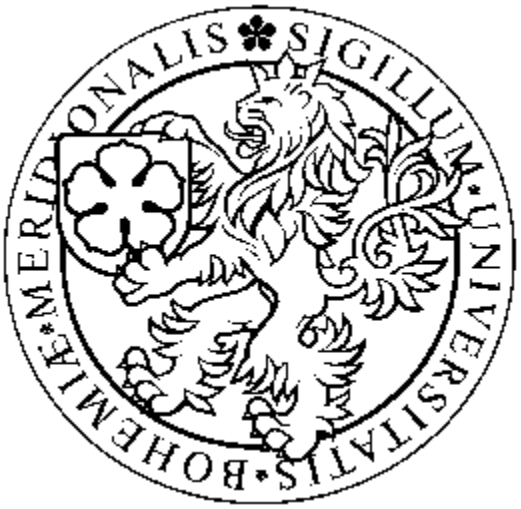
University of South Bohemia in České Budějovice

Faculty of Science
Department of Molecular Biology and Genetics

The role of adenosine receptor in self-activation of immune cells



Bachelor Thesis

Klára Kopicová

Supervisor: doc. Mgr.Tomáš Doležal Ph.D.

České Budějovice, 2017

Kopicova, K., 2017: The role of adenosine receptor in self-activation of immune cells. BSc. Thesis, in English, 38 p., Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic.

Annotation

Adenosine receptor plays very important role in regulation of immune response. Its signalling may lead to initiation of metabolic switch, which suppresses energy consumption by peripheral tissues (selfish behaviour of immunes system). In this thesis I attempt to test the potential role of adenosine signalling in self-activation of immune cells which could alternatively explain its role in systemic energetic switch in model organism *Drosophila melanogaster*.

Acknowledgment

At the first place I would like to thank my supervisor doc. Mgr. Tomáš Doležal Ph.D. for giving me the opportunity to work in his laboratory and his introduction to the fruit fly world. Further, I am grateful for his effort to keep us students self-reliant, his never ending patience, kindness and positive approach, which hasn´t changed even under press during last Easter holiday.

This work wouldn´t have been arising so easily without our lab manager RNDr. Martina Slapničková, her daily smile and almost mother care. In general the whole fly lab was a big aid, showing me good tricks how to handle the fruit fly difficulties.

Finally I would like to thank my entire family for their wide-ranging support, encouragement and deep affection for nature, which brought me up to Faculty of Science in České Budějovice.

Abstract

Extracellular adenosine (e-Ado) is a signal molecule with low physiological concentration, which can rapidly increase during stress or tissue damage.
e-Ado works further as a systemic hormone, which can regulate metabolism to suppress energy consuming processes in one part of organism and support releasing energy supplies in other parts. In order to the full operation of this e-Ado-regulation cascade it is necessary to express adenosine receptors on the cell surfaces.

This project aims to test the cell-autonomous role of adenosine receptor, present on the membrane of immune cells, within systemic Ado–signalling causing the metabolic switch. Such cell-autonomous signalling could play a role in self-activation of immune cells, which would mean the initiation of their development and proliferation during infection. This in turn could affect the systemic metabolism.

Fruit fly *Drosophila melanogaster* is used as a model organism, the immune response here is induced by the parasitic wasp *Leptopilina boulardi* infection. To test the cell-autonomous role and generate mutant clones for adenosine receptor within wild-type organism, mitotic recombination within the hematopoietic lineage is used.

Index

1. Introduction

1.1 The metabolism under stress

Inflammation, ischemia, hypoxia, infection, tissue damage - these and other similar stress impulses, competent directly or indirectly attack any organized system of the living cells, are responsible for disruption of homeostasis, the systemic stillness that manifests throughout the system.

Each stress impulse must be quickly recognized and supressed (immune system). If any part of body-compartment had been damaged, it has to be renewed soon. Because regeneration processes are energetically dependent, the basic step for system regeneration and quick return to homeostasis is the increase of supply to such repair mechanisms. Within organism energy occurs, most importantly, obliged in anhydride, N-glycosidic and phosphate bonds of adenosine triphosphate molecule (ATP).

1.1.1 Systemic energy regulation

The level of free affordable energy in system (for example glycaemia) is hormonally regulated.

Insulin is a steroid hormone, whose binding to the insulin receptor stimulates glucose uptake; its antagonist is glucagon. If the amount of glucose in circulation overgrows the standard, excesses are converted into storage polysaccharide – liver glycogen (glycogenesis). Pathological glycaemia increase (hyperglycaemia) or decrease (hypoglycaemia) may play an important role in metabolic signalling. For example, when the glucose concentration decreases pathologically (hypoglycaemia), the ratio of glucagon to insulin is increasing in plasma, which can tend to liver glycogen metabolising (glycogenolysis) later.

Taken together, the metabolic activity must be supported that the state of balance would regenerate. It means the local strengthening of the energy supply that is regulated by insulin functioning.

As an important component of this energetic management serves a specific type of the systemic over-switch; at its end some cell lines secondary lose their sensitivity for insulin, while some other cell lineage (in damaged regions) are to be preferred in the energy income.

One of the possible (and for initiating of the entire energetic management responsible) candidates might be the adenosine molecule.

1.1.2 Adenosine molecule

Adenosine, a purine nucleoside generated by the dephosphorylation of adenine nucleotides, plays an important role as a potent physiological and pharmacological regulator of many functions.

It was first reported to inhibit the inflammatory actions of neutrophils nearly 30 years ago and since then the role of adenosine and its receptors as feedback regulators of inflammation has been well established (Hasko et Gronstein, 2013).

Adenosine (Ado) composed of a molecule of adenine attached through β-N9-glycosidic bond to ribose sugar molecule is present in all living cells. Its physiological concentration, maintained under normal conditions from 20 to 200 nM, (tested in mammal hippocampal slices) (Latini *et al*., 1999), can rapidly increase during stressful impulses like inflammation, ischemia or hypoxia into the micromolar range (Fredholm, 2010).

1.1.3 Adenosine transport and regulation

Cell membrane damage causes ATP leaking out of the tissue followed by ATP degradation. By a cascade of Ectoenzymes – Apyrases/NTPDases and Ecto-5'-nucleotidases (Fenckova *et al*., 2011) – during inflammatory response - an extracellular Adenosine (e-Ado) is produced (Dolezal, 2015).

Adenosine in the extracellular compartment may be transported through connexin/pannexin, other channels or through protein or hormone-transporting vesicles (Novakova, 2011). Further it signalizes through G-protein coupled receptor AdoR (Dolezal *et al*., 2005; Dolezelova *et al*., 2007; Kucerova *et al*., 2012) and at the end, e-Ado is degraded by adenosine deaminases – ADGFs (Dolezal *et al*., 2005; Zurovec *et al*., 2002).

Intracellular adenosine is exported from/to cells via equilibrative nucleoside transporters (DmENT1, DmENT2 an DmENT3) or during apoptosis or necrosis (Hasko et Gronstein, 2013).

e-Ado serves as an evolutionary ancient signalling molecule and contribute to the regulation of various biological processes, including hormone action, neural function, platelet aggregation, lymphocyte differentiation, regulation of cardiovascular and ion channel activity and modulation of immune response (Novakova, 2011).

The level of e-Ado needs to be tightly regulated by converting to inosine by adenosine deaminase and further metabolized to uric acid. There are two types of adenosine

deaminases, ADA1 and ADA2 (Maier et al., 2005). ADA1 can be localized both in intracellular and extracellular space while ADA2 is secreted enzyme.

1.1.4.1 Adenosine receptor (ADR)

Adenosine signalling occurs through purinergic G-protein coupled adenosine receptors. The receptors structure contains seven interspecies conserved transmembrane domains with an extracellular amino (N) terminus and intracellular carboxy (C) terminus that is subject to phosphorylation (Novakova, 2011).

In humans, four adenosine receptor (ADR) subtypes were described and denoted as A1, A2A, A2B and A3. Adenosine receptors are expressed in a variety of cell types and their activity is determined by concentration of extracellular adenosine. A1, A2A and A3 receptors are activated by low adenosine concentration between 0.01 μM and 1 μM that correspond to physiological level, whereas A2B receptor activation requires pathophysiological conditions where adenosine levels exceed 10 μM concentration (Fredholm *et al,* 2001; Fredholm, 2007).

A1 and A3 inhibit adenylate cyclase, whereas A2A and A2B stimulate this enzyme. The distribution of the receptors on different cells varies and thus e-Ado may have various effects on physiology (Dolezal, 2015).

1.1.4.2 Adenosine receptor in Drosophila melanogaster (DmAdoR)

A single AR was found in a model organism *Drosophila melanogaster* (DmAdoR) based on its homology to the human ARs. The closest homolog of *Drosophila* DmAdoR in human is A2A receptor. It has about 38.3% identity in the 350 base long N-terminal part. Other ARs show following rank order of amino acid conservation: A1 having 36.2%, A2B – 35.2% and A3 – 34.5% identity with DmAdoR (Kucerova *et al*, 2012).

DmAdoR is expressed mainly in the optic lobes of the brain, ring gland, imaginal discs and salivary glands of the third instar *Drosophila* larvae (Dolezelova, 2007; Kucerova *et al*, 2012). Ado responses are highly dependent on the level of receptor expression in a particular cell type (Kucerova *et al*, 2012).

1.1.5 Studies of adenosine signal pathway

Immune cells dramatically change their metabolism upon activation, leading to increased aerobic glycolysis akin to the Warburg effect (Cheng *et al*., 2014; Delmastro-

Greenwood and Piganelli, 2013). Expression analysis of glycolytic genes, glucose and trehalose transporters, and 14C uptake by immune cells suggest a similar behaviour for the differentiating *D. melanogaster* immune cells upon wasp attack (Dolezal, 2015).

Taken together, the immune cells can autonomously regulate the systemic metabolic switch based on their acute energy needs. Previous studies have shown that e-Ado increases circulating glucose via adenosine receptor signalling (Zuberova *et al*., 2010). While the circulating glucose has increased during infection in control larvae, this increase was suppressed in *adoR* mutant larvae (*adoR* mutants were three times less successful at neutralizing the wasp eggs and surviving to adult flies). It indicated that AdoR was indeed necessary for the energy redistribution during infection (Bajgar *et al*., 2015).

Although AdoR signaling showed to be crucial for effective immune defense (most likely due to systemic metabolic switch induction), its cell-autonomous role hasn´t been tested yet. What if the presence of adenosine receptor on the membrane of immune cells is required only for their self-activation, while the systemic activation (during infection), subsequently responsible for the energy redistribution, is controlled by other signals?
What if lamellocytes in the *adoR* mutant simply hadn´t been developed fully? Because of this, their amount in circulation could have been reduced, which might have influenced the mutant survival in the end.

1.2. Drosophila melanogaster – model organism

Fruit fly *Drosophila melanogaster* (*D. melanogaster*) belongs to the class Insecta, order Diptera, family Drosophilidae. It represents the lower complexity model system with only three equilibrate nucleoside transporters and only single adenosine receptor (Kucerova *et al*, 2012). To its biggest advantages further confers a short generation time (around 10 days at 25 degrees Celsius) quite cheap way of breeding and the opportunity to use many various genetic approaches *in vivo.*

Flies have four pair of chromosomes (X, 2, 3, 4), usually represented as lines and circles for arms and centromeres. The size of the X, 2 and 3 are roughly comparable, whereas chromosome 4 is only about one-fifth as large (Greenspan, 2004).

Sex determination in *D. melanogaster* is based on ratio of X chromosomes to autosomal set. In males, one X with two autosomal sets gives a ration 0.5, whereas females have a ration 1.0.

The Y chromosome contains few genes and is not required for most aspects of male development, only for proper sperm motility (Greenspan, 2004).

The energy regulation seems to be the most prominent role of e-Ado in flies while in higher organisms, as mammals, situation is complicated by various immunomodulatory roles of e-Ado (Dolezal, 2015). Therefore the *D. melanogaster* presents a perfect genetic tool to clarify the importance of single cell components (for instance the cell membrane structure, various receptor families, signal ligands etc.), their functionality and role in different metabolic cascades.

1.2.1 The role of e-Ado in immune response to infection in *D. melanogaster*

To establish the metabolic stress in *D. melanogaster* the parasitoid wasp *Leptopilina boulardi* has been used. It falls into the class: Insect, the order: Hymenoptera, the family: Figitidae.

Parasitoid wasp´s egg, injected into *D. melanogaster* third-instars, is recognized by circulating pro-hemocytes (plasmatocytes), whose later activation and proliferation leads to their differentiation into specialized immune cells – lamellocytes. The whole cascade is associated with increased glycolysis and glucose consumption in proliferating and differentiating precursors of lamellocytes (Bajgar *et al.*, 2015). Lamellocytes must appear in the circulation within 24 hours, starting to encapsulate the parasitoid egg. Within 48 hours,

the encapsulated egg must be destroyed by melanisation otherwise a parasitoid larva will hatch and will consume the host (Dolezal, 2015).

Plasmatocytes, recognizing the within the gut folds hiding parasitoid egg, send unknown signal, which activates proliferation and differentiation of specialized immune cells – lamellocytes – in lymph gland (Dolezal, 2015).

To fully differentiate, encapsulate and destroy (by melanisation) the parasitic egg, lamellocytes needs to be energetically advantaged, so e-Ado suppresses metabolism of other tissue by AdoR signalling (Dolezal, 2015).

1.3 Testing AdoR self-activation role in immune cells

Within the testing of AdoR self-activation role in immune cells, AdoR wild and *adoR* mutant clones must have been created; within one organism, in vivo.

After parasitoid infection the immune cells are activated, develop and proliferate. If AdoR presence plays any role in immune cells development, we should be able to observe the significant difference between numbers of full developed lamellocytes with and without AdoR.

Similar numbers of both lamellocyte types would show that their development isn´t conditioned by adenosine receptor expression and support the requirement of systemic adenosine signalling or the immune system energetic selfish behaviour.

1.3.1 Genetic tools - mitotic recombination

Exchange and separation of genetic material is characteristic for meiosis division. During the meiotic crossing over and subsequent segregation the genetic information is randomly mixed. So the genetic variability is increasing and giving further to the next generation. However, similar scrambling of genetic material occurs even in somatic cell line.

When e. g. radiation causes the DNA breaks, the natural repair mechanism - mitotic recombination - combine and stick missing parts together, so new genetic combinations are arising. However, it is complicated, rather impossible, to regulate this from outside. That’s why different genetic tools have been developed to induce genetic exchange in targeted regions.

1.3.1.1 Homozygous cells from heterozygous cell

Genetic mitotic recombination was first defined by studies of *D. melanogaster*, again on the basis of the observation that genes on different copies of homologous chromosomes can reassert during meiosis (Cooper, 2000).

Mitotic recombination causes the switch of homologous chromosome parts in pre-defined sections and leads to homozygosity of originally heterozygous genes.

Many of these artificially induced mutations are homozygous lethal in embryonic or early larval stages of development (Blair, 2003), which can make the later selection according to phenotype easier.

A low level of mitotic recombination occurs spontaneously in flies. This rate can be increased to a useful, although still low, level by exposing flies to X-rays, gamma-rays or using Gal4-UAS system (Duffy, 2002).

Segregation of the four strands occurs in an equational, typically mitotic mode in respect to the fibre points (Blair, 2003).

In this project the mitotic recombination was used to induce the division of AdoR heterozygous mother cell into *AdoR+* and *adoR* mutant homozygous daughter cells.

1.3.1.2 Clones marking

One problem caused by structural changes on chromosome is the random location of new arising homo- or hemizygous cells within the tissue. These cells therefore need to be marked.

More recently, constructs encoding several non-endogenous, histologically identifiable tags have been inserted into the genome – besides other markers the green fluorescent protein (GFP) (Blair, 2003).

In some cases, this *marker*/*marker* sister of the *mutant*/*mutant* cell can be easily identified and used not only as a control for the effects of the sister *mutant*/*mutant* cells, but as an indication of the location of the recombination event within the tissue too (Blair, 2003).

1.3.1.3 FRT mediated mitotic recombination

In order to utilize the FRT/FLP recombination system to induce mosaicism for specific (e. g. *AdoR*) gene regions, through the *D. melanogaster* genome, it is essential to generate a set of specific strains that each carries an FRT sequence near the centromere of a chromosome arm (Xu and Rubin, 1993).

When X-irradiation is used to induce mitotic recombination, radiation damage causes considerable cell death. In contrast, it has not been observed any obvious developmental defects associated with the induction of mosaicism using the *P[ry+, hs-neo, FRT]* elements (Xu and Rubin, 1993).

Incorporated FRTs are used to exchange stretches of DNA, including targeted mutation, located behind these two FRTs via the FLPase activation (Blair, 2003).

1.3.2 Targeted gene expression

The mosaic techniques uses combination of two different systems, both derived from yeast. The first part enables the specific DNA sections recombination between FRTs regions, activated by the FLP recombinase (FLPase), described above. The second part uses the Gal4 transcription factor to drive the expression, that are coupled to the UAS enhancer sequence, to specific cell lineage (Blair, 2003), in our case to immune cell line.

1.3.2.1 Gal4 – UAS system

In 80´s two methods were employed most widely to manipulate the time or location of gene expression. The first was to drive expression of a gene from a heat shock promoter. The second technique was to drive expression of a gene using the transcriptional regulatory sequences from a defined tissue-specific promoter. But if the gene product to be expressed was toxic to the organism, it was impossible to establish stable transgenic lines carrying the chimeric gene (Brand et Perrimon, 1993).

To overcome these difficulties Andrea Brand and Norbert Perrimon (1993) developed a new biochemical method for directing gene expression in Drosophila, Gal4 - UAS system, enabling gene expression in a different cell, or at a different time of development.

1.3.2.2 Gal4 sequence

Gal4 (and Gal80) gene sequences, encoding a protein of 881 amino acids, are required for the regulation of the galactose-inducible genes in *Saccharomyces cerevisiae.*

Gal4 has been shown to activate transcription, not only in Drosophila, but also in plants and in mammalian cells, besides to activate the mouse mammary tumor virus promoter (Kakidani et Ptashne, 1988).

In flies, minimal Gal4 activity is present at 16°C, while 29°C provides a balance between maximal Gal4 activity and minimal effects on fertility and viability due to growth at high temperature (Duffy, 2002).

1.3.2.3 UAS element, the regulation of gene expression

The method separates the target gene under UAS sequence from its transcriptional activator (Gal4 protein) in two distinct transgenic lines. Only when these two lines are crossed is the target gene turned on, respectively activated in different cell- and tissue-types, and the phenotypic consequences can be conveniently studied (Brand et Perrimon, 1993).

Expression of the gene of interest, the responder, is controlled by the presence of the UAS element, in this case five tandemly arrayed and optimized Gal4 binding sites (Duffy, 2002).

Thanks to UAS sequence and Gal4 driver - transcriptional activator (Gal4), binding itself to UAS sequence, it is possible to regulate the location of targeted gene expression (Brand et Perrimon, 1993). So it is possible to initiate mitotic clone proliferation within targeted region, immune cell lineage.

2. Summary

Goal of this project is to test a possible role of adenosine signalling in immune cell activation in a cell-autonomous manner.

H_1: Establishment of wild-type and mutant clones within specific (immune) cell line

Using hematopoietic line – specific Srp-Gal4 coupled to UAS-Flipase and FRT sites, combined with GFP markers and *adoR* mutation - we induced a mitotic recombination leading to formation of homozygous wild type and mutant sister clones of immune cells from AdoR+/*adoR*- heterozygous parental cell.

H_2: Counting the amount of proliferating lamellocytes after parasitic infection

Infection initiates the development of all 3 types of immune clones: heterozygous cells, homozygous wild-type and *adoR* mutant sister clones.
If the presence of AdoR plays any important role in the self-activation and next proliferation of immune cells, the missing adenosine receptor on immune cell membrane would cause a decrease of number of circulating lamellocytes compared to wild type lamellocytes.

3. Methodology

3.1 Fruit flies stocks

During our experiment it has been worked with many of the well-known genetic modified fruit fly genome varieties.

They were hold, as the experimental fruit fly stocks, on 5% cornmeal diet (8% corn-meal; 1% agar; 4% yeast; 5% sacharose) at 18 degrees Celsius, while for experiments themselves the experimental diet with lower share of nutrients has been used (5,4% corn meal; 0,6% agar, 2,8% yeast; 5% sacharose).

Fruit fly´s lines were kept under low temperature (18°C), for needs of the experiment (laying eggs, larvae development) parental lines were moved into incubators (25°C).

To gain the final fruit fly´s genotype, needed for the experiment, following fruit fly´s lines were used (Tab. I).

Tab. I: Drosophila´s stocks used for genetic crosses.

Number of stock	Name in text	Genotype	Chromosome
Srp-Gal4, FRT mutant (X; III)			
1419	Srp-Gal4 (on X)	Srp-Gal4	X
1376	TM3 GFP	w*; +*/+*;adgf-a[kar]*/TM3 GFP Ser	III
1375	TM6B	w*; +*/+*;adgf-a[kar]*/TM6B	III
1226	*adoR*	w*; adoR	III
1307	FRT	w; P{ry[+t7.2]=neoFRT}82B	III
1309	UAS-FLP; GFP	yw UAS-FLP; P{ry[+t7.2]=neoFRT}82B {w[+mC]=Ubi-GFP.D}83	X+III
1542	UAS-FLP (on III)	w[1118]; P{y[+t7.7] w[+mC]=20XUAS-FLPD5.PEST}attP2	III
565	UAS-GFP (on II)	yw; P{$w^{+mW.hs}$=UAS-GFP}	II
1595	Srp-Gal4; FRT	wSrp-Gal4; P{ry[+t7.2]=neoFRT}82B	X+III
Srp-Gal4, FRT mutant (III)			
1588	FRT	w*; P{ry[+t7.2]=neoFRT}82B	III
1586	Srp-Gal4 (on III)	w*; Srp-Gal4	III
Srp-Gal4, FRT, *adoR* mutant (III)			
1221	Wild-type control	w*	X

As the table I shows, not all used constructs were present either on the second or third chromosome, but on the sex chromosome as well. So it was necessary to pay attention how the inserted construct (the induced mutation) travels across generations.

Fruit fly female stages had to be selected earlier, 2 or 3 hours after hatching, to eliminate the dangerous of contamination in next steps. Only virgin female stages, too young to mate with

their new hatched brothers, can come through the new genetic crossing (with new cross partners) and ensure that the next generation won´t be carrying the old parental features instead of the new ones (derived from meiotic recombination and segregation).

3.2 Genetic crossing

3.2.1 Srp-Gal4, FRT GFP

In the first step it was essential to gain a specific genotype containing Srp-Gal4 and FRT sequence whose cross to UAS-FLP; FRT GFP lineage would induce the development of 3 types of immune cells in hematopoietic line marked by GFP as follow GFP/GFP; GFP/- and -/- with different fluorescence intensity.
We started the genetic cross with Srp-Gal4 driver (on X) and FRT site (on III), using the TM3GFP (on III) and TM6B (on III) as balancers (Fig. 1).

After 5 crossing steps we finally gained the targeted female genotype carrying homozygous Srp-Gal4 (on X) and FRT 82B site (on III) which could be crossed to line carrying Flipase; FRT site and GFP marker (see further).

Genetic Crossing: Srp Gal4, FRT (X; III)

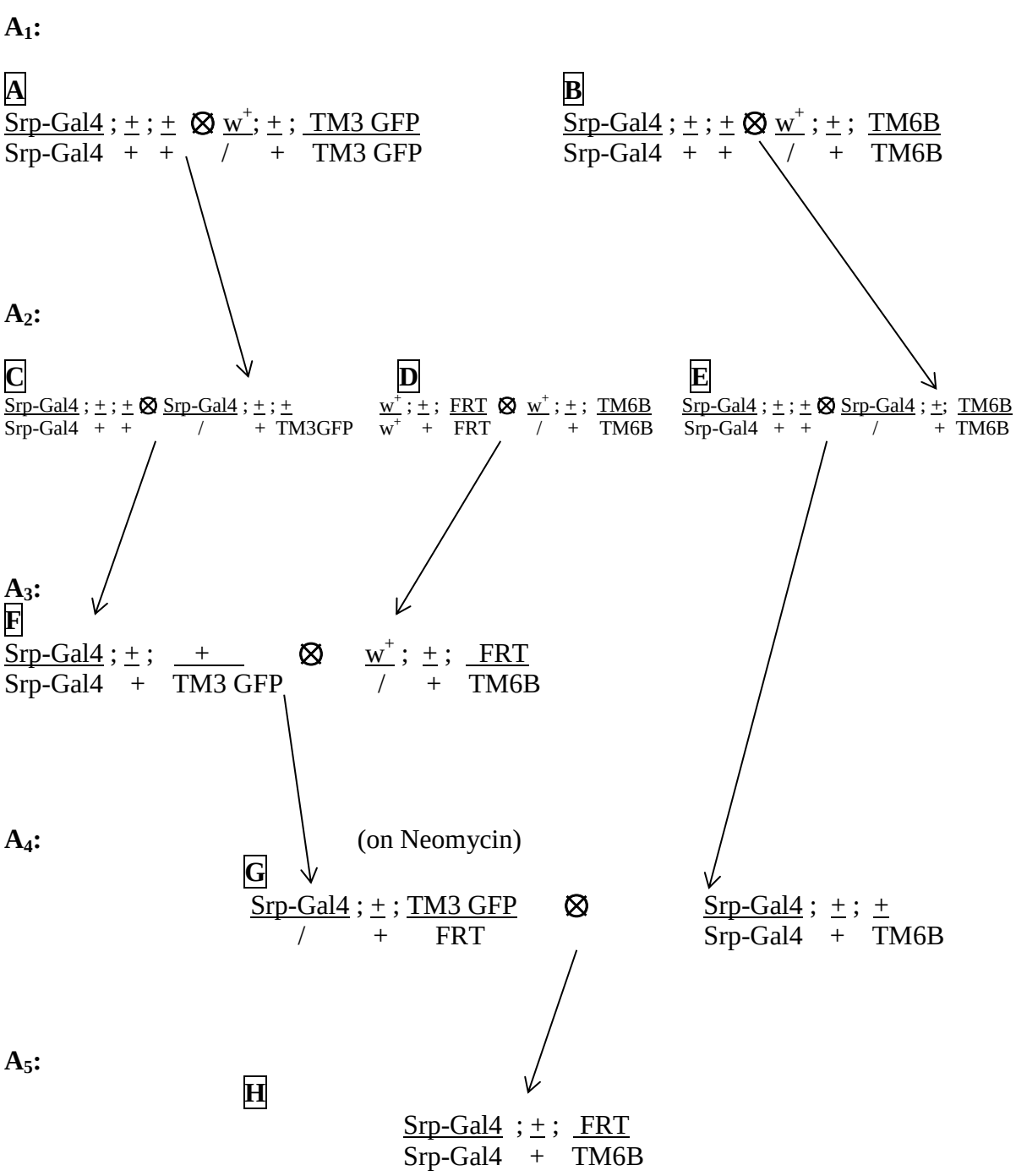


Fig. 1: The crossing schema for Srp-Gal 4 and FRT combination.

3.2.2 Srp-Gal4, FRT, *adoR* mutation

To produce *adoR* mutant clones, we have created the fruit fly lineage carrying Srp-Gal4, FRT 82B constructs and *adoR* mutation (Fig. 2). We used a different approach to hold all sequences on one chromosome (on III).

Genetic Crossing: Srp-Gal4, FRT *adoR* mutant (III)

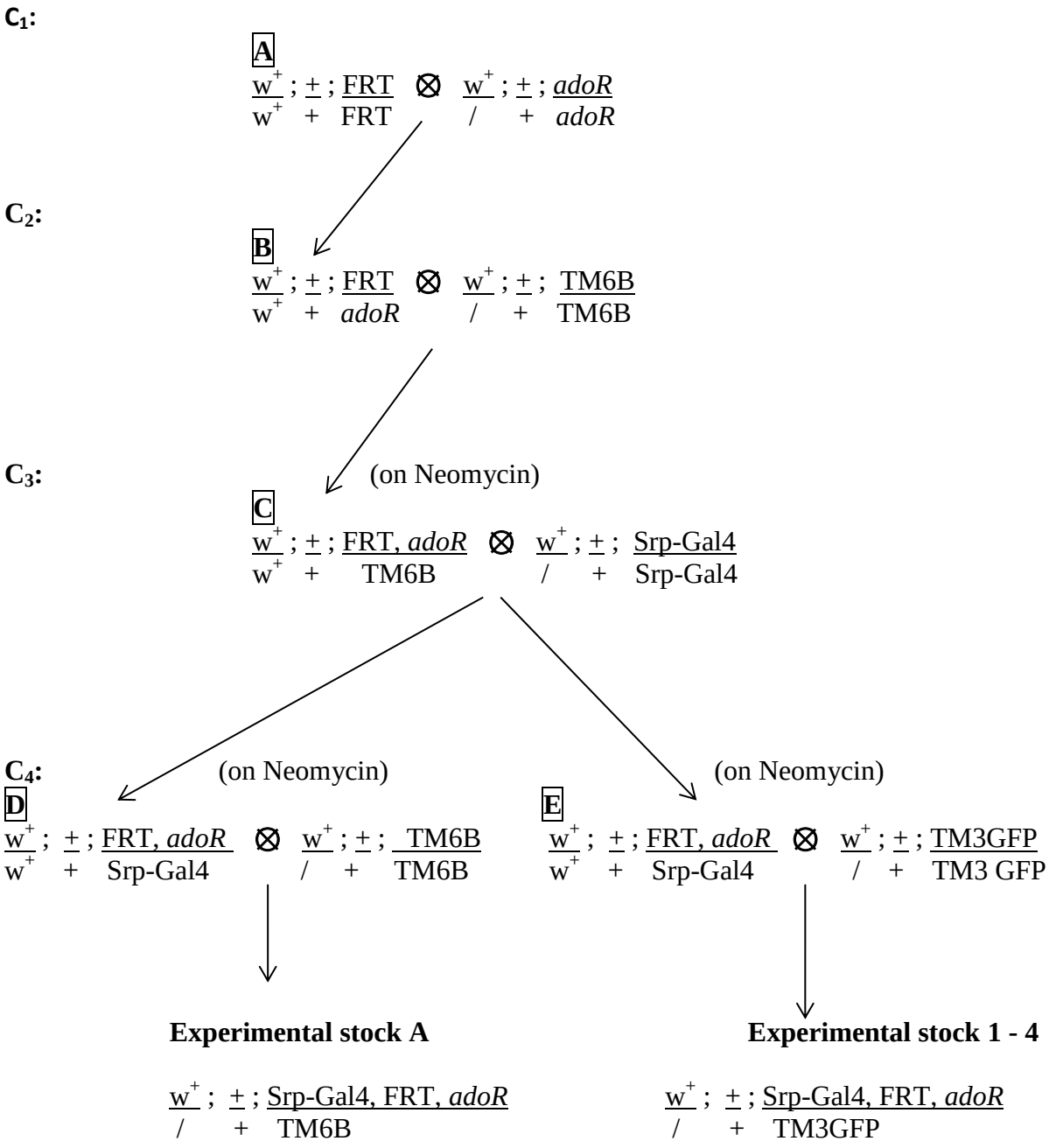


Fig. 2: The Crossing schema for Srp-Gal4, FRT and *adoR* combination.

3.3 Verification of resulting Drosophila lines

Firstly Srp-Gal4 and FRT combination (X, III) and all five newly, single-crossed experimental stocks (III) (A, 1-4) had to be tested for presence of the Srp-Gal4, FRT sites and the *adoR* mutation.

3.3.1 Control cross – presence of Srp-Gal4 construct

Srp-Gal4, FRT as well as Srp-Gal4, FRT, *adoR* homozygous recombinant individuals were crossed with UAS-GFP (on II) (Fig. 3) and their descendants were observed under GFP microscope. The fluorescent signal coming out of larvae tissues (lymph gland, fat body or circulating hematocytes) proved that UAS sequence is present and induces GFP expression in hematopoietic lineage.

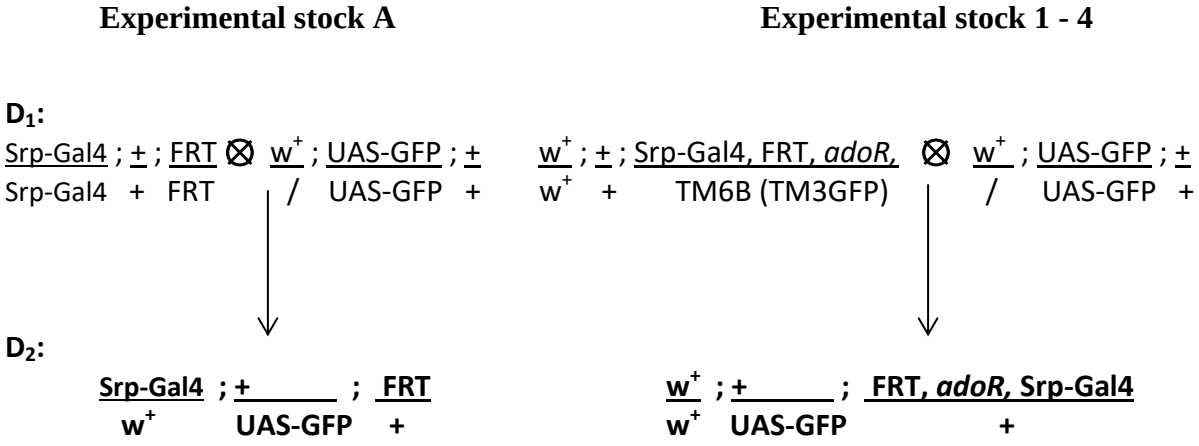


Fig. 3: The crossing schema for Srp-Gal4 and FRT combination with UAS-GFP and Srp-Gal4, FRT, *adoR* combination with UAS-GFP.

3.3.2 Control cross – presence of FRT construct

As Golic and Lindquist (1989) showed the site-specific recombination system of the yeast 2 μm plasmid (the FLP recombinase and its target the FRT sequence) can function in *D. melanogaster* (Xu and Rubin, 1993). Two copies of the *hs-neo* gene were placed in the *P[ry+, hsneo, FRT]* construct to facilitate the genetic selection for this element (Xu and Rubin, 1993).

Flies carrying the *P[ry+, hs-neo, FRT]* element can be selected by their resistance to G418 (Geneticin, GIBCO laboratories). G418-containing medium was made as follows: a

few holes were made in standard fly medium with toothpicks, and 0.2-0.3 ml of 25 mg/ml freshly made G418 solution was added per 10 ml of medium and the vials were allowed to air-dry for several hours. G418 is stable in medium stored at 4-18°C for more than 2 weeks.

To visualize the disadvantage of the FRT absence we have always planted two fruit fly lineages on Neomycin antibiotic. The first one – FRT positive: Srp-Gal4, FRT (X,III) and Srp-Gal4, FRT, *adoR* (III) – developed fully, meanwhile the second one – FRT negative: UAS-GFP (III) didn´t develop at all.

3.3.3 Control PCR – presence of the *adoR* mutation

The *adoR* mutation, caused by an insertion of the miniwhite construct (4.8 kb) in the third exon of the AdoR sequence, is responsible for incorrect transcription of the AdoR gene (lying on IIIrd chromosome). In consequence of that the targeted protein (former adenosine receptor) doesn´t appear on the cell membrane.

To demonstrate the existence of miniwhite construct and detect the presence of *adoR* mutation three Polymerase Chain Reactions (PCR) overall had to be conducted.

Firstly AdoR –RTR and w11678 primers were used to detect 2.16 kb long fragment which consists of A part of the AdoR third exon and the initiate section of the miniwhite construct.

Secondly sp3 and AdoR - RTF primers were used to detect 2.16 kb long fragment which consists of the terminal section of miniwhite construct and B part of the AdoR third exon 3Bct (Fig. 4).

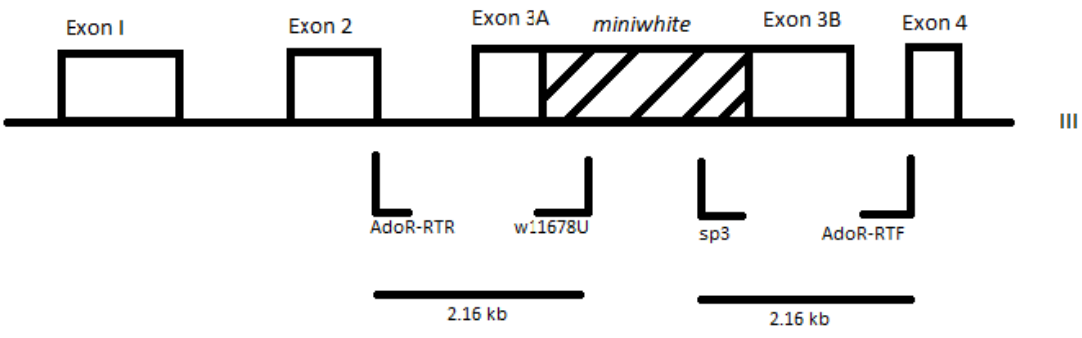


Fig. 4: Schema of the *adoR* mutation presence including primers needed for its detection.

The last PCR reaction (primers D1 SARC/W) played a role of the positive control for genetic material presence.

Required primers were designed with help of webpage ensemble.org (Fruit fly, AdoR category) and the computer programme Geneious, their oligonucleotide sequences are listed in table II.

Tab. II: Overview of the utilised primers, their sequences and melting temperatures.

Designation	**Oligonucleotide sequence**	**Melting temperature (°C)**	**Concentration**
AdoR-RTF	CTA CCG GGT CAT CAT CAA ACA G	63	10***μ***M
AdoR-RTR	CGT GCC TCA ACC AAA TGG GTG	63	10***μ***M
sp3	GAG CTG CCA GTT TTT ATG AG	55	10***μ***M
w11678u	TCA TCG CAG ATC AGA AGC GG	59	10***μ***M
D1 SAR C	GTA CGA GGA ATC ACG CTC	57	10***μ***M
D1 SAR W	AGG TTC TCA TCC ACA GTG G	57	10***μ***M

For all three types of PCR screening, described above, flies of interest were individually squeezed using a pipette tip in 50 ***μ***l squishing buffer (10mM Tris-HCl pH 8,2; 1mM EDTA; 25 mM NaCl) freshly supplemented with Proteinase K (final concentration 200 ***μ***g/ml) and incubated at 37°C for 30 min. Proteinase K was then inactivated by incubation at 95°C for 2 min.

PCR reactions were set up in 20 ***μ***l (10X ThermoPol Reaction Buffer; 2,5 mM dNTPmix; 10 ***μ***M primerF/R; 200 ng/***μ***l DNA template) and targeted fragments amplified using NEB Taq DNA Polymerase (5U/***μ***l). Annealing temperature for RTF/sp3 PCR was 55°C, for RTR/w11678u PCR was 61°C and for D1 SAR C/W PCR was 53°C. The amplification step was repeated 35 times.

10 ***μ***l of each PCR reaction was run on 1% agarose gel and the rest (10 ***μ***l) was stored in the fridge (-20°C).

3.4 Mitotic recombination and infection

As soon as the experimental genotypes were designed and tested, it was possible to initiate the mitotic recombination (cross with UAS-FLP on III) (Fig. 5) and continue with infections. Infection stimulates the immune response, which leads to pro-hemocytes proliferation and next immune cells development.

3.3.1 Experimental cross – UAS-FLP (on III) construct

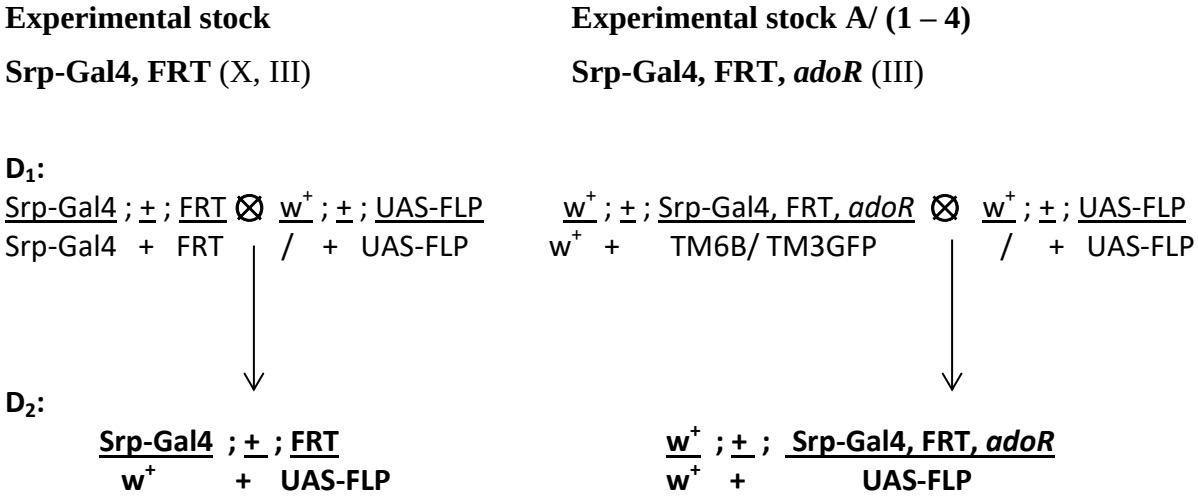


Fig. 5: The schema of experimental crossing Srp-Gal4, FRT and Srp-Gal4, FRT, *adoR* combinations with UAS-FLP.

3.3.2 Fruit fly selection and infection

For needs of infection we selected around 50-60 of early *D. melanogaster* third-instars (72-hours-old). To prevent the absolute larvae damage we used the middle strong infection. Compared to the amount of larvae instars it consisted only of the half amount of three to five-days-old wasps (25-30 wasps). The infection took around 30 minutes.

After 24-hours-long incubation period (at room temperature) infected larvae could have been dissected and we could judge the strength of the fully running immune response (by circulating lamellocytes counting).

3.3.3 Control infections

To be sure what impact do our individual crosses have for lamellocytes development we compared our results with the influence, what does the infection have on different types of genotypes:

1. Heterozygous Srp-Gal4, FRT, *adoR/*TM6B lineage (crossed with UAS-FLP, FRT GFP; selecting heterozygous FRT GFP/TM6B without recombination) presuming the lower (heterozygous) intensity of lamellocytes fluorescence (giving the standard for heterozygously shining *adoR*-mutated lamellocytes);

2. Srp-Gal4, FRT without *adoR* mutation (crossed with UAS-FLP; FRT; causing the recombination) presuming the lower or higher intensity of GFP in lamellocytes development (giving the standard for homozygously shining lamellocytes, having adenosine receptor fully developed);

3. Homozygous Srp-Gal4 and UAS-FLP lineages only without by Flipase/Srp-Gal4 induced mitotic recombination, presuming no sister clones.

Each of these controls should exclude any side mutation effects due to homozygosity of chromosome that could affect lamellocytes differentiation.

4. Results

4.1 Verification of construct controls

From previous genetic crosses we gained five independent fruit fly lineages (A, 1-4) that had to be tested one by one for Srp-Gal4 and FRT constructs and for *adoR* mutation presence. These tests must have been conducted before infection to intercept possible errors and prevent later misinterpretation.

4.1.1 Srp-Gal4 - construct control

Srp-Gal4, FRT (X, III) and all experimental stocks (A, 1-4) Srp-Gal4, FRT, *adoR* (on III), homozygous mutants, were crossed with UAS-GFP (on II) and their third-instar offspring was dissected and observed under GFP microscope (Fig. 6).

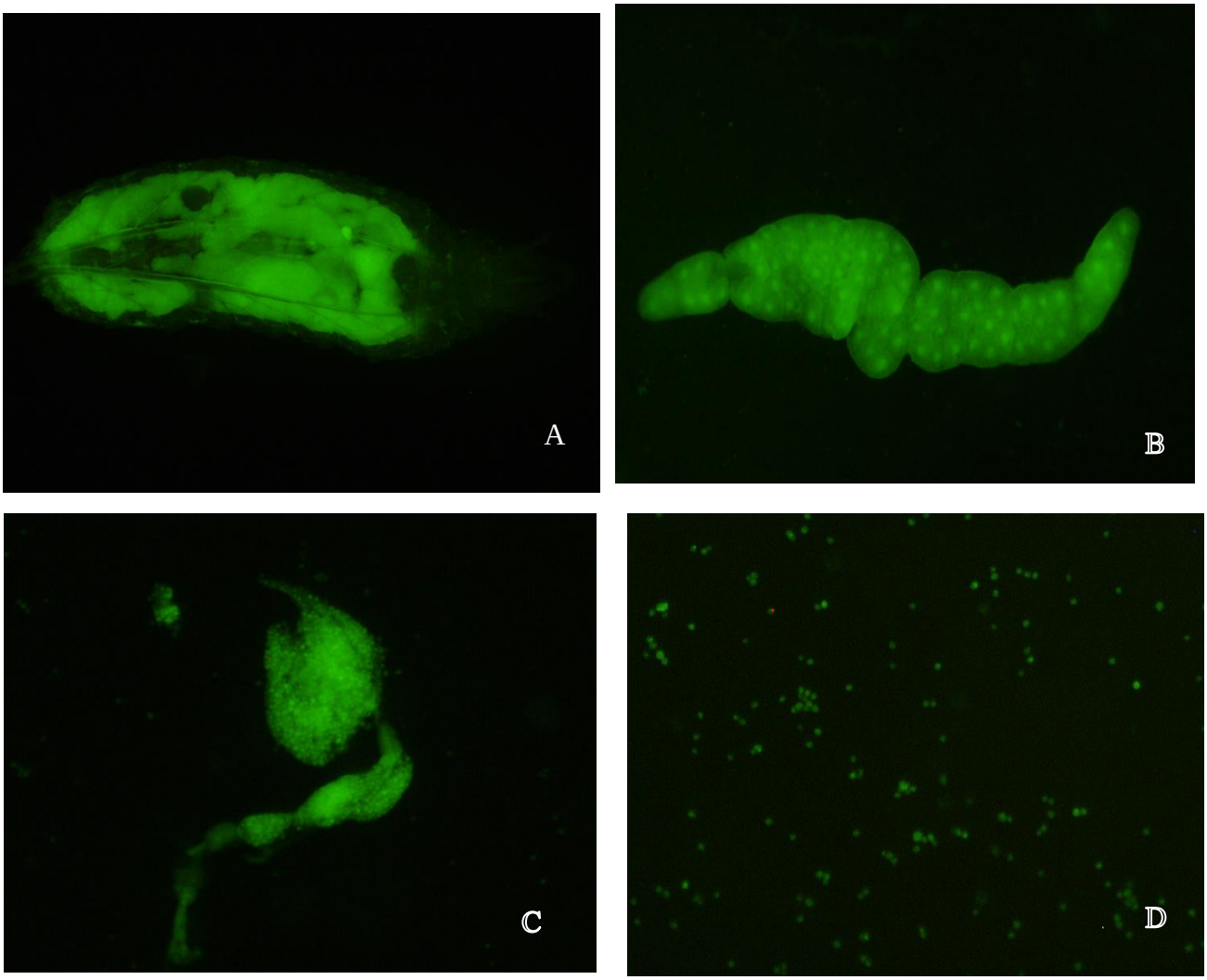


Fig. 6: GFP expression in fat body (A), fat body in detail (B), lymph gland (C) and in free circulating hematocytes (D).

The fluorescent signal coming out of larvae tissues (lymph gland, fat body or circulating hematocytes) (Fig. 6) showed the same intensity within all tested samples and proved that UAS sequence is present and induces GFP expression specifically, in hematopoietic lineage only.

4.1.2 FRT – construct control

The presence of FRT construct has been tested on the basis of neomycin resistance. As soon as we compared the survival of two newly hatching fruit flies lines (FRT positive ones: Srp-Gal4, FRT (X, III) and Srp-Gal4, FRT, *adoR* (III) – and secondly – FRT negative UAS-GFP (III)), we could judge, whether samples carried antibiotic resistant or not. One hundred percentage survival testified that FRT construct hasn´t been missing.

4.1.3 PCR – *adoR* construct control

With help of PCR we detected the presence of *adoR* mutation, more precisely demonstrated the existence of two inserted miniwhite constructs supressing the wild AdoR expression.

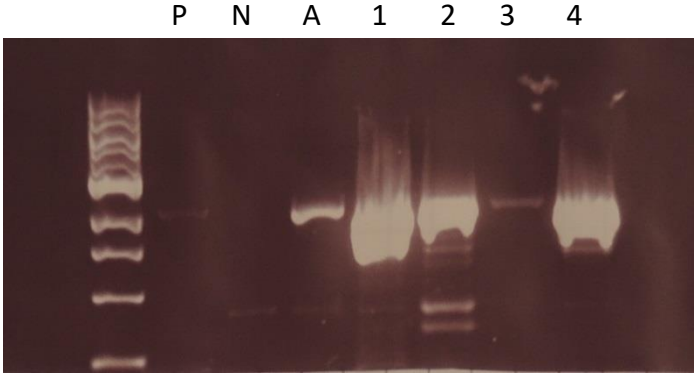


Fig. 7: Detection of miniwhite fragment using SP3-RTF primers.

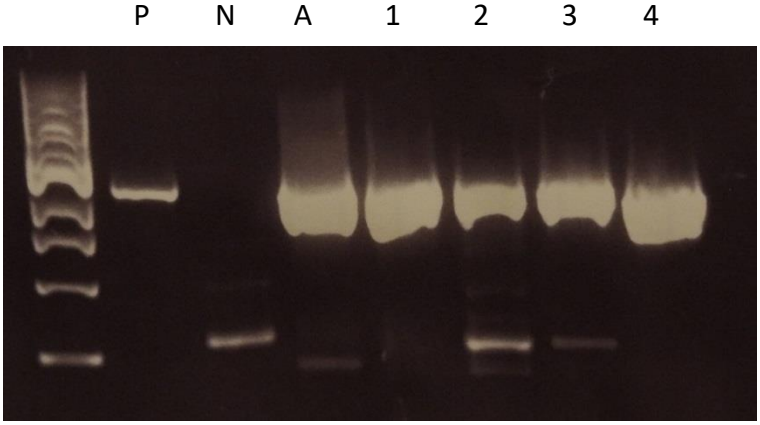


Fig. 8: Detection of miniwhite fragment using w11678u-RTR primers.

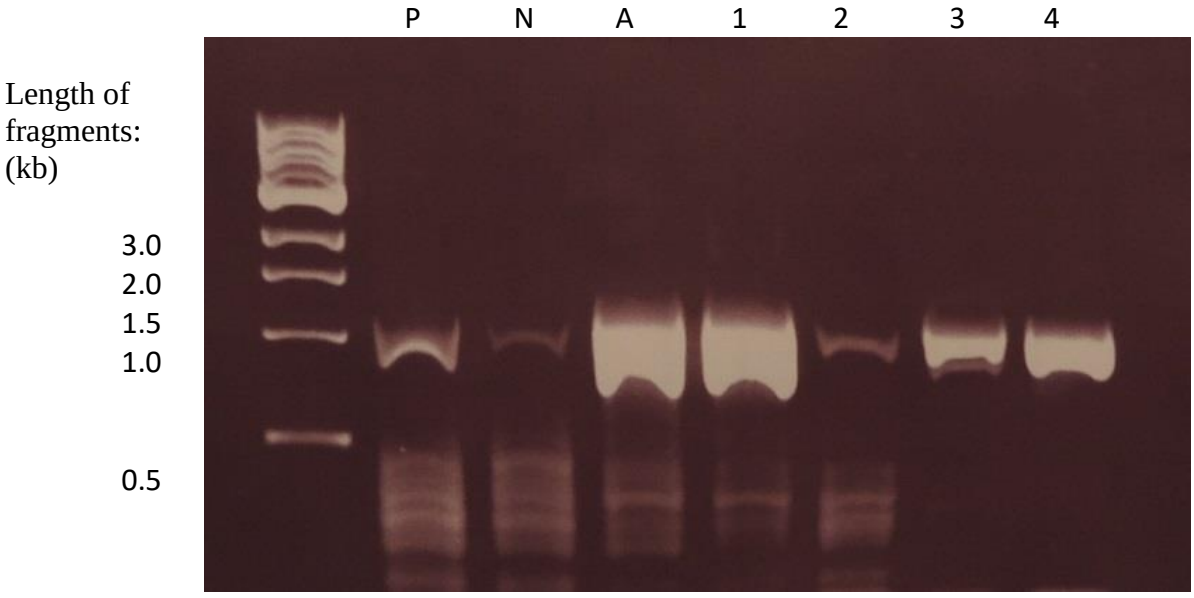


Fig. 9: Detection of Taq-Polymerase activity using different D1SAR_c/w primers.

PCR with 4 primers (Tab. II) was used to detect the presence of miniwhite coding sequences in all genotype constructs: P – *adoR* mutant (positive control); N – wild fruit fly lineage (negative control); A – Srp-Gal4, FRT, *adoR* (balanced with TM6B); 1-4 – Srp-Gal4, FRT, *adoR* (balanced with TM3GFP). Primers D1SAR_c and D1SAR_w were used as positive controls of correct Taq-Polymerase activity.
PCR reactions (Fig. 7-9) proved the presence of miniwhite constructs in all tested samples.

4.2 Crossing controls (before infection)

Because we wanted to test the clones establishment of proliferating plasmatocytes, not influenced by infection, we took pictures of free circulating Srp-Gal4, FRT prehemocytes, developing after mitotic recombination (induce by UAS-FLP (on III)).

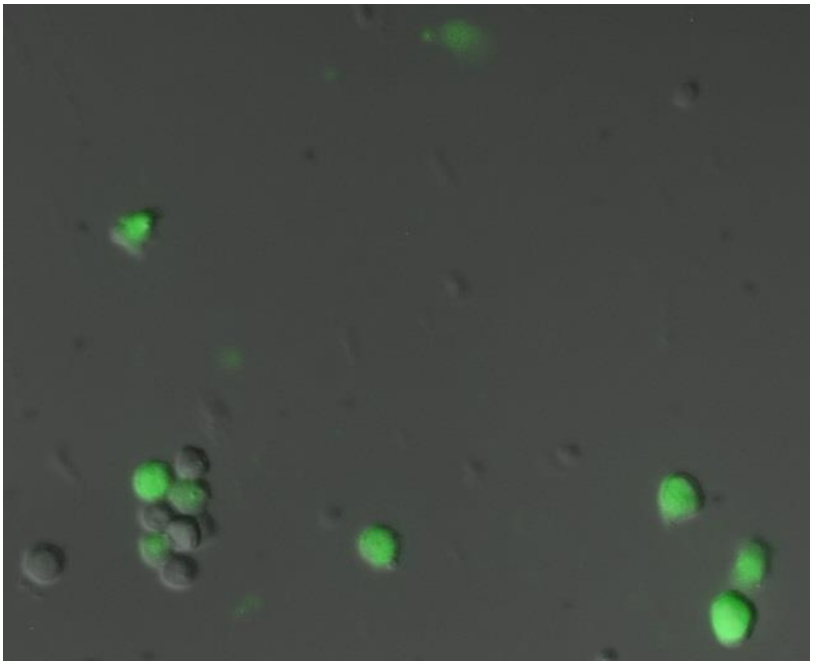


Fig. 10: Immune cell clones establishment after mitotic recombination (*D. melanogaster*, Srp-Gal4, FRT (III) crossed to UAS-FLP (III).

DIC image overlaid with GFP fluorescence image (Fig. 10) shows plasmatocytes (circulating macrophage-like immune cells) with no GFP (6), lower GFP intensity (2 in 8-cell cluster) and higher GFP intensity (6) demonstrating a formation of homozygous clones (with either no GFP or doubled GFP) from heterozygous parent cells (lower GFP).

4.3 Crossing controls (after infection)

To be sure what impact do our individual crosses have for lamellocytes development we tested all five different varieties of the final genotype and after infection we compared reciprocal similarities and deviations.

4.3.1 Srp-Gal4, FRT, *adoR*/TM6B crossed with UAS-FLP; FRT GFP

To gain the more precisely concept of how intense the fluorescence of heterozygous Srp-Gal4, FRT, *adoR* clone expression might be, we infected the heterozygous Srp-Gal4, FRT, *adoR* /TM6B lineage, previously crossed with UAS-FLP (Fig 11).

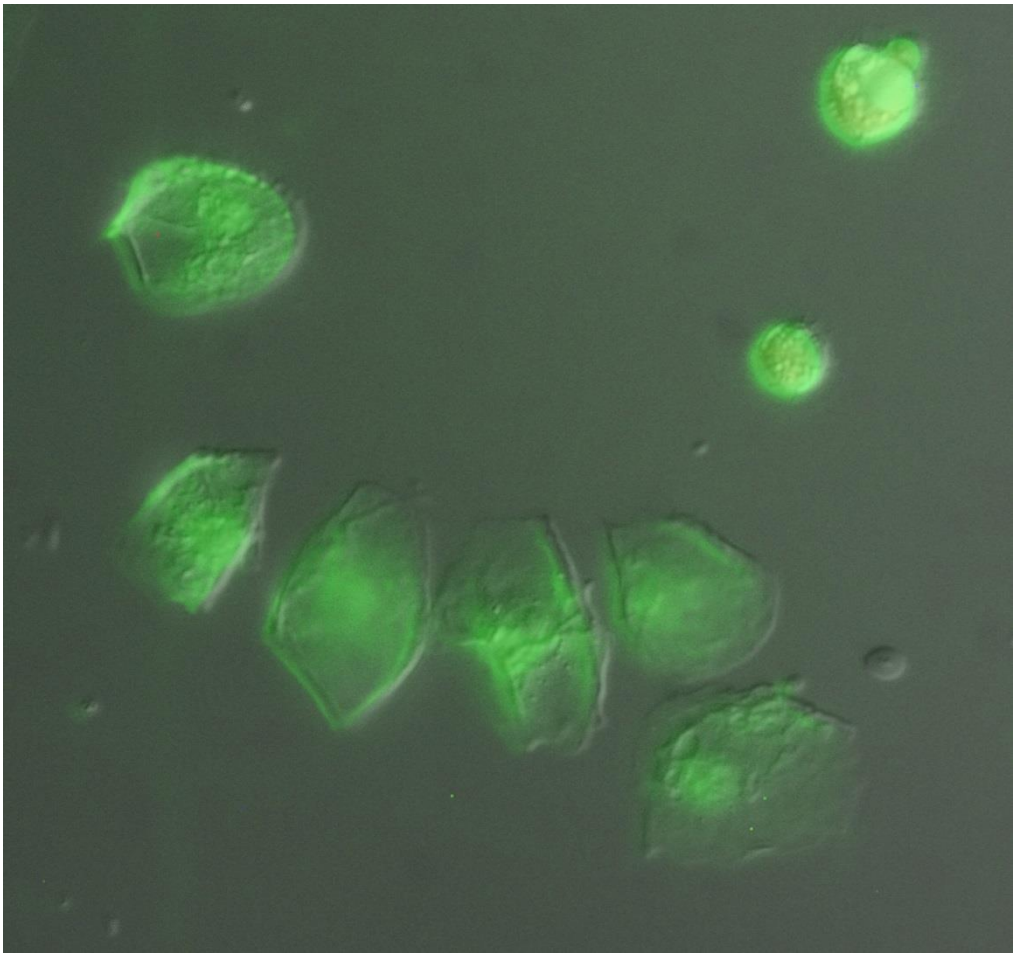


Fig. 11: The fluorescence intensity of heterozygous FRT GFP/TM6B expression in lamellocytes (5 larger flat cells).

Because heterozygous GFP lamellocytes display the uniform intensity of heterozygous GFP expression, at the end these figures could have been used as model. So they have made the selection of lamellocytes, heterozygous and homozygous GFP fluorescent, easier.

4.3.2 Srp-Gal4, FRT crossed with UAS-FLP (no *adoR*)

To gain the more precisely concept of how intense the fluorescence of only homozygous Srp-Gal4, FRT (without *adoR* mutation) clone expression might be, we infected the homozygous Srp-Gal4, FRT lineage, previously crossed with UAS-FLP (Fig 12).

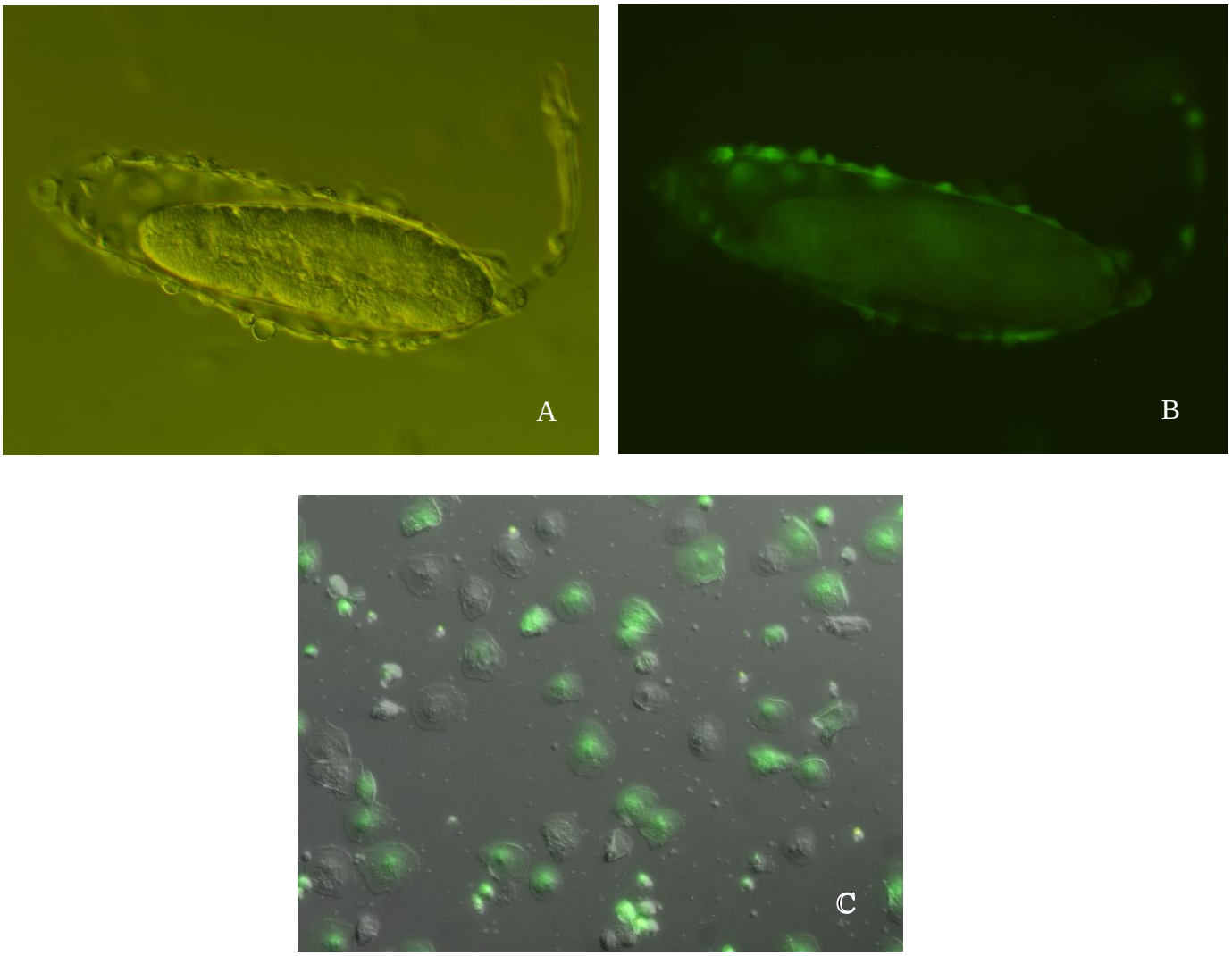


Fig. 12: The fluorescence intensity of homozygous Srp-Gal4, FRT (without *adoR* mutation) observing by egg encapsulation (A - DIC20; B - GFP fluorescence) and lamellocytes proliferation (C - DIC20, overlaid with GFP fluorescence).

It is possible to see (Fig. 12) that there is a significant difference between dark, not GFP shining and green, GFP shining lamellocytes.

However, it is difficult to clearly distinguish lamellocytes, which are homozygous for GFP (doubled form of GFP expression), and should have been shining more intensively green, from heterozygous for GFP carrying only one GFP copy.

4.3.3 Homozygous Srp-Gal4 and UAS-FLP chromosomes

To exclude the possible impact of homozygous Srp-Gal4 or UAS-FLP constructs-carrying chromosomes, which are formed in clones after mitotic recombination, on lamellocytes development we have tested the immune response of these homozygous lineages as well.

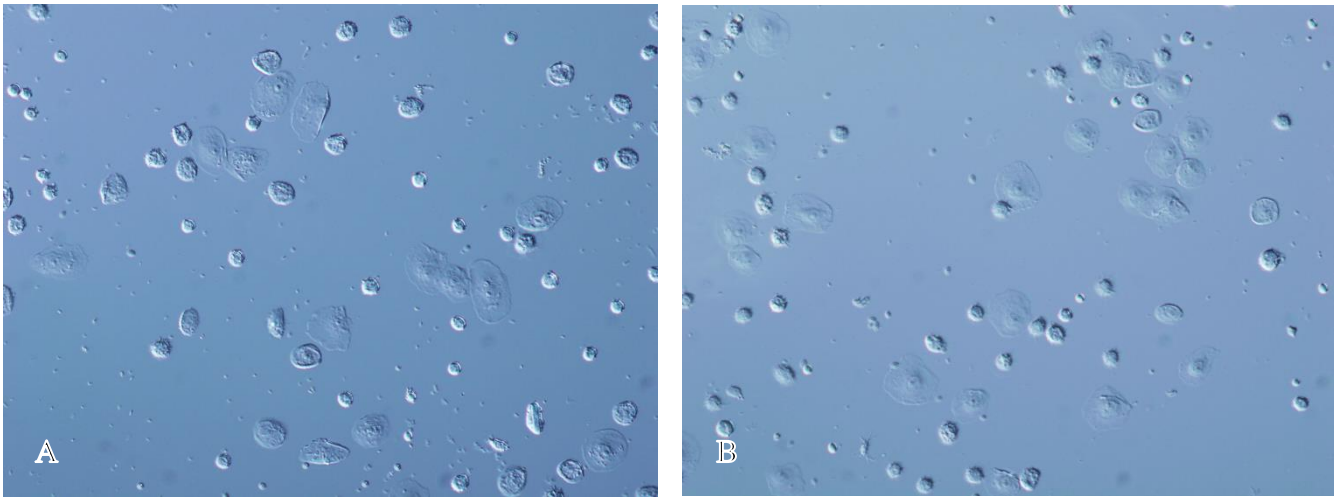


Fig. 13: Lamellocytes formation in Srp-Gal4 (A) and UAS-FLP (B) individuals 18 hours after infection.

Because we observed the normal lamellocytes formation (Fig. 13), we can confirm that homozygous Srp-Gal4 or UAS-FLP chromosomes don´t affect lamellocytes production.

4.4 *adoR* mutant clones

Finally we could start the own experiment and compare the difference between number of proliferating *adoR* and wild-type AdoR lamellocytes**.**

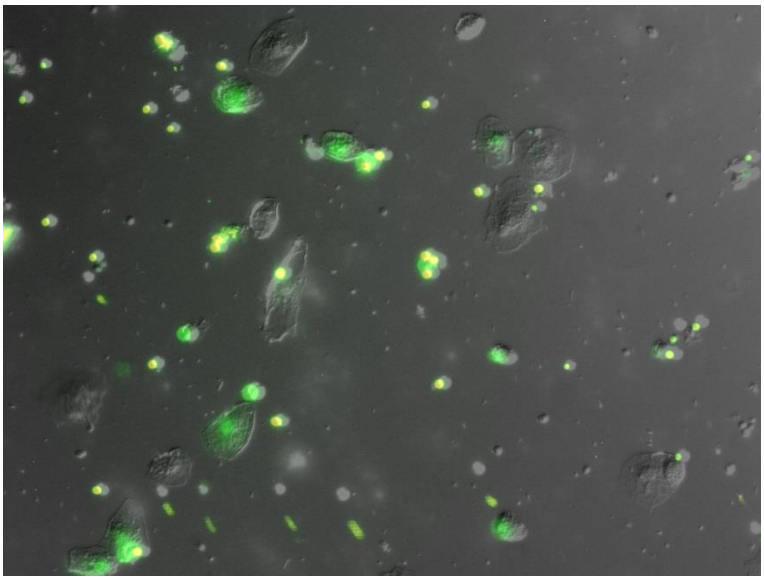


Fig. 14: Profile of proliferating *adoR* (dark) and wild/ AdoR (GFP shining) lamellocytes, taken from Srp-Gal 4, FRT, *adoR* (on III) from the experimental stock 1.

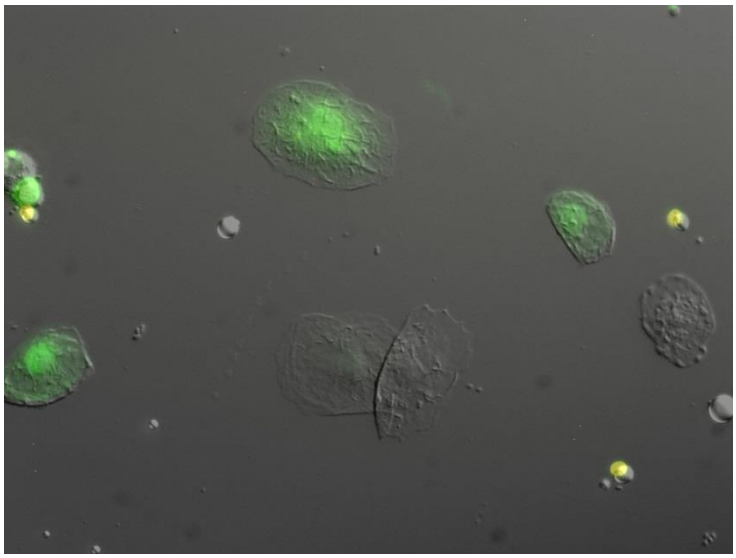


Fig. 15: Profile of proliferating *adoR* (dark) and wild/ AdoR (GFP shining) lamellocytes, taken from Srp-Gal 4, FRT, *adoR* (on III) from the experimental stock A.

As figures above demonstrate (Fig. 14 - 15), *adoR* mutant clones of lamellocytes (no GFP) are formed upon infection.

Because we were not able to distinguish their sister homozygous GFP clones from the non-recombinant heterozygous GFP lamellocytes absolutely, and so could not directly compare their numbers either, it was rather appropriate to compare the average rate of *adoR* mutant clones formation with the rate of GFP-negative clone formation in lines without *adoR* mutation (see below).

4.5 Statistics

We counted total 294 lamellocytes from control larvae and 332 lamellocytes from the *adoR* mutant clones (Tab. III) bearing larvae within 5 independent experiments:

Tab. III: Number of proliferating dark, no GFP, *adoR*-less lamellocytes and green, GFP shining, wild, AdoR expressing lamellocytes.

Lamellocytes:	**With GFP expression**	**Without GFP expression**	**Total number**
Control	201	93	294
***adoR* mutant**	230	102	332
Sum	431	195	626

In the table III are noticed parameters confirming our hypothesis that *adoR* presence plays no role in number of proliferating lamellocytes (Fisher test, P value = 0,8627, (alpha < 0.05)); these results have been graphical captured with help of GraphPad Prism software (Fig. 16) and Statistica 13 (Fig. 17).

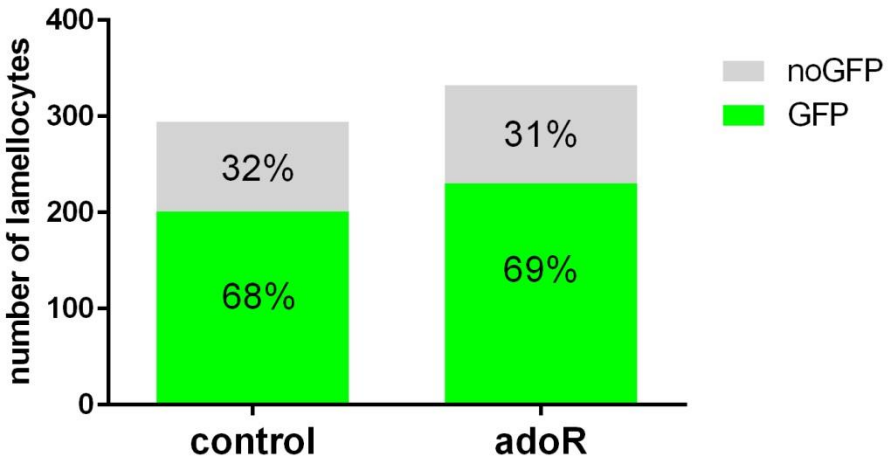


Fig. 16: Rate of proliferating (no GFP/GFP) lamellocytes within control compared to the rate of proliferating no GFP/GFP lamellocytes in *adoR* mutant larvae.

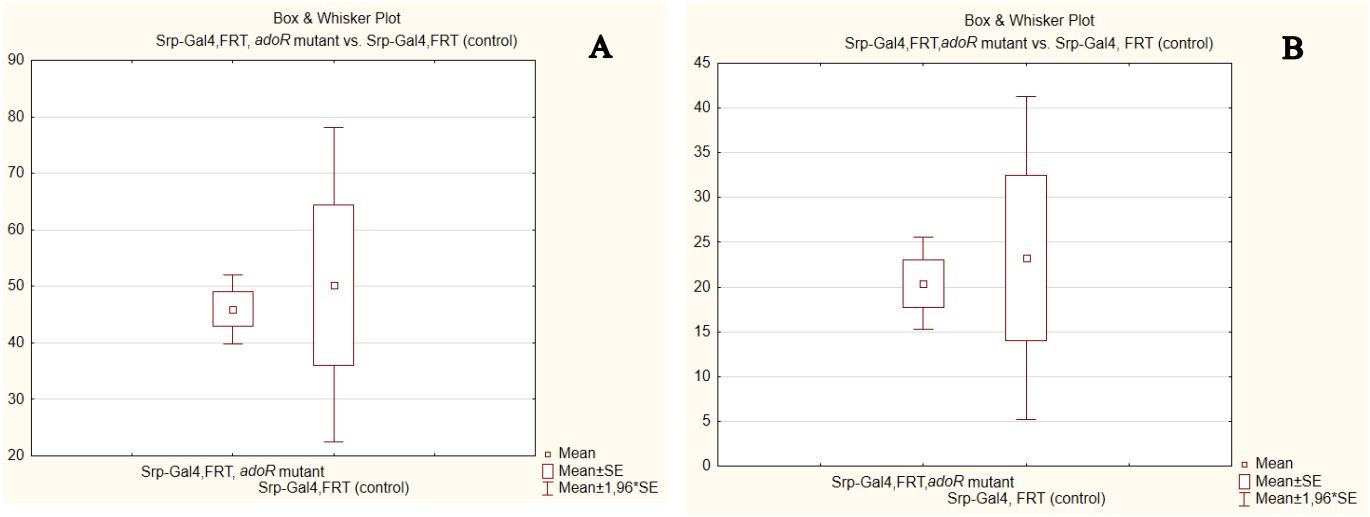


Fig. 17: Comparison of GFP shining lamellocytes numbers (t-test, P value = 0,752736, (alpha < 0.05), df =7) (**A**) and numbers of not GFP shining lamellocytes (t-test, P value = 0,750613, (alpha < 0.05), df =7) (**B**); *adoR* mutant against Srp-Gal4, FRT control.

In both cases, the rate of clone formation missing GFP expression (either wild-type or *adoR* mutant) was approximately 30%. By comparing of both rates of clone formation in t-test within 5 independent experiments (Fig. 16) and in Fisher´s exact test (Fig. 17), when all counting were combined, no significant difference in the formation rates has been demonstrated.

5. Discussion

To answer the main question of this project, if there is a cell-autonomous role of adenosine receptor signalling on immune cells, we tested a Flipase-induced mitotic recombination in hematopoietic lineage that would produce somatic *adoR* mutant clones.

5.1 Establishment of wild-type and mutant clones in hematopoietic lineage

There are several ways how to induce mitotic recombination within somatic lines. Employment of Gal4-UAS system coupled to tissue-specific Gal4 driver allows us target the mitotic recombination to specific somatic cell lines. According to our knowledge it has not been employed for the hematopoietic cell lineage yet, so we first had decided to test hematopoietic-specific induction of mitotic recombination.
This was important in case we wanted to check the potential cell-autonomous role of AdoR signalling in immune cells and not to affect other tissues.

Systemic AdoR signalling was shown to be important for rapid proliferation and differentiation of lamellocytes (Bajgar et al. 2015). However, thus affecting other tissues might have complicated the AdoR signalling testing.

We used Srp-Gal4 driver which is expressed in the hematopoietic lineage (throughout its development) and is coupled with UAS-Flipase to produce somatic clones (marked by GFP, present on chromosome III with FRT site).
We demonstrated that this system induces mitotic recombination in ca. 60% of hemocytes. 1/3 of hemocytes become GFP-negative clones, on contrary the rest is represented by sister clones with one or two GFP copies, which are unfortunately hard to distinguish from each other. Nevertheless, these results demonstrate quite high efficiency of recombination induced by our system.

5.2 The difference between proliferating types of lamellocytes

To study the importance of the adenosine receptor signalling in immune cell, we used the same Srp-Gal4, UAS-FLP system and added the *adoR* mutation. We had to combine three constructs, Srp-Gal4, FRT site and the *adoR* mutation to chromosome III. Subsequently it was tested, whether all 5 designed experimental lineages carry all needed

genetic constructs. As the resulting fluorescence of larvae tissues showed, all tested lineages carried Srp-Gal4 construct (Fig. 6). Thanks to successful larval development on Neomycin present in diet, we were assured, that even FRT construct hasn´t been missing. The presence of *adoR* mutation construct was successfully proved by PCR reaction (Fig. 7-9).

To test the role of AdoR in lamellocytes production, third-instars with the *adoR* mutant clones were infected by parasitoid wasps. We compared the numbers of proliferated lamellocytes to control carrying no *adoR* mutation.

The number of proliferated lamellocytes, missing adenosine receptor, hasn´t changed dramatically (compared to the wild-type lamellocytes, as the statistic confirmed), which supported our entrance hypothesis, that presence of adenosine receptor doesn´t influence significantly the fruitfulness of prohemocytes self-activation.

Because it was difficult to recognize lamellocytes carrying two copies of GFP marker (sister clone of the *adoR* homozygous mutant, GFP-negative lamellocytes) from the heterozygous non-recombinant lamellocytes carrying one GFP copy, we had to compare the percentage of the adoR-mutant clones to the rate of wild-type GFP-negative clones obtained from animals not carrying adoR mutation.

Ideally, if we could recognize lamellocytes with one and two GFP copies, we could compare numbers of the adoR mutant clones with the number of their sister clones within one animal and these numbers should be the same if there is no AdoR signalling cell-autonomous effect on production of lamellocytes.

6. Conclusion

Firstly, my work gave a proof about the possibility and simplicity of wild-type and mutant clone-establishment in one specific cell line. Using UAS-Gal4 system combined with FRT-Flipase activation can facilitate mutation studying, because it enables to compare the mutated and wild-type phenotype within one living organism, under uniform conditions.

Secondly, the difference between numbers of *adoR* and control lamellocytes hasn´t been showed as a significant, which means that adenosine receptor doesn´t play any important part in lamellocytes production during parasitoid wasp infection.

In contrary, it seems that AdoR presence is required on ambient tissue surfaces, where it must be prepared to react with extracellular signals to induce a metabolic switch. This fact is important for further investigation to the questions, what kind of immune activating metabolic pathways is adenosine molecule involved in.

The list of used acronyms

ATP	Adenosine triphosphate molecule
Ado	Adenosine molecule
e-Ado	Extracellular adenosine molecule
AdoR	Adenosine receptor
Ado–signalling	Adenosine signalling
adoR	The mutation of adenosine receptor
Srp	Serpent gene in *D. Melanogaster* - transcriptional activator
UAS	Upstream Activating Sequences
Gal4	Yeast protein of 830 Amino Acids. - transcriptional activator
FLP	Flipase, lipid enzyme - transmembrane transporter
FRT	Short Flipase recognition target
GFP	Green Fluorescent Protein
PCR	Polymerase Chain Reaction

7. References

1. Bajgar, A., Kucerova, K., Jonatova, L., Tomcala, A., Schneedorferova, I. et al. (2015). Extracellular Adenosine Mediates a Systemic Metabolic Switch during Immune Response. *PLOS Biology*. 13(4): e1002135.

2. Blair, S. (2003). Genetic Mosaic Techniques for Studying Drosophila. *Development*. 130(21):5065-72.

3. Brand, A.H., Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 118(2): 401-415.

4. Buch, S., Melcher, C., Bauer, M., Katzenberger, J., and Pankratz, M.J. (2008). Opposing effects of dietary protein and sugar regulate a transcriptional target of Drosophila insulin-like peptide signalling. *Cell Metabolism*. 7 (4), 321–332.

5. Buck, L.T. (2004). Adenosine as a signal for ion channel arrest in anoxia-tolerant organisms. *Comparative Biochemistry and physiology, Part B, Biochemistry and Molecular Biology*. 139(3), 401–414.

6. Cooper, GM. (2000). Recombination between Homologous DNA Sequences. *The Cell: A Molecular Approach.* (2nd edition). Sunderland (MA): Sinauer Associates. ISBN-10: 0-87893-106-6

7. Delmastro-Greenwood, M.M., and Piganelli, J.D. (2013). Changing the energy of an immune response. *American journal of clinical experimental immunology*. 27;2(1):30-54.

8. Dolezal, T., Dolezelova, E., Zurovec, M., and Bryant, P.J. (2005). A role for adenosine deaminase in Drosophila larval development. *PLOS Biology 3(7),* e201.

9. Dolezal T. (2015). From Drosophila model to human disease and back again. *Habilitation thesis*. Faculty of Science, University of South Bohemia in Ceske Budejovice, Ceske Budejovice, Czech Republic.

10. Dolezelova, E., Nothacker, H.-P., Civelli, O., Bryant, P.J., and Zurovec, M. (2007). A Drosophila adenosine receptor activates cAMP and calcium signaling. *Insect Biochemistry and Molecular Biology*. 37(4):318-29.

11. Duffy, J. B. (2002). Gal4 system in Drosophila: a fly geneticist´s Swiss army knife. *Genesis*. 34(1-2): 1-15.

12. Fenckova, M., Hobizalova, R., Fric, Z.F., and Dolezal, T. (2011). Functional characterization of ecto-5'-nucleotidases and apyrases in Drosophila melanogaster. *Insect Biochemistry and Molecular Biology*. 41(12):956-67.

13. Fredholm, B.B., Irenius, E., Kull, B., Shulte, G. (2001). Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochemical Pharmacology*. 61(4):443-8.

14. Fredholm, B.B. (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death and differation*. 14(7):1315-23.

15. Fredholm, B. B. (2010) Adenosine receptors as drug targets. *Experimental Cell Research*. 316 (8), 1284–1288.

16. Giniger E., Varnum S.M., Ptashne M. (1985). Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell.* 40(4):767-74.

17. Greenspan, R. J. (2004). *Fly pushing: the theory and practice of Drosophila genetics*. (2nd edition). Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. ISBN 0879697113.

18. Hasko G., Cronstein B. (2013). Regulation of Inflammation by Adenosine. *Frontiers in Immunology*. 4(85): eCollection 2013

19. Cheng, S.-C., Quintin, J., Cramer, R.A., Shepardson, K.M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H.A., Rao, N.A., Aghajanirefah, A., et al. (2014). mTOR- and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. *Science.* 345(6204):1250684

20. Kakidani H., Ptashne M. (1988). GAL4 activates gene expression in mammalian cells. *Cell*. 52(2):161–167.

21. Kucerova, L., Broz, V., Fleischmannova, J., Santruckova, E., Sidorov, R., Dolezal, V. and Zurovec, M. (2012). Characterisation of the Drosophila Adenosine Receptor: The Effect of Adenosine Analogs on cAMP Signalling in Drosophila Cells and Their Utility for in Vivo Experiment. *Journal of Neurochemistry.* 121(3):383-95

22. Latini, S., Bordoni, F., Pedata, F., Corradetti, R. (1999). Extracellular adenosine concentrations during *in vitro* ischaemia in rat hippocampal slices. British Journal of Pharmacology. 127(3):729–739.

23. Laughon, A., Gesteland, RF. (1984). Primary structure of the Saccharomyces cerevisiae GAL4 gene. *Molecular and Cellular Biology*. 4(2): 260–267.

24. Maier, S.A., Galellis, J.R., and McDermid, H.E. (2005). Phylogenetic Analysis Reveals a Novel Protein Family Closely Related to Adenosine Deaminase. *Journal of Molecular Evolution*. 61(6):776-94.

25. Novakova, M. (2011). Regulation of extracellular adenosine level by ADGF-A in *Drosophila melanogaster*. *Ph.D. Thesis*, Faculty of Science, University of South Bohemia in Ceske Budejovice, Ceske Budejovice, Czech Republic.

26. Xu, T., Rubin, M.G. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development*.117: 1223-1237.

27. Zurovec, M., Dolezal, T., Gazi, M., Pavlova, E., and Bryant, P.J. (2002). Adenosine deaminase-related growth factors stimulate cell proliferation in Drosophila by depleting extracellular adenosine. *Proceedings of the National Academy of Science of the USA*. 99(7):4403-8.

28. Zuberova, M., Fenckova, M., Simek, P., Janeckova, L., and Dolezal T. (2010). Increased extracellular adenosine in Drosophila that is deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. *Disease Model and Mechanisms*. 3(11-12): 773–784.