expression on the nutrient-rich diet, sugar-rich diet, and restricted diet compared to the standard diet, 10:10 g/dl. There was no significant change measured in *Akh* expression. P value of 0.0564 was obtained after the statistical analysis for all diets of *Akh*, which is higher than the significance level (Figure 6E). One-way anova bonferroni post hoc test was performed. The 10:10 g/dl diet was used as a

standard diet in the experiments and its expression level was set as 100% represented as 1 on the graph (Figures 6A-E).

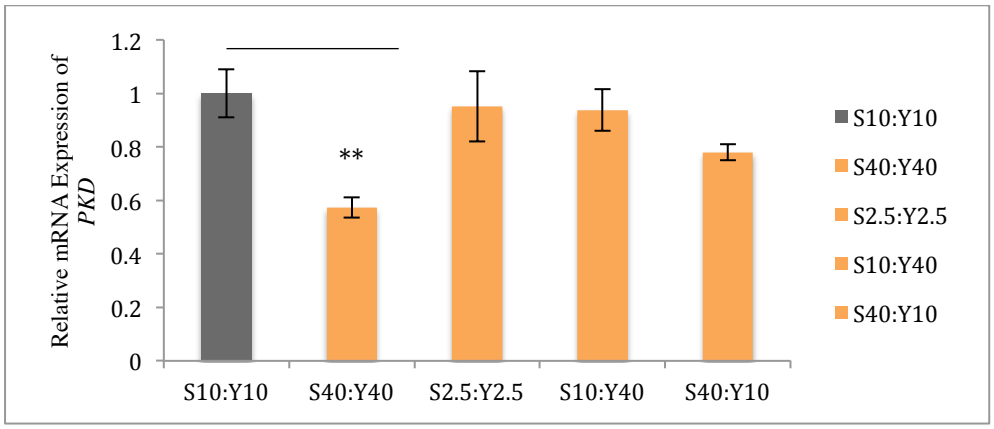


**Figure 6A) Nutrient dense diet reduces protein kinase D (*PKD*) transcription in the flies heads.** *D. melanogaster CSORC* flies were fed for five days on five various dietary regimes, a restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), a standard diet (10:10 g $dl^{-1}$ sugar: protein), high calorie diet (10:40 g $dl^{-1}$ sugar: protein), high sugar diet (40:10 g $dl^{-1}$ sugar: protein) and a nutrient dense diet (40:40 g $dl^{-1}$ sugar: protein). The mRNA was isolated from the heads of the flies, cDNA was synthesized and a quantitative RT-PCR was performed. Five replicates were used per condition and each replicate consisted of 50 flies' heads. All values are given as mean ± SE. A double asterisk sign (**) denotes high significant difference to control ($p<0.0045$) which is less than the significant level. One-way anova with bonferroni post hoc test was performed. The *CSORC* strain in the (10:10 g $dl^{-1}$ sugar: protein) diet was applied as a standard diet and the expression level was set to level 1 in the diet. The black bar indicates the base case in the figure.

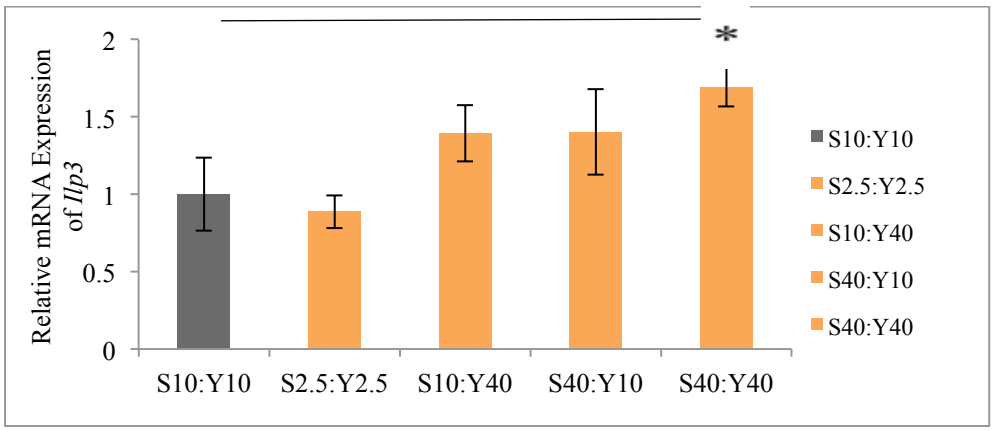

**Figure 6B) Nutrient dense diet elevates *Ilp3* transcription in the flies brain.** Wild-type *CSORC* flies were fed for five days on five various dietary regimes, restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), a standard diet (10:10 g $dl^{-1}$ sugar: protein), high calorie diet (10:40 g $dl^{-1}$ sugar: protein) high sugar diet (40:10 g $dl^{-1}$ sugar: protein) and a nutrient dense diet (40:40 g $dl^{-1}$ sugar: protein). The mRNA was extracted from flies heads, cDNA was synthesized and quantitative RT-PCR was carried out. Five replicates were used per condition and each replicate consisted of 50 flies heads. All values are given as mean ± SE. When compared to control a signficant difference was found. P<0.05 was used as a significant difference. One-way anova with bonferroni post hoc test was performed. The (10:10 g $dl^{-1}$ sugar: protein) diet was used as a base case. The expression level was set as 100% represented as 1 in the figure and is shown by a black bar on the graph.

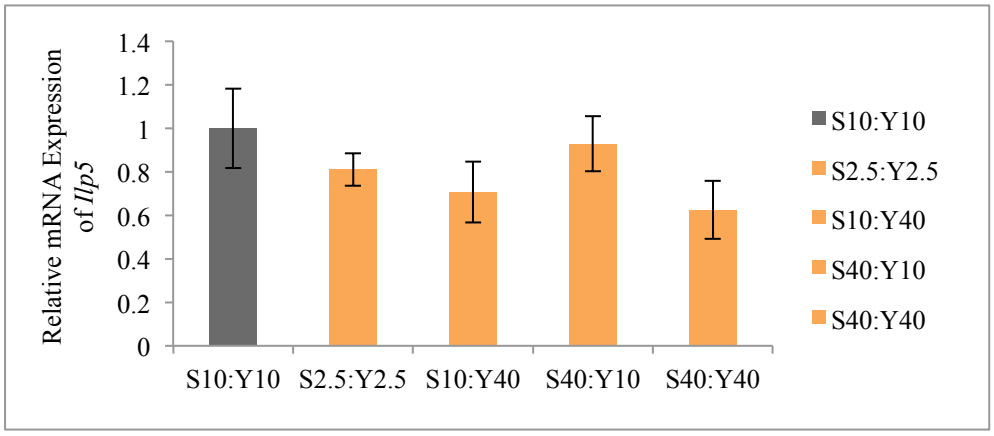


**Figure 6C) *Ilp5* transcription was not affected by various dietary regimes in the fly heads.** *D.melanogaster CSORC* flies were fed for five days on five various dietary regimes, a restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), a standard diet (10:10 g $dl^{-1}$ sugar: protein), high calorie diet (10:40 g $dl^{-1}$ sugar: protein), high sugar diet (40:10 g $dl^{-1}$ sugar: protein) and a nutrient dense diet (40:40 g $dl^{-1}$ sugar: protein). The mRNA was isolated from flies heads, cDNA was synthesized and a quantitative RT-PCR was run. Five replicates were used per condition and each replicate consisted of 50 flies heads. All values are given as mean ± SE. No significant difference was found to control. P value <0.05 was used as a significance level. One-way anova with bonferroni post hoc test was performed. The control sample was compared with the four experimental samples and a p value of 0.3531 was obtained which is bigger than the significance level. The (10:10 g $dl^{-1}$ sugar: protein) diet was used as a standard diet and its expression level was set as 100% indicated as 1 on the graph. The black bar shows a standard diet on the graph.

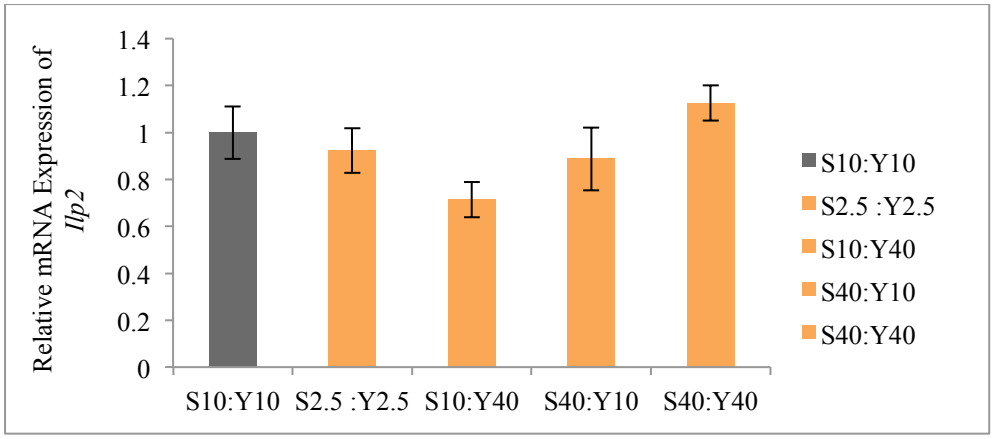


**Figure 6D) *Ilp2* transcription was not affected by different dietary regimes in flies heads.** *D. melanogaster CSORC* male flies were fed for five days on different dietary regimes, a restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), a standard diet (10:10 g $dl^{-1}$ sugar: protein:), caloric rich diet (10:40 g $dl^{-1}$ sugar: protein), high sugar diet (40:10 g $dl^{-1}$ sugar: protein) and a nutrient thick diet (40:40 g $dl^{-1}$ sugar: protein). The mRNA was isolated from flies' heads, cDNA was synthesized and a quantitative RT-PCR was carried out on cDNA of flies' heads. Five replicates were used per condition and each replicate consisted of 50 flies heads. All values are given as mean ± SE. No significant difference was found to control. $P<0.05$ was used as a significance level. One-way anova with bonferroni post hoc test was performed. The (10:10 g $dl^{-1}$ sugar: protein) diet was used as a base case in the experiment and its expression level is set to level 1. The black bar represents a *CSORC* standard diet in the figure.

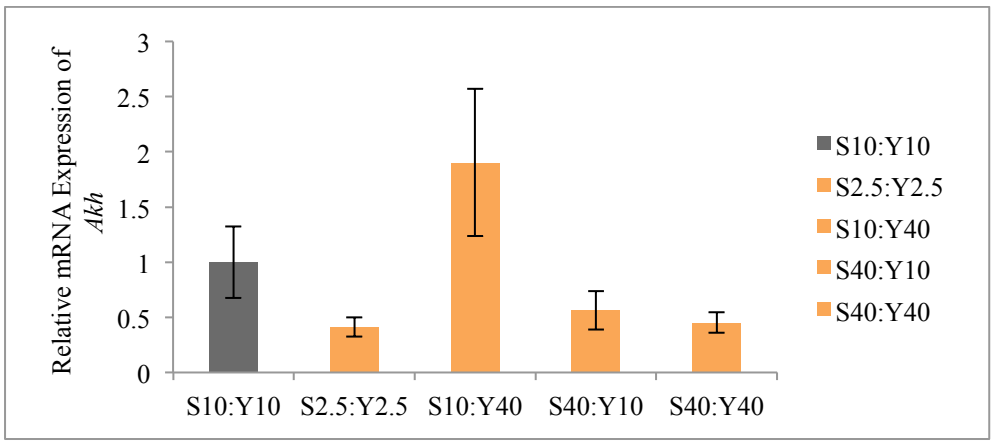


**Figure 6E) *Akh* transcription was not affected by different dietary regimes in the fly heads.** *D. melanogaster CSORC* male flies were fed for five days on different dietary regimes, a restricted diet (2.5:2.5 g $dl^{-1}$ sugar: protein), a standard diet (10:10 g $dl^{-1}$ sugar: protein), high calorie diet (10:40 g $dl^{-1}$ sugar: protein),

high sugar diet (40:10 g $dl^{-1}$ sugar: protein) and a nutrient thick diet (40:40 g $dl^{-1}$ sugar: protein). The mRNA was isolated from flies' heads, cDNA was synthesized then a quantitative RT-PCR was carried out on cDNA of flies' heads. Five replicates were applied for each genotype and each replicate consisted of 50 flies heads. All values are given as mean ± SE. No significant difference was found to control. $P<0.05$ was used as a significance level. One-way anova with bonferroni post hoc test was performed. The *CSORC* standard diet (base case) is represented with a (10:10 g $dl^{-1}$ sugar: protein) diet in the experiment and its expression level is set to level 1 in the diet. The black bar shows a base case on the graph.

**Effect of starvation on *PKD* and *ILPs* transcription:**

We have investigated the effect of starvation on *PKD* and *Ilps* expression, flies were starved for 24 and 48 hours then they were frozen for 1 day. Afterward, flies' heads were removed and mRNA was isolated from flies' heads. The cDNA was synthesized and a qPCR was performed on cDNA of flies' heads. The mRNA expression level was analyzed for different genes. There was no significant change observed in the expression of *PKD or Ilp*2 (*PKD*, $p<0.0817$, Figure 7A, *Ilp*2, 0.2100, Figure 7B, one-way anova bonferroni post hoc test). To investigate the function of important genes involved in energy metabolism, mRNA levels of fly glucagon *Akh* and of the brain expressed *Ilps (Ilp*3 and *Ilp*5*)* were measured. After 24 hours starvation the expression of *Ilp*5 was down regulated and a significant change was observed (*Ilp*5: $p<0.0324$, Figure 7C), but after 48 hours starvation there was no significant change observed compared to *CSORC* control (un-starved). The expression of *Ilp3* was down regulated after 48 hours starvation (*Ilp*3: $p<0.0002$, 7D), but there was no significant change recorded in the expression of *Ilp3* after 24 hours starvation. The expression of *Akh* was up regulated after 24 hours starvation and a significant change was recorded compared to the control (un-starved), but there was no change observed after 48 hours starvation compared to control (Figure 7E).

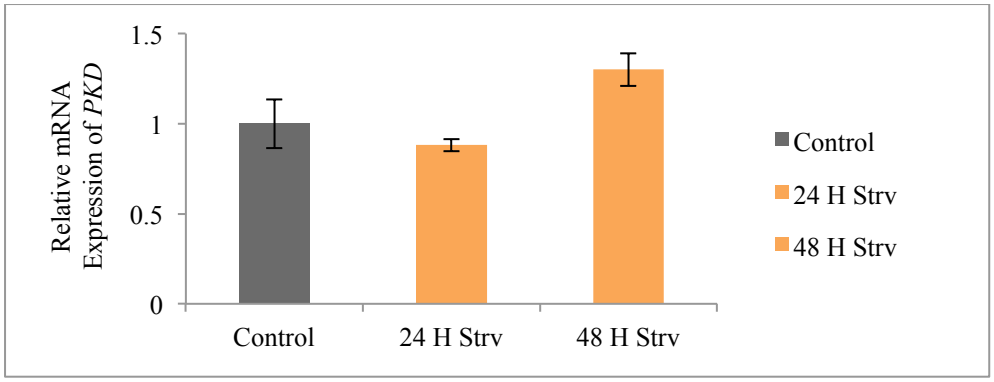


**Figure 7A). *PKD* transcription was not significantly affected by starvation in the fly brain**. *D.melanogaster CSORC* flies were starved for 24 and 48 hours. The control flies (*CSORC*) were not starved. The mRNA was isolated from flies' heads, cDNA was synthesized and a qPCR was carried out. *PKD* mRNA expression level was compared to control (un-starved). Five replicates were applied in the control and in starvation samples and each replicate consisted of 50 flies heads. All values are given as average ± SE. There was no significant change observed in the expression of *PKD* compared to control (un-starved). P<0.05 was used as a significance level. One-way anova with bonferroni post hoc test was performed.

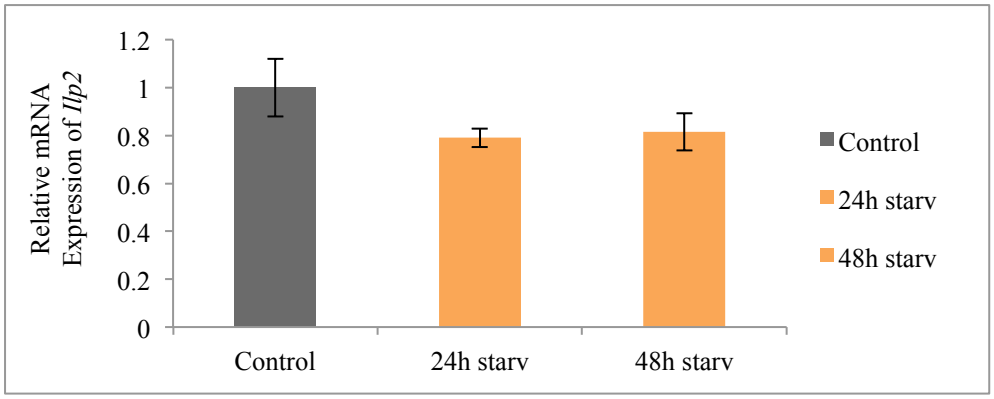


**Figure 7B). Starvation did not affect transcription of *ILP2* in the fly brain**. *D. melanogaster CSORC* flies were starved for 24 and 48 hours. The control flies (*CSORC*) were not starved. The mRNA was extracted from flies' heads, cDNA was synthesized and a qPCR was performed. *Ilp2* mRNA expression level was not affected compared to control (un-starved). Five replicates were applied per condition and each replicate consisted of 50 individuals heads. All values are given as averages ± SE. P<0.05 was applied as a significance level. One-way anova with bonferroni post hoc test was applied.

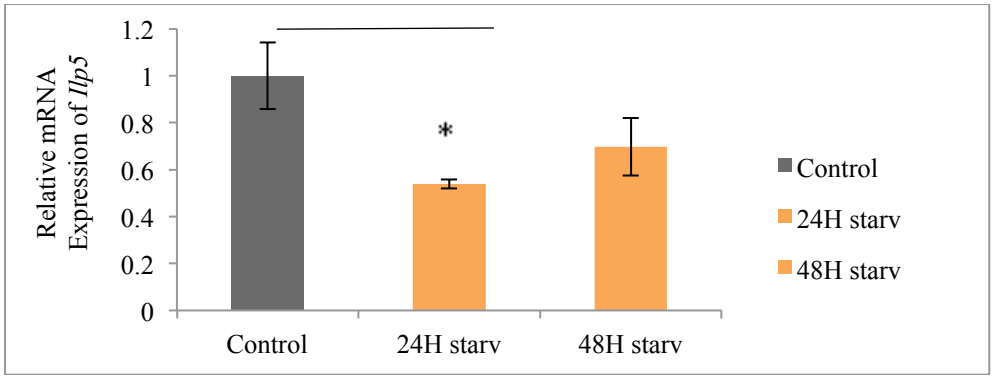


**Figure 7C). Starvation reduces *Ilp 5*- transcription in the fly brain.** *D. melanogaster CSORC* flies were starved for 24 and 48 hours. The control flies (*CSORC*) were not starved. *Ilp5* mRNA expression level was reduced in 24 hours starvation compared to control (un-starved). There was no significant change observed in the expression of *Ilp5* after 48 hours starvation compared to control. Five replicates were used per condition and each replicate consisted of 50 individuals heads. All values are given as average ± SE. Asterisk sign (*) denotes significant difference in the graph. P<0.0324 was obtained after statistical analysis which is smaller than the significance level. P<0.05 was used as a significance level. One-way anova with bonferroni post hoc test was performed.

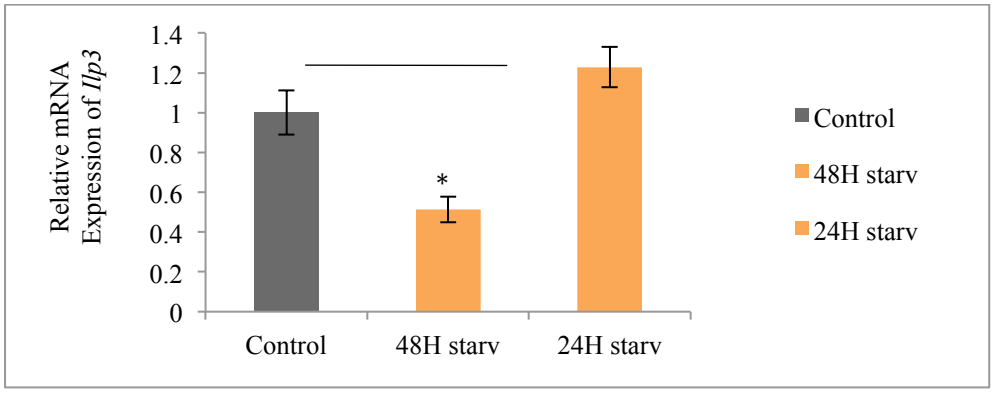


**Figure 7D) Starvation reduces *Ilp 3*- transcription in the fly brain.** *D. melanogaster CSORC* flies were starved for 24 and 48 hours. The control flies (*CSORC*) were not starved. No significant change occurred in the expression of *Ilp3* after 24 hours starvation compared to control. *Ilp3* mRNA expression level was reduced in 48 hours starvation compared to the control (un-starved). Five replicates were used per condition and each replicate consisted of 50 individuals heads. All values are given as average ± SE. A single asterisk (*) denotes significant difference to the un-starved control obtained after statistical analysis (p<0.0002) and the value is small than the significance level. P<0.05 was used as a significance level. One-way anova with bonferroni post hoc test was performed.

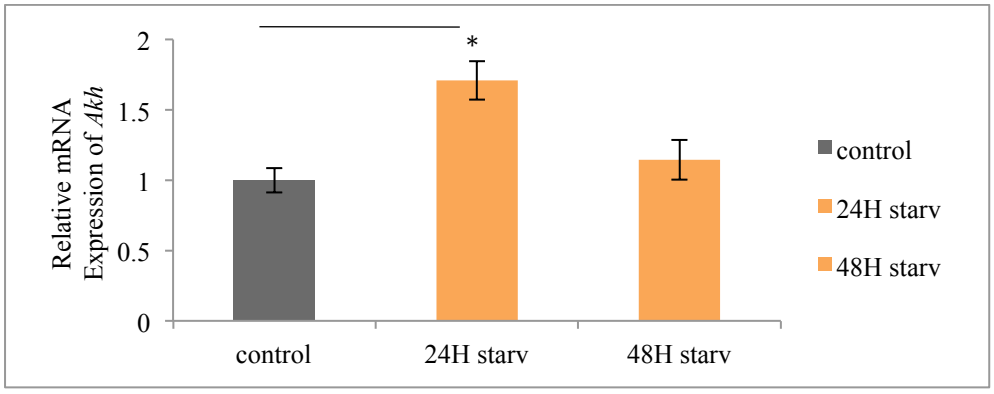


**Figure 7E). Starvation up-regulates *AKH* transcription in the fly brain.** *D. melanogaster CSORC* flies were starved for 24 and 48 hours. The control flies (*CSORC*) were not starved. *Akh* mRNA expression level was up-regulated after 24 hours starvation but there was no change recorded in the expression of *Akh* after, 48 hours starvation compared to the control (un-starved *CSORC*). Five replicates were applied per condition and each replicate consisted of 50 individuals heads. All values are given as average ± SE. $P< 0.05$ was applied as a significance level. One-way anova with bonferroni post hoc test was performed.

**Discussion:**

We have reported above on the transcription of some insulin-like peptides (*ILPs*) in *D. melanogaster*. *PKD* is the key element for the production of insulin for normal starvation resistance, and when misregulations in the insulin-signaling pathway occur, individuals get serious health complications incorporating obesity, cardiovascular diseases and type-2 diabetes.

All the *Ilps* 2, 3 and 5 are co-expressed in the neurosecretory cells in the protocerebrum portion called *Pars intracerebralis (PI)* of adult and larvae flies' brains and in mammalian β cells. When a high amount of sugar is consumed, as a result of secretion of circulating insulin in the body, the rest of the sugar (carbohydrates) that is not required to the body is stored in the form of lipids in a fly fat body and in mammalian adipose tissue [24, 25]. Therefore, it is assumed that the production of insulin is directly proportional to the content of lipid in the flies' fat body; lower lipid content could be caused by a reduction in insulin production and hence lower the flies' starvation resistance and result in the lowest survival time [26]. It was demonstrated that the *PKD* knock down flies *Elav-GAL4/PKD*$^{RNAi}$ have shown a minimum survival time compared to the control, i.e. *Elav-GAL4/PKD*$^{RNAi}$ were more starvation vulnerable and died earlier than controls i.e. *Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$ (Figure 4). Furthermore, three various time points (0 hours, 6 hours and 12 hours) were used for the two over-expression genotypes, *PKD* knock down *Elav-GAL4/PKD*$^{RNAi}$ and the two controls *Elav-GAL4/Yw* and *Yw/PKD*$^{RNAi}$ to measure their lipids content. The *PKD* knock down flies *Elav-GAL4/PKD*$^{RNAi}$ have shown a lower lipid content at all the three time points compared to the controls (Figure 5). Previously, it was shown that the removal of the IPCs in *D. melanogaster* results in the prolongation of starvation resistance and high lipid storage, which is contrast to our data [5, 27, 28]. However *Ilps* are not the only hormones generated by IPC, IPC also express genes that control the feeding behaviors and the metabolism of lipids [29]. Our

further interest was in the knockdown of *PKD*, to observe whether *PKD* reduction affects other genes involved in insulin production in *Drosophila melanogaster*. The reduction of *PKD* has not affected the transcription levels of *Ilps* 2, 3, and 5, [30] (Figure 3A, 3B, and 3C). Furthermore, the transcriptions level of *Akh* and *Ilp*6 was also not affected after reduction of *PKD* in *D. melanogaster* (Figure 3D, 3E). Another experiment was performed on wild-type *CSORC* flies to confirm whether the expression of *PKD* is affected by various concentrations of foods and different environmental conditions mentioned in materials and method. Only a protein rich diet was accompanied by about 40% reduction in *PKD* expression in the fly heads (Figure 6A). Previously it has been shown when flies were overfed that the activity of insulin in flies' fat body was reduced [31]. The expression of *Ilp*3 at high protein content in the medium was increased and reached a statistically significant level (Figure 6B).

The *ILPs* 2, 3 and 5 are co-expressed in the protocerebrum region called *Pars intracerebralis (PI)* of adults and larvae. The expression of *ILPs* 2, 3 and 5 are independently controlled whereas *dilp*5 and *dilp*3 are controlled via nutrients. Therefore, due to starvation the amount of detectable transcripts was minimized after 24 hours of starvation for *Ilp*5 and 48 hours for *Ilp*3 however *Akh* was increased after 24 hours of starvation (Figure 7C, 7D, 7E). *Ilp*2 was not affected after starving the flies for 24 and 48 hours. Starvation resistance is related to *Ilps* signaling and IPCs [32, 33].

In 2008, the World Health Organization (WHO) reported that there were more than 1 billion overweight people throughout the world. At least 300 million people are obese and should be treated clinically. Obesity and overweight can cause chronic diseases, such as type-2 diabetes, cancer, cardiovascular, and high blood pressure diseases. In 1980, it was reported that obesity had increased 3 times in the world as a result of the consumption of high levels of sugar and saturated fats. Lower physical activity of the individual has been observed which is common in the western world.

Obesity contributing factors can be mutations in multiple genes, however a mutation in a single gene can also contribute to obesity. For example adipose tissue secrets a hormone called leptin, when this hormone is released in small amounts it causes severe obesity in an individual [34]. The first gene called *fat mass* or short *FTO* gene was identified as a result of the GWAS study [35].

There are several vertebrate models such as frogs, rodents and fish used as animal models in order to find out the function of these genes and enhance our knowledge of human biology [36].

We have used *D. melanogaster* a strong model organism that has certain advantages over vertebrates. Firstly, *D. melanogaster* has a short generation time and low maintenance cost compared to other vertebrate models. The total life span of *D. melanogaster* is around eight weeks with a generation time of about fourteen days. Secondly, *D. melanogaster* has fewer ethical issues related to in vivo studies. Thirdly, *D. melanogaster* can help us understand and analyze the function of disease genes, similar to the obesity-linked genes in a whole fly. Therefore discoveries in flies can offer a good initiative and can then lead to a more specialized research in higher animals [37].

**Conclusion:**

In this report we have uncovered that reduction in *PKD*-expression resulted in reduced starvation resistance in *Drosophila melanogaster*, thus *PKD* contributes to starvation resistance. This reduction in *PKD* has also caused reduced insulin signaling. A future perspective is to uncover whether *PKD* is important for the proper production of all insulin releasing peptides or is it particular to one brain developed peptides.

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