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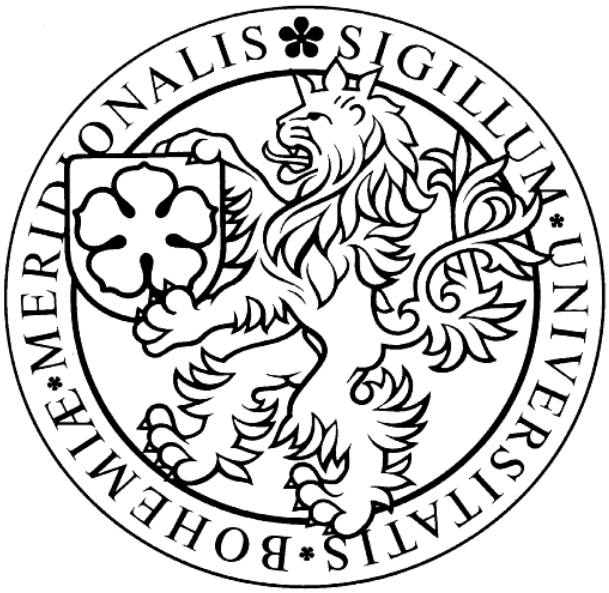

Bachelor thesis

Study of Dco role in *Drosophila melanogaster* hematopoiesis

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Annotation:

The main goal of this work was to test, whether an RNA interference (RNAi) construct can be used to knock down the gene *Disc overgrown* (*Dco*) in *Drosophila melanogaster* and what effects this knock down of *Dco* has on the hematopoiesis, the phenotype and the development of *D. melanogaster*.

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České Budějovice, May 2011

Tamara Bernadette Aigner

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Last but not least I thank my beloved family and friends, who are on my side at all times.

Abstract:

The main goal of this work was to test, whether an RNA interference (RNAi) construct can be used to knock down *Dco* in *Drosophila melanogaster* and what effect it would have on hematopoiesis of Drosophila.

Disc overgrown (*Dco*) in *Drosophila melanogaster* is the homologue of the human *Casein Kinase I epsilon* (*CKIε*). In *CKIε* somatic mutations were found in breast cancer tissue. These mutations, when incorporated into the fruit fly's *Dco* gene, lead to striking overgrowth phenotypes in imaginal discs, but interestingly also to overproliferation of hemocytes, suggesting a role of *Dco* in hematopoiesis. In this study it was tested, whether *Dco* could be knocked down using a combination of Gal4-UAS system with RNAi. First, the actin-Gal4 was used to induce systemic Dco-RNAi to be able to compare the resulting phenotype with the null-*dco* mutant. Simultaneous expression of both Dco-RNAi construct and *Dicer* (*Dcr*) driven by actin-Gal4 reproduced the discless phenotype of the null-*dco* mutant, demonstrating a functional system. In systems without *Dcr* only minor effects could be observed, suggesting that *Dcr* is needed in stoichiometric amount to obtain a perfectly working system.

Then *Hml*-Gal4 was used to drive the expression into differentiated hemocytes. It was tested whether there is a difference in the number of hemocytes in the genetically modified compared to wild type larvae. The *Hml*-Gal4 induced Dco-RNAi did not cause severe changes in fly development and the number of hemocytes was normal.

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1. Introduction

1.1 Goal of this thesis

Somatic mutations often play an important role in all stages of human carcinogenesis. Unfortunately we miss affordable and practicable techniques to identify the affected genes. Therefore model organisms as *Drosophila melanogaster* are predestined to find such mutations in candidate genes, which are related to cancer. The information gained with the help of flies can be used to develop new possibilities to fight against cancer - a disease, which affects one out of three persons in his or her life (M. Cotman, Z. M. Kukovic, 2008).

Fuja *et al.* (2004) found a high rate of somatic mutations in the gene encoding *Casein Kinase Iε* (CKIε) in human breast cancer tissue. In fruit flies a homologue of this gene exists, called *Disc overgrown* (*Dco*). Doležal, Kucerová, Neuhold and Bryant (2010) tested modified *dco* genes in vivo, containing the most frequent point mutations of human *CKIε*, namely L39Q and S101R. Inserting L39Q resulted in "a striking overgrowth phenotype in flies" (Doležal *et al.*, 2010, p.1) and S101R "modifies the mutant phenotype so that the affected tissue disintegrates, mimicking more aggressive forms of breast cancer" (Doležal *et al.*, 2010, p.1). Doležal *et al.* further discovered that the mutant larvae had an abnormal number of hemocytes – Drosophila "blood cells" –, suggesting that this gene may play an important role in leukaemia. To investigate the role of *Dco* in hematopoiesis a specific knock-down of *Dco* in hemocytes would be an important tool. Therefore it was important to establish a genetic tool for a tissue-specific Dco-RNA interference (RNAi) in flies. The larvae with ubiquitous Dco-RNAi were prepared to test the RNAi by comparing the effects of the *Dco* knockdown with the null *dco* mutant. Tissue-specific knockdown including knockdown in hemocytes was subsequently analyzed.

1.2 *Drosophila melanogaster* in general

In the 20th century a model organism for testing evolutionary theories and for dealing with the nature and function of genes was chosen: the fruit fly *Drosophila melanogaster*.
Thomas H. Morgan was the first to grow Drosophila in 1908 without any concrete knowledge about genes, their localization and their function, besides the mendelian rules, in which he additionally did not believe. Since then more and more was found out about Drosophila, its

genetics and therefore about genetics in general. That is the point, why Drosophila is still used nowadays; many genes and mechanisms are conserved in different organisms and can be observed even in humans. It's proven that more than 70 % of the proteins, which are involved in human diseases, exist in Drosophila (G. M. Rubin, et al., 2000; E. Bier, 2005; L. T. Reiter and E. Bier, 2002).

Other important advantages are the large numbers of offspring, the cheap food, the little spatial requirements, the possibility for easy observation and manipulation, the robustness against plagues and pathogens and the huge number of genetic tools available. (Hugo Stocker and Peter Gallant, in Dahmann 2008, p. 27-44)

1.2.1 Body plan

The flies are three millimetres in length – females are a little bit larger than males. Females weigh about 1.4 mg and males about 0.8 mg. This difference is mainly due to the ovaries in the abdomen of the females. The size and the weight are influenced by environmental conditions such as temperature and food quality and by their genotype. (Hugo Stocker and Peter Gallant, in Dahmann 2008, p. 27-44)

1.2.2 Live cycle

In the ideal case the live cycle of fruit flies takes ten days:

The females are fertilized and lay hundreds of eggs in the food. Embryogenesis occurs within the eggs and under standard laboratory conditions – 25°C and 70 % humidity - the first larvae hatch after about 24 hours. The larvae moult twice and therefore it passes through three larval stages in four days. During this time the main task is feeding. On the fifth day, after increasing approximately 200-fold in weight (possible due to endoreplication), the larvae stop feeding and leave the food to find a dry place for pupariation. The first flies eclose after four days of metamorphosis. (Hugo Stocker and Peter Gallant, in Dahmann 2008, p. 27-44)

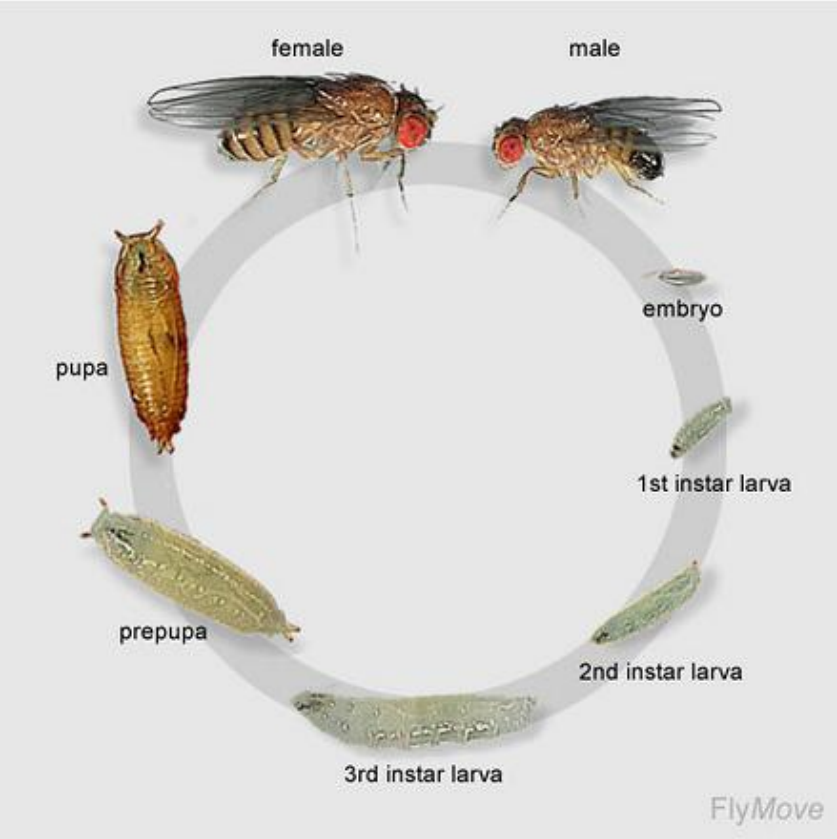

Fig. 1. Life cycle of male and female *Drosophila melanogaster* (courtesy of Christian Klambt in FlyMove:

http://flymove.uni-muenster.de/Genetics/Flies/LifeCycle/LifeCyclePict/life_cycle.jpg; 18.05.2011)

1.2.3 Handling & Crossing

The flies are kept in glass vials filled with food and enclosed with a cotton plug. The food is a mixture of water, agar, sugar, corn-meal, yeast and fungicides. The food is the most important factor for successful fly-pushers. The plug is not just a barrier to avoid the escaping of the flies, as it is made of cotton, it also provides the flies with fresh air.

To observe the flies under the microscope, they are anaesthetized with carbon dioxide and put on a fly pad. This fly pad is a porous plate, where CO_2 effuses to keep them "asleep". On this plate they survive several minutes, but if they are exposed to CO_2 for more than 20 minutes, the flies die. Other risks are loss in fertility and dehydration, the latter one can be minimized by passing the CO_2 through a bottle of water first.

The flies seem to be very fragile, but they are not. They can be easily moved with a small paintbrush to find out the sex and to distinguish between the genetic markers. For the transfer of the selected flies from the fly pad to a vial an air bulb adapted with a plastic tip can be used. After the transfer, the vial is laid on the side to avoid sticking of the flies to the food.

It is very important to mark the vials immediately after adding the flies. Usually the genotype of the females and males and the date is written on the vial.

For an acceptable number of progeny ten virgins and three to five males per vial are enough. To ensure that the progeny will have the desired genotype the collected females must be virgins. Under standard conditions flies start mating about eight hours after eclosion. Newly emerged flies have a very pale pigmentation and a dark spot called meconium in the abdomen. Therefore it is best to collect fresh virgins in the morning, when most of the flies emerge; then collect a few hours later to ensure that all females are virgins. The flies should be transferred to a fresh vial every two to three days.

Last but not least: All work has to be done in a clean environment to avoid plagues!

1.2.4 Imaginal discs

Great parts of the body wall of the adult fly such as wings, legs and eyes are generated from larval imaginal discs during metamorphosis. Imaginal discs are autonomously developing parts, which are defined during embryogenesis and proliferate during the three larval stages. After the definition of the monolayered epithelia during embryogenesis, each disc invaginates into the inside of the embryo. A small stalk forms the connection between the imaginal discs and the epidermis. The flattened, saclike discs consist of two distinct sides: the one, which forms the imaginal disc epithelium and the other, which develops into the peripodial plane. The latter one is built up from very thin, flat epithelial cells, whereas the first one has a columnar shape. The apical side of those imaginal disc cells face the lumen, which is formed between these two cell types.

During the larval development the imaginal disc cells proliferate tremendously and in the end each imaginal disc is made of thousands of cells. Thus, as a consequence of the lack in space, each imaginal disc epithelium folds in a characteristic manner. The final assembly of the imaginal discs to form the adult body takes place in the pupa.

The imaginal discs do not participate in larval life; what makes them especially interesting to scientists is that they can be manipulated genetically or physically without having any impact on the survival of the larva. They are used to study pattern formation in a cellular context, signal transduction and several cell biological processes e.g. polarity and endocytosis. (Thomas Klein, in Dahmann 2008, p. 253-263)

1.2.5 Hemocytes

Drosophila melanogaster defends itself by innate immune mechanisms. The innate immune system consists of two complementary facets, the humoral and the cellular immunity (M. Meister and M. Lagueux, 2003). The latter one is enabled by circulating hemocytes, which recognize and neutralize non-self or noxious objects through phagocytosis or encapsulation and melanization (N. Remillieux-Leschelle, P. Santamaria and N. B. Randsholt, 2002). An infection by bacteria or fungi leads to an activation of humoral immunity and therefore to a massive production of antimicrobial peptides and other effectors in the fat body – the liver counterpart in insects (M. Meister and M. Lagueux, 2003).

Haematopoiesis in Drosophila can be divided into two phases during development and results in three hemocyte linages, comparable with the myeloid lineages in mammals (M. Meister and M. Lagueux, 2003). In the beginning hemocytes are produced in the second half of embryogenesis in the procephalic mesoderm (Tepass *et. al*, 1994). Plasmatocytes, one of the hemocytes types, act as macrophages, which eat up apoptotic cells (Franc *et. al*, 1996, 1999) and are the main part of the circulating hemocytes. A second type of hemocytes, crystal cells, are produced nearby the anterior region of the gut, their function in the embryo is still unknown. Just about 5 % of all larval circulating cells are crystal cells. The name comes from crystalline inclusions, which correspond to enzymes necessary for humoral melanization. The third hemocyte linage in larvae is made by lamellocytes. They are large flat cells able to encapsulate invaders, which are too large to be phagocytosed by plasmatocytes. Lamellocytes are produced only in case of parasitic infestation and thus are rarely observed in healthy flies. (M. Meister and M. Lagueux, 2003)

The precursors of the lymph glands form in the lateral mesoderm and then migrate dorsally at the end of embryogenesis. They create there the first paired lobes of the lymph glands (Rugendorff *et al.*, 1994), the organ where the greatest part of the haematopoiesis takes place during all larval stages (Shrestha and Gateff, 1982; Rizki and Rizki, 1984; Lanot *et. al*, 2001). At the end of larval development, the lymph glands consist of variable number of paired lobes, which are located along the dorsal vessel. Undifferentiated precursor cells are located in the posterior lobes, whereas differentiated hemocytes are found in the more anterior lobes. These differentiated hemocytes are released into the circulation (M. Meister and M. Lagueux, 2003).

At the beginning of metamorphosis a huge amount of active phagocytes, pupal macrophages, are released from the lymph glands. These macrophages are very important in

tissue remodeling, because they phagocyte the larval structures, which are not needed in the adult flies (Lanot *et. al*, 2001).

The specification of the hematopoietic linage and the proliferation and differentiation of hemocytes are strictly controlled. Therefore mutations in genes, regulating the lymph gland cell proliferation and hemocytes numbers in the body, cause overgrowth of the hematopoietic organ and overproliferation of hemocytes. (N. B. Randsholt *et. al*, 2002)

1.3 *Drosophila melanogaster* Genetics

1.3.1 Chromosomes, Sex determination, Balancers and Markers

Drosophila has four chromosome pairs, where the first pair determines the sex and the last three are autosomes. Females have two X-chromosomes and males have either an X-chromosome and a Y-chromosome or just an X-chromosome, as the Y-chromosome is important only for proper sperm motility. The chromosomes differ significantly in their sizes, the third is the largest followed by the second, then the first and by far the smallest is the fourth chromosome.

A big advantage in males is that no meiotic recombination takes place. Therefore one can ensure the genotype of the progeny. This is not the case in females and here the most important genetic tool in fruit flies comes into play: balancer chromosomes. On the one hand balancers contain multiple inversions to suppress meiotic recombination and on the other hand they carry dominant mutations, which cause easily visible phenotypes and are lethal or cause sterility in homozygotes. Such mutations are called markers and are important to distinguish the phenotype and thus the genotype of the progeny. (Hugo Stocker and Peter Gallant, Christian Dahmann 2008, p. 27-44; R. J. Greenspan, 2004)

1.3.2 RNAi (*Dcr*, RISC)

The RNA interference (RNAi) mechanism can be used to knock down genes. The mechanism of RNAi works as follows: The endonuclease *Dcr* cuts double-stranded RNA (dsRNA) into short pieces known as small interfering RNA (siRNA) (E. Bernstein, A. A. Caudy, S. M. Hammond and G. J. Hannon 2001). Then the large RNA-induced silencing complex (RISC) is loaded with the antisense strand of the siRNA. This huge complex is targeted to the mRNA and is bound via base-pairing between the antisense siRNA and the mRNA. The RISC protein contains at least one member of the argonaute protein family and

thus it acts very likely as an endonuclease, cutting the mRNA, which is then subsequently degraded. (S. M. Hammond, E. Bernstein, D. Beach and G. J. Hannon 2000)

In living organisms like plants, worms and flies the RNAi mechanism is used as a protection mechanism. On the one hand it is an anti-viral "immune defense" mechanism to protect the genome from viral infections. On the other hand it secures the stability of the genome by keeping mobile elements silent. But in this case the RNAi machinery is not always efficient, as not all transposons are kept under control; some are still able to jump and cause deleterious effects in the genome. In vertebrates, including humans, it is still unclear, how the mechanism works and if it is relevant at all. (A. Z. Fire, C. C. Mello, 2006)

The mode of action of RNAi provides a great experimental tool to repress specific genes in model organisms like Drosophila. It can be used to silence certain genes and to study the resulting phenotype and therefore to find out the function of individual genes. This knowledge could become a useful approach in future in the medical field of gene therapy to cure inherited diseases. (A. Z. Fire, C. C. Mello, 2006)

1.3.3 Gal4-UAS system

Saccharomyces cervisiae grown on galactose instead of glucose expresses several genes to metabolise the galactose. Gal1 and Gal10 are two of those genes, lying close to each other on the yeast chromosomes. The transcription of GAL1 and GAL10 is controlled by a sequence between them, called upstream activating sequence or short UAS. UAS contains four Gal4 binding sites, each being a palindrome of eight base pairs with an A:T base pair in the middle. Gal4 is an 881 amino acids long protein, which is encoded by an unlinked gene. Experiments have shown that the first 73 amino acids are enough for binding to UAS. Those amino acids contain a zinc finger motif for binding to DNA. (D. P. Snustad, M. J. Simmons, 2010)

Gal4-UAS in flies is a bipartite system of the driver and the responder. The driver is the transgenic line, which expresses the Gal4 in a known temporal or spatial pattern. Gal4 expression is regulated by endogenous enhancers, whereof almost 7000 exist. The responder contains the UAS-dependent transgene (*Fig. 2*).

Gal4 and UAS can be brought together by a simple genetic cross. This allows a remarkable flexibility of the system, what is one considerable reason for the use of UAS-Gal4 system. The progeny then produces the transgene only in the cells expressing the Gal4 protein, also called ectopic expression. This technique is for example used to: "(1) induce a cell fate change" (R. L. Davis, H. Weintraub and A. B. Lassar, 1987; in Dahmann 2008, p.

79); “(2) induce altered cell fates in neighbouring cells” (T. Bouwmeester, S. Kim, Y. Sasai, B. Lu, and E. M. De Robertis, 1996; in Dahmann 2008, p. 79); and “(3) alter the cell’s physiology”(T. D. Southall, S. Terhzaz, P. Cabrero, et al., 2006; in Dahmann 2008, p. 79). Gal4-UAS system can also be used to induce tissue-specific knockdown by activating RNAi under Gal4-UAS control (E. Enerly, J. Larsson and A. Genesis, 2002). These examples demonstrate that the Gal4-system is a very sophisticated tool in Drosophila genetics.

As an example for the working principle of this system one can assume that the enhancer in the driver is the enhancer for actin. Then Gal4 is produced in all cells, where actin is produced. Therefore the target protein, for instance GFP, is produced in all cells where actin is produced. By observing the progeny, it can be found out that the GFP is produced in all cells. From this observation one can follow that actin is produced in all cells.

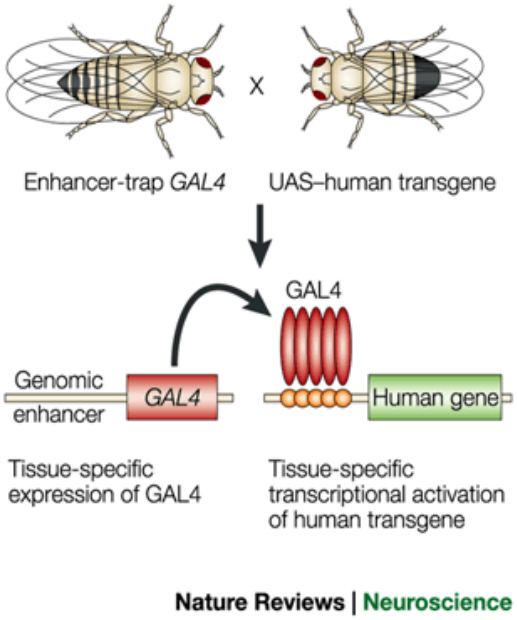

Fig. 2: Principle of GAL4-UAS system (Miratul M. K. Muquit and Mel B. Feany (2002), Modelling neurodegenerative diseases in Drosophila: a fruitful approach?, Nature Reviews Neuroscience 3, 237-243; http://www.nature.com/nrn/journal/v3/n3/fig_tab/nrn751_F2.html; 18.05.2011)

1.4 CKIε & *dco* genes

Casein kinase I epsilon (*CKIε*) is a member of the *CKI* family. This family of serine/threonine specific protein kinases consists of seven mammalian *CKI* isoforms namely α, β, γ1, γ2, γ3, δ and ε. All these kinases have highly conserved kinase domains and especially *CKIδ* and *CKIε* are very similar. (Rowles *et al*., 1991; Fish *et al*. 1995; Zhai *et al*., 1995) These CKI isoforms are mainly co-factor independent and use ATP as a phosphate donor (P. T. Tuazon, J. A. Traugh, 1991; H. Flotow *et al*., 1990).

CKIε plays important roles in membrane transport processes, circadian rhythm, cell division, apoptosis, cell differentiation and in tumor suppression with the suppressor p53 and the oncoprotein mdm2. (U. Knippschild *et al*., 2005) The role of CKIε in some of these mechanisms is explained in the following text in more details.

The circadian clock is responsible for the signal transduction pathway integrating external signals, the central oscillator generating the circadian signal and the signal transduction pathway manifesting circadian periodicity (U. Knippschild *et al*., 2005). The main clock genes are *CLOCK* and *BMAL1* as positive regulators and *PERIOD* (*PER1-3*) and *CRYPTOCHROME* (*CRY1-2*) as negative regulators (D. P. King, J. S. Takahashi, 2000; S. M. Reppert, D. R. Weaver, 2002). If CKIε phosphorylates PER a conformational change takes place and masks a nuclear localization signal (E. Vielhaber, *et al*., 2000). Then CRY forms a complex with CKIε and PER, which accumulates nuclear and protects PER from degradation (M. Akashi, *et al*., 2002; E. Vielhaber, *et al*., 2000; P. L. Lowrey, *et al*., 2000). Furthermore this CRY-PER-CKIδ/ε complex inhibits the transcriptional activity of the DNA bound BMAL1-CLOCK complex and therefore the transcription of PER and CRY (M. Akashi, *et al*., 2002). CKIδ/ε can also modulate the functions of CRY and BMAL1 by phosphorylation (E. J. Eide, *et al.*, 2002).

The tumor suppressor p53 and its oncoprotein mdm2 were found to be the key signal integrator molecules. Furthermore it was found that changes in their phosphorylation can abolish their functions and result in an uncontrolled growth of cells (D. W. Meek and U. Knippschild, 2003). CKIδ/ε is responsible for the phosphorylation of p53 at certain serines and threonines (U. Knippschild, 1997).

Centrosomes play an important role in cell division and CKIδ/ε seem to be involved in their regulation (J. E. Sillibourne, *et al*., 2002) since an inhibition of CKIδ/ε as well as non-functional p53 lead to centrosome amplification and reduplication (A. J. Minn, L. H. Boise and C. B. Thompson, 1996; A. F. Wahl, *et al*., 1996; P. Tarapore and K. Fukasawa, 2002);

this further leads to genomic instability (D. W. Meek, 2000; P. Tarapore and F. Fukasawa, 2000), spindle failures and inhibition of mitosis (L. Behrend, *et al*., 2000).

From these examples one can already conclude that overexpression or misexpression of *CKIε* can cause severe effects in a living organism. Fuja et al. (2004) observed a correlation between immunohistological staining intensity of CKIε and tumor differentiation in breast cancer tissue, suggesting that CKIε plays a role in cancerogenesis. According to K. Masuda *et al.* (2003) an increased nuclear CKI kinase activity was found in acute myeloid leukemia (AML) patients.

The *Disc overgrown* (*Dco*) gene in Drosophila was discovered by Jursnich *et al*. (1990). It was found that Dco is necessary for normal development and growth control in the imaginal discs in Drosophila. Several recessive lethal alleles exist, one of them – homozygous dco^3 – resulted in an extended larval period and overgrowing imaginal discs. (Jursnich *et al*., 1990) Nine years later Zilian *et al*. (1999) demonstrated that *Dco* is identical to the *Double-time* gene (*Dbt*), which plays an important role in the circadian rhythm. Further they showed that Dco/Dbt is a homolog of CKIδ/ε and that it does not just affect the circadian rhythm, but it is important also for cell survival and growth control in imaginal discs. Similar results were found by Kloss *et al.* (1998), who detected an identity of 86 % of the kinase domains of *CKIε* and *Dbt*.

Disc overgrown is important to keep the level of apoptosis low, because it activates the apoptosis inhibitor DIAP1 and suppresses the apoptotic activity of head involution defective (Hid) (J. Guan *et al.*, 2007). Thus *dco*-null mutant larvae are completely discless and cannot metamorphose to the adult fly. Therefore knocking down *Dco* in certain tissues may lead to important information on how gene therapy should be designed to cure cancer patients.

2. Materials and Methods

2.1 Fly stains & Crossing scheme

The flies were grown on standard culture media in glass vials at 25 °C as described in section 1.2.3 Handling & Crossing. All flies were provided by Tomáš Doležal.

In the following crossing schemes the following abbreviations are used:

"G" stands for parental generation

"F" stands for daughter generation of interest

The following markers were used to distinguish between flies with different genotypes:

abbreviation	**name**	**effect**
CyO	CurlyO	The fly has curly wings.
e	ebony	The fly is black.
GFP	Green fluorescence protein	The fly shines green under the fluorescence microscope.
Hu	Humeral	The fly does not have the two characteristic hairs on the shoulder.
kar	adgf-a[kar]	Recessive lethal mutation on chr. III.
Sco	Scutoid	Less than four hairs are on thorax.
Ser	Serrate	Parts of the wings are missing – the wings are notched at the tip.
Tb	Tubby	The larvae are tubby.

TM3 GFP Ser is a balancer chromosome three, containing the markers GFP, ebony and Serrate. TM6B is also a balancer chromosome three, containing the markers Tb and Hu. (Flybase, 1999)

The enhancers used for the driver of the Gal4-UAS system were the enhancers for *actin* (*Act*), *hemolectin* (*Hml*) and *engrailed* (*En*). Actin is part of the cell skeleton and therefore it is produced in all cells, hemolectin is produced in differentiated hemocytes called plasmatocytes and engrailed is, besides other tissues, produced in one half of the wing.

2.1.1 RNAi^{dco} flies

$G_{1.1}$:

$$w; \frac{RNAi^{dco}}{RNAi^{dco}} \text{ x } \frac{Act\ Gal4}{\text{TM6B}}$$

$F_{1.1}$:

$$\frac{RNAi^{dco}}{Act\ Gal4}$$

$G_{1.2}$:

$$w; \frac{RNAi^{dco}}{RNAi^{dco}} \text{ x } w; \frac{En\ Gal4}{En\ Gal4}$$

$F_{1.2}$:

$$\frac{RNAi^{dco}}{En\ Gal4}$$

These two crosses were performed to test, whether RNAi^{dco} has an effect on the fly development and tissue even without *Dcr*. The flies were kept at an elevated temperature of 28 °C, because higher temperatures increase Gal4/UAS activation (Doležal – personal communication).

2.1.2 RNAi^{dco} & *Dcr* flies

$G_{1.1}$:

$$\frac{UAS{-}Dcr}{UAS{-}Dcr}; \frac{+}{+} \text{ x } \frac{Sco}{CyO\ GFP}; \frac{kar}{TM3\ GFP\ e\ Ser}$$

$F_{1.1}$:

$$\frac{UAS{-}Dcr}{Sco}; \frac{+}{TM3\ GFP\ e\ Ser}$$

$G_{1.2}$:

$$\frac{+}{+}; \frac{UAS{-}RNAi^{dco}}{UAS{-}RNAi^{dco}} \text{ x } \frac{Sco}{CyO\ GFP}; \frac{kar}{TM6B}$$

$F_{1.2}$:

$$\frac{+}{CyO\ GFP}; \frac{UAS{-}RNAi^{dco}}{TM6B}$$

G_2:

$$♂ \frac{UAS{-}Dcr}{Sco}; \frac{+}{TM3\ GFP\ e\ Ser} \text{ x } ♀ \frac{+}{CyO\ GFP}; \frac{UAS{-}RNAi^{dco}}{TM6B}$$

F_2:

$$\frac{UAS{-}Dcr}{CyO\ GFP}; \frac{UAS{-}RNAi^{dco}}{TM3\ GFP\ e\ Ser}$$

G_3:

$$\frac{UAS-Dcr}{CyO\ GFP};\frac{UAS-RNAi^{dco}}{TM3\ GFP\ e\ Ser}\ \text{x}\ \frac{UAS-Dcr}{CyO\ GFP};\frac{UAS-RNAi^{dco}}{TM3\ GFP\ e\ Ser}$$

F_3:

$$\frac{UAS-Dcr}{UAS-Dcr};\frac{UAS-RNAi^{dco}}{UAS-RNAi^{dco}}$$

$G_{4.1}$:

$$♂\ \frac{UAS-Dcr}{UAS-Dcr};\frac{RNAi^{dco}}{RNAi^{dco}}\ \text{x}\ ♀\ \frac{Hml\ Gal4\ UAS-GFP}{Hml\ Gal4\ UAS-GFP};\frac{+}{+}$$

$F_{4.1}$:

$$\frac{Hml\ Gal4\ UAS-GFP}{UAS-Dcr};\frac{UAS-RNAi^{dco}}{+}$$

$G_{4.2}$:

$$♂\ \frac{UAS-Dcr}{UAS-Dcr};\frac{RNAi^{dco}}{RNAi^{dco}}\ \text{x}\ ♀\ \frac{Act\ Gal4}{Act\ Gal4};\frac{+}{+}$$

$F_{4.2}$:

$$\frac{Act\ Gal4}{UAS-Dcr};\frac{UAS-RNAi^{dco}}{+}$$

$G_{4.3}$:

$$♂\ \frac{UAS-Dcr}{UAS-Dcr};\frac{RNAi^{dco}}{RNAi^{dco}}\ \text{x}\ ♀\ \frac{En\ Gal4}{En\ Gal4};\frac{+}{+}$$

$F_{4.3}$:

$$\frac{En\ Gal4}{UAS-Dcr};\frac{UAS-RNAi^{dco}}{+}$$

2.2 Dissection of larvae

Drosophila melanogaster larvae were dissected to check, whether the imaginal discs (larval tissues from which the adult wings, legs and other appendages origin) and the brains are normal or show some abnormalities. A third instar larva in the vial was chosen and carefully transferred to a spot plate filled with phosphate buffered saline (PBS) buffer using a needle. The buffer was used to avoid bursting of cells and to hinder the larvae in crawling away. The tail was cut off at the very end by squeezing the tail of the larva with one pair of forceps and using the second pair of forceps for cutting. Then the larva was inverted. Therefore the larva was taken at its “neck” with a pair of forceps and then inverted by pushing the head inside with a second pair of forceps. The larva was “roled” on the second pair of

forceps and later put off. The fat tissue, tracheae et cetera was removed to get a free sight at the imaginal discs and brain lobes.

2.3 Hemocyte counts

Two wandering third instar larvae were washed in a PBS buffer solution and then ripped in 30 µl Drosophila Ringer. Therefore the two forceps were deposited at the belly of the larva, which was then ripped and inverted to get all circulating hemocytes out of the body. Then the solution was mixed and 11 µl were transferred to a counting grid. The number of hemocytes per larvae was estimated by counting the number of cells on the surface of the grid on inverted microscope. The number of hemocytes was counted at least six times for each genotype. As a reference the number of hemocytes in wild type flies was determined.

3. Results

3.1 RNAi^{dco} Drosophila

First flies with the combinations *En* Gal4-UAS; UAS-RNAi^{dco} and *Act* Gal4-UAS; UAS-RNAi^{dco} were grown at elevated temperature of 28 °C. In the cultured flies with the *En* and the *Act* combination already first signs of the working RNAi^{dco} machinery could be observed. When dissecting some larvae with the *En* combination both wings discs were present, but in three out of six cases one wing disc was smaller and parts were missing and/or misshaped. Most larvae metamorphosed to adult flies.

In the larvae with the *Act* combination the imaginal discs seemed normal, but the larval period took very long, longer than the larval period of the tubby *TM6B* larvae, which did not have the Gal4 driver. In the end most of the pupae were not able to metamorphose to the adult state and died in the pupa state. The few flies, which were able to metamorphose to the adult state, hatched much later than the *TM6B* flies and seemed very weak.

3.2 RNAi^{dco} & *Dcr* Drosophila

Flies with the combinations *Hml* Gal4-UAS GFP / UAS-*Dcr*; UAS-RNAi^{dco}, *En* Gal4-UAS / UAS-*Dcr*; UAS-RNAi^{dco} and *Act* Gal4-UAS / UAS-*Dcr*; UAS-RNAi^{dco} were grown. In general addition of *Dcr* enhanced the effect of RNAi^{dco} in all combinations.

The larvae were dissected to determine, whether the larvae showed differences in the development of the imaginal discs and brain lobes. In the wild type larvae and the larvae with the *Hml* combination all tissues necessary to metamorphose were present and seemed normal (Figure 4).The larvae containing the *Act* combination were totally discless, just the eye-lobes and brain stem were present (Figure 5). Therefore none of the larvae was able to pupate, as proper developed imaginal discs are essential for the metamorphosis. There were just a few larvae with the *En* combination, therefore just one larva was dissected. It had a mutated wing disc and a smaller wing disc (Figure 6).

In the *Hml* and *En* combinations the fly development was decelerated and they produced less progeny. The larvae with the *Hml* construct did not show a significant change in number of hemocytes. Whereas the knock-down of *Dco* in the larvae containing the *Act* combination showed in average an elevated number of hemocytes. Another point is that the

standard deviation of the hemocyte count of the genetically modified flies is higher compared to that of wild type flies. These results are presented in Table 1 and Figure 3. The blood cells of the *En* flies were not counted.

The larvae and flies with the *Hml* construct also produced the green fluorescence protein (GFP) promoted by *Hml*. The comparison of the intensity in fluorescence of the hemocytes of larvae with induced Dco-RNAi and of the hemocytes of the wild type larvae showed, that the hemocytes of the genetically modified larvae shined less intensively than the hemocytes of the wild type larvae (wild type see Figure 7 and 8, *Hml* combination see Figure 9 and 10).

A lot of hemocytes in the larvae containing the *Act* combination were observed, while digesting some material (Figure 11).

Table 1: The table shows the estimation of the number of hemocytes per larvae per count, the average and the standard deviation.

	Act combination	*Hml* combination	Wild type
1	19950	12750	6750
2	20100	3600	5700
3	13500	6750	6150
4	27450	7200	7350
5	27600	4800	11100
6	22800	4500	5850
7	5550		
8	5250		
9	11850		
Average	17117	6600	7150
Standard deviation	8524	3312	2030

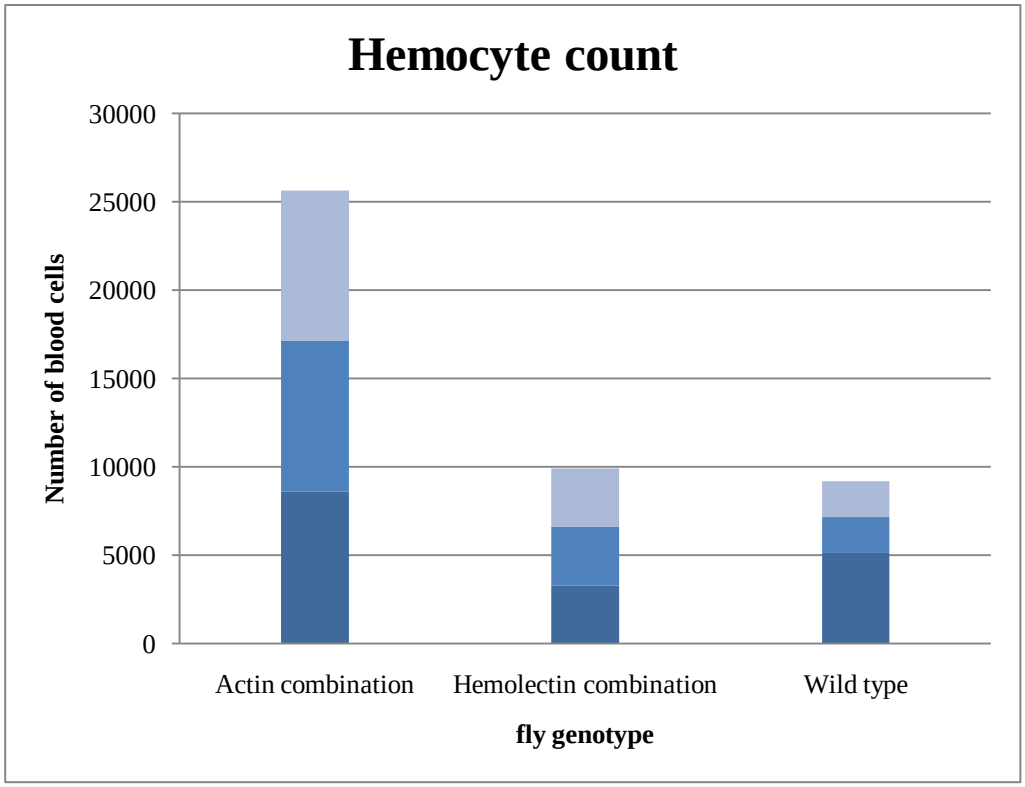

Figure 3.: The bar chart shows the number of blood cells in larvae of the different genotypes inclusive average and standard deviation. The first border represents the average minus the standard deviation, the second the average and the third the average plus the standard deviation.

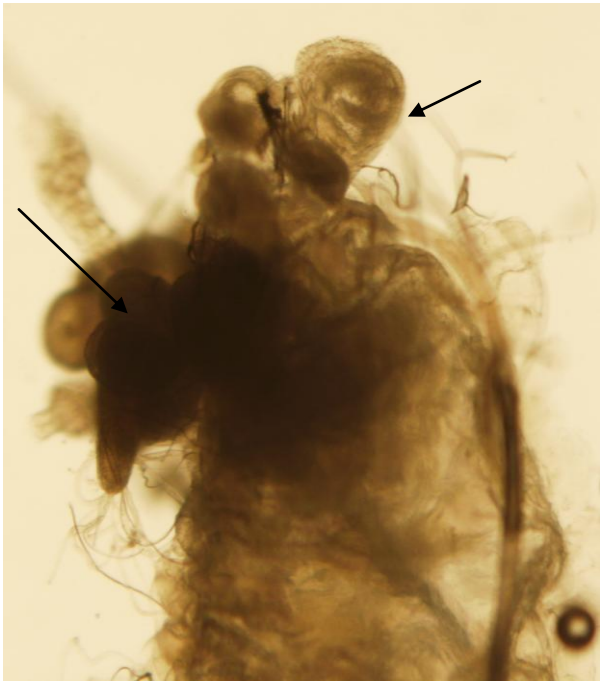

Figure 4.: The picture shows the head of an inverted larvae with the wild type genotype. The imaginal discs and the all brain lobes can nicely be seen.

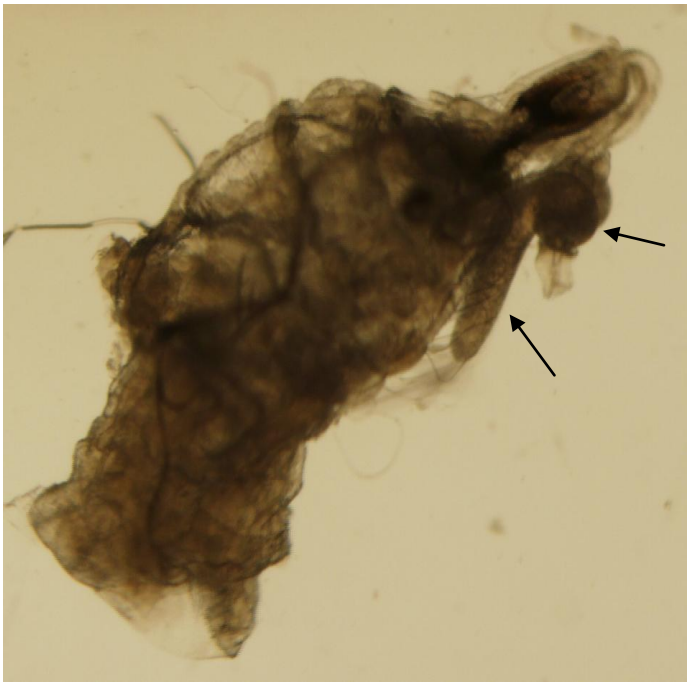

Figure 5.: The picture shows the head of an inverted larvae with the *Act* GAL4-UAS / UAS-*Dcr*; UAS-RNAi^{dco} genotype. The imaginal discs and the parts of the brain are missing, just the eye lobes and the brain stem are present.

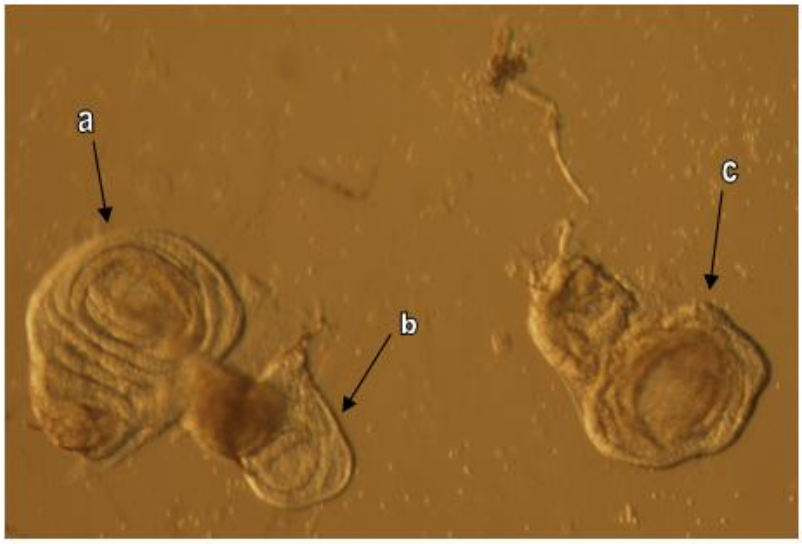

Figure 6: The picture shows three different wing discs:

a) normal wing disc of wild type larva
b) small wing disc of the larva with the *En* combination
c) mutated wing disc of the same larva

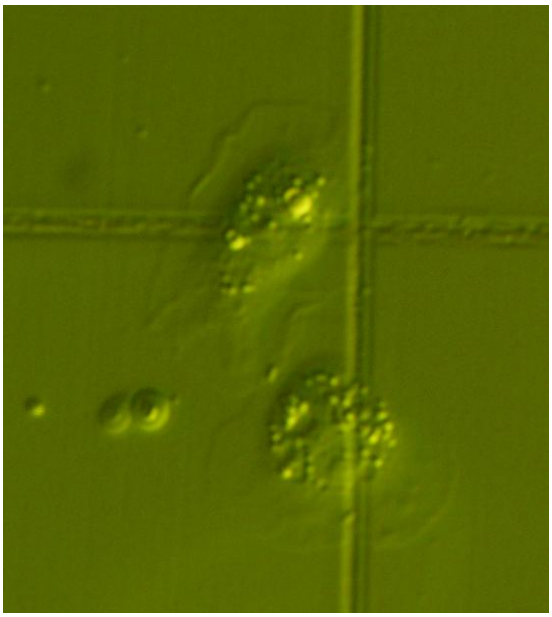

Figure 7: This picture shows hemocytes of *Hml* Gal4-UAS GFP larvae under DIC contrast.

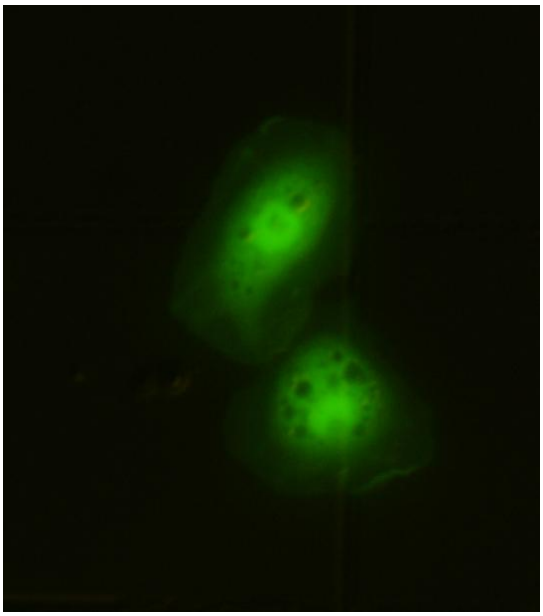

Figure 8: This picture shows hemocytes of *Hml* Gal4-UAS GFP larvae under the GFP filter.

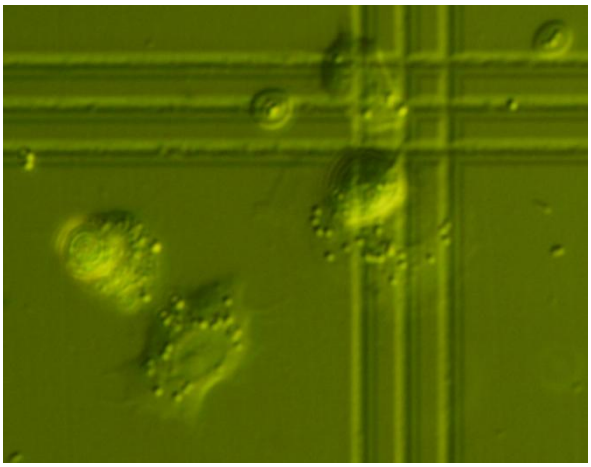

Figure 9: This picture shows hemocytes of *Hml* GAL4-UAS GFP / UAS-*Dcr*; UAS-RNAi^{dco} larvae.

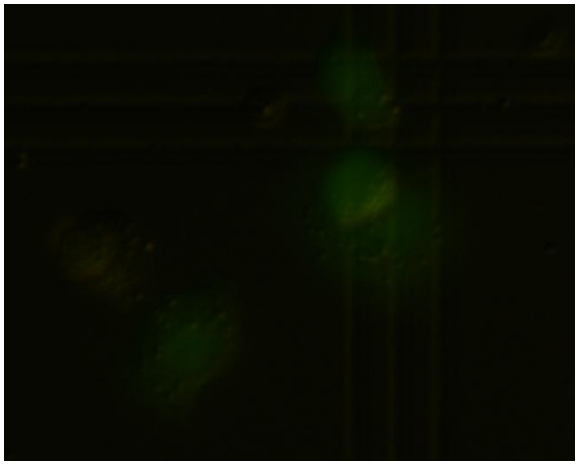

Figure 10: This picture shows hemocytes of *Hml* GAL4-UAS GFP / UAS-*Dcr*; UAS-RNAi^{dco} under the GFP filter.

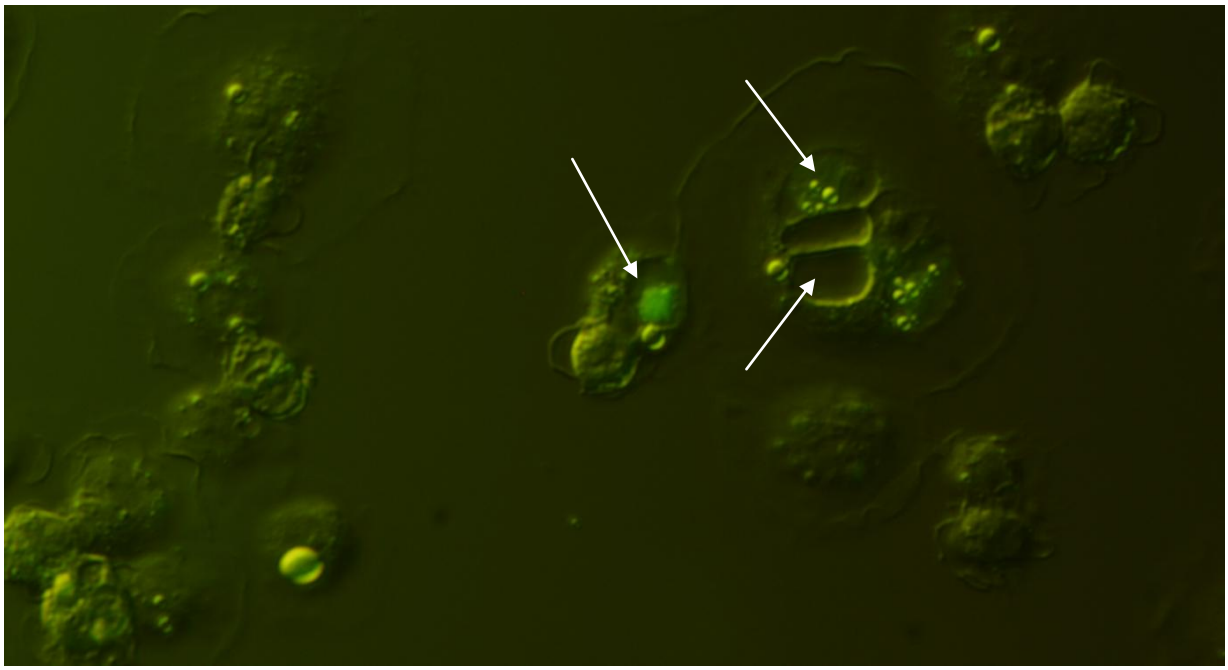

Figure 11: This picture shows hemocytes of larvae with the *Act* combination eating other materials.

4. Discussion

This work demonstrates that the combination of the Gal4-UAS system and Dco-RNAi can be used to knock down *Dco* in Drosophila. An effective Dco-RNAi is achieved only in combination with *Dcr*. The combinations without *Dcr* showed just minor effects, suggesting that *Dcr* is needed in stoichiometric amount to obtain a perfectly working $RNAi^{dco}$ machinery. These minor effects observed in the combinations without *Dcr* could be due to small amounts of naturally occurring *Dcr*.

Larvae containing the *Hml* Gal4-UAS GFP / UAS-*Dcr*; UAS-$RNAi^{dco}$ combination did not show any significant difference in the appearance of the imaginal discs compared to wild type larvae. Just the decelerated development and the smaller amount of progeny confirm that those flies are in general weaker than wild type flies.

In contrast to that the modification with the *Act* promotor showed a severe change in fly development. The larvae missed great parts of the imaginal discs and did not metamorphose. This proves that Dco-RNAi perfectly worked and that Dco indeed has a tremendous effect on the development of fly tissue. These findings perfectly agree with the outcome of the work of Guan *et al.* (2007). As the larvae with this genotype looked the same as the larvae with the *dco*-null mutation, one can conclude that *Dco* was really knocked down in all larval tissues.

The amount of hemocytes in the larvae containing the *Hml* combination was normal in comparison to the wild type larvae. This could be explained due to the fact that hemolectin is produced in the latest stage of haematopoiesis and therefore knocking-down the *Dco* at this stage might not affect the overall hemocytes number.

The elevated average amount of hemocytes in the larvae with the *Act* combination could be due to the high amount of apoptotic tissue and a lot of macrophages are built to remove these dying parts. The high amount of digesting macrophages observed, support this theory.

The less intensive GFP signal observed in the hemocytes of the *Hml* construct suggests that these cells either loose this differentiation marker or the fully differentiated hemocytes are undergoing changes (e.g. apoptosis), which are associated with the GFP degradation.

Another interesting aspect is the high standard deviation, especially in the *Act* construct. The fact, that *Drosophila* is a living organisms and that it is therefore pretty sensitive to small environmental changes, definitely plays a role. Additionally the age of the

larvae could be of great importance. It is possible that older larvae have a higher amount of blood cells than younger ones or vice versa. The delay in development makes exact staging of the larvae containing the *Act* combination difficult. Larvae with the *Hml* combination had also an extended larval life time. This would explain why the hemocyte count of the *Act* construct shows such a high standard deviation and why even the standard deviation of the hemocyte count of the *Hml* construct is higher than the one of the wild type flies.

More larvae and flies with the *En* promotor must be observed to be able to draw a conclusion about this genetic combination.

5. Conclusion

This work demonstrates that the RNAi^{dco} machinery induced by Gal4-UAS system and used in combination with *Dcr* perfectly works and that it can be used to knock-down *Dco* in a tissue specific manner. It was also shown that the knock-down of *Dco* in certain tissues has an effect on Drosophila development and phenotype. Knocking-down the *Dco* in fully differentiated hemocytes by a hemolectin driver did not affect the total number of hemocytes, but changed the expression of the hemolectin marker. Further studies knocking down the *Dco* in hematopoietic stem cells by using the presented tool might help to uncover the role of *Dco* in hematopoiesis in future.

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