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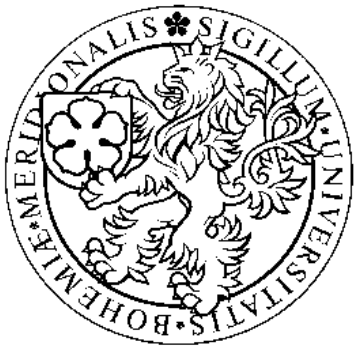

Study of Ectonucleotidases and Adenosine Deaminases in *Drosophila*

Bachelor thesis

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Annotation

Extracellular adenosine triphosphate and extracellular adenosine are important regulatory molecules in the human immune system. The concentrations of these molecules are in turn regulated by ectonucleotidases and adenosine deaminases. In this thesis I attempt to test the gene silencing efficiency of RNA interference for three different genes coding for such enzymes in the model organism *Drosophila melanogaster*.

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Linz, 15.08.2013

Preuer Kristina

Acknowledgement

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Abstract

Extracellular adenosine triphosphate (e-ATP) and extracellular adenosine (e-ado) play an important role as signalling molecules modulating immune response. High concentrations of e-ATP are acting pro-inflammatory whereas its counterpart e-ado is down regulation excessive immune response. The levels e-ATP and e-ado are regulated by enzymes called ectonucleotidases and adenosine deaminases, respectively.

Drosophila melanogaster possesses genes coding for proteins which have the same enzymatic function. Silencing these genes by RNA interference (RNAi) is one of the possible approaches to investigate the role of the two regulatory purine molecules in the signaling pathway of the immune system.

In this study we tested the efficiency of RNAi induced gene silencing by expression reporters, quantitative real time PCR and showed that the RNAi is highly efficient in knocking down the NT5E-1 ectonucleotidase gene by reducing the levels of RNA to 2.5 % compared to the corresponding control. Furthermore we observed the spatial localization of NT5E-1 close to the cell surface by confocal microscopy, supporting the findings of a previous study that this enzyme is anchored to the cell membrane.

Abstrakt

Extracelulární ATP (e-ATP) a extracelulární adenozin (e-Ado) hrají důležitou úlohu v imunitní odpovědi. Zvýšené koncentrace e-ATP vyvolávají zánětlivou odpověď, zatímco e-Ado naopak tlumí přílišnou imunitní reakci. Hladiny e-ATP a e-Ado jsou regulovány enzymy ektonukleotidázou a adenozin deaminázou.

Drosophila melanogaster je nositelkou genů, které kódují proteiny se stejnou enzymatickou funkcí. Umlčení těchto genů pomocí RNA interference (RNAi) je jedním z možných přístupů pro studium signalizačních úloh těchto dvou purinových molekul v imunitním systému.

V této práci jsme testovali efektivitu umlčení metodou RNAi pomocí expresních reportérů, kvantitativní Real-Time PCR a ukázali jsme, že RNAi je vysoce efektivní při umlčení ektonukleotidázy NT5E-1 redukcí hladiny její RNA na 2,5 % v porovnání s příslušnou kontrolou. Dále jsme ověřili pomocí konfokální mikroskopie lokalizaci NT5E-1 na buněčném povrchu, čímž jsme podpořili předchozí zjištění o lokalizaci tohoto enzymu.

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1. Introduction

1.1Adenosine Signalling Pathway

Already in 1929 Drury and Szent-Györgyi recognized the signalling potential of adenosine compounds, when studying there effects on the mammalian heart muscle. Adenosine and adenosine triphosphate are generally present at low physiological concentrations in the extracellular space. In general studies showed that in humans extracellular adenosine (e-ado) has approximately 10-fold lower concentrations then extracellular adenosine triphosphate (e-ATP), 40–80 nM and 400–700 nM respectively (as reviewed in Bours et al. 2006). However, during tissue damage, inflammation, ischemia or hypoxia these concentrations are rising rapidly (Bodin, Burnstock 1998; Latini, Pedata 2001; Lazarowski et al. 2003). Adenosine can either be released by the cell itself or is a product of the degradation of e-ATP (Yegutkin 2008). The degradation of e-ATP to e-ado is shown in Fig. 1.

1.1.1 Extracellular Adenosine Triphosphate

E-ATP and e-ado are important regulatory molecules acting through purinergic receptors initiating signalling pathways. High concentrations of e-ATP cause different actions in mammals important for the immune system. They lead to the differentiation of T helper 17 cells (Atarashi 2008), which for example play a role in inflammatory bowel disease (Raza et al. 2012). Additionally e-ATP chemotactically attracts macrophages (Elliott 2009) as well as immature dendritic cells (Idzko et al. 2002). Whereas low concentrations of e-ATP lead to down regulation of pro-inflammatory cytokines, release of immune-suppressive cytokines and distorted maturation of dendritic cells (La Sala et al. 2001, Wilkin et al. 2002)

ATP is dephosphorylated through ecto-nucleotidases to generate adenosine. First, cluster of differentiation (CD) 39 hydrolyzes ATP to ADP and subsequently to AMP. Finally, the last phosphate group of AMP is cleaved by CD 73 to yield the nucleoside. (Wang et al. 1996, Zimmerman 1992).

1.1.2 Extracellular Adenosine

In mammals adenosine can bind to four different transmembrane bound adenosine receptors, which are coupled to GTP-binding proteins, A1, A2A, A2B and A3. E-ado is mainly acting via the A2A receptor (Ohta, Sitkovsky 2001). Elevated levels of the nucleoside, in contrast to higher levels of the nucleotide, lead to the inhabitation of T-cell activation, proliferation and expansion, having therefore an immunosuppressive effect (Huang et al. 1997, Deaglio 2007).

Adenosine levels are controlled by adenosine deaminase (ADA). ADA is an enzyme which is catalyzing the deamination reaction of adenosine to inosine (Conway, Cooke 1939). The enzyme can be present in at least three different forms, ADA1, ADA1+CP and ADA2. ADA1 is the monomeric form, ADA1+CP is a dimer of ADA1 which is connected by a third protein (Hirschhorn, Ratech 1980). The ADA2 dimer is present in various tissues, however its contribution to the total ADA activity is lower compared to the two other forms (Van der Weyden, Kelley 1976). ADA2 belongs to the family of adenosine deaminase related growth factors proteins (ADGF). Zavialov et al. (2005) suggested that ADA2 is a specialized form of ADA, which is activated in sites of inflammation or tumourigenesis, where eleveated adenosine levels and acidic pH are present.

As the fine tuning of our immune system is essential, malfunctioned regulation of the above described pathway can lead to several diseases and syndromes for example a lack of adenosine deaminase is a cause of severe combined immunodeficiency syndrome (Zhang 2008).

However, due to the complexity and variety of pathways in the human body where e-ATP and e-ado are involved, it is a useful approach to first study these important molecules in a simplified model like *Drosophila melanogaster*.

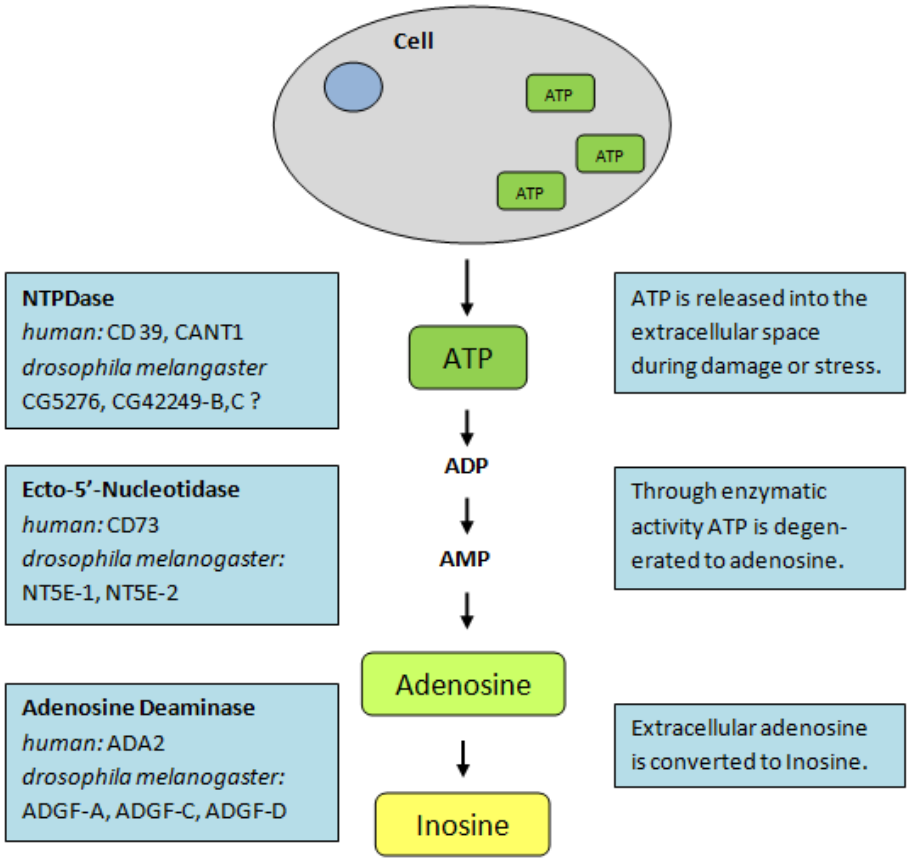


Figure 1: Degradation of extracellular ATP

1.2 Drosophila Melanogaster

The great potential of *Drosophila melanogaster* serving as a model organism was firstly recognized by Thomas Hunt Morgan. He experimentally proofed the chromosome theory of inheritance with his work on *Drosophila melanogaster* white-eye mutants (Morgan 1910). *Drosophila melanogaster* is the model organism of choice for many scientists, because of several reasons – fast and prolific reproduction, only 4 chromosomes, cheap and easy breeding, well known and sequenced genome to name but a few. One of the most important features of a model organism is its comparability to humans. Although there are other model organisms which are closer related to humans, however, there are many mechanisms which are conserved also between invertebrates and vertebrates. Approximately 75 % of human diseases genes match *Drosophila melanogaster* sequences (Reiter et al. 2001), therefore making it such a strong model for research. Additionally due to the long tradition of using fruit flies as a model organism a wide pool of free information as well as a large array of genetic and molecular methods are available.

1.2.1 Balancers and Marker

One of the most important genetic tools are so-called balancers. Balancer chromosomes suppress meiotic recombination between homologous chromosomes. For easy identification of the presence of a balancer chromosome certain markers are used. Markers are dominant mutations with an easily observable phenotype. Additionally balancers carry recessive lethal or recessive sterile mutations, which prevent losing the mutation of interest in a stock. There are balancers available for 3 drosophila chromosomes; the fourth chromosome does not undergo meiotic recombination, therefore no balancer is needed. Curly of Oster (CyO) and TM6B are two typically used balancers for the second and third chromosome, respectively. CyO leads to a bended wings (curly) and larvae carrying the TM6B chromosome can be visually identified by their short tubby shape. There are a wide variety of genetic markers available effecting leading to different phenotypes. Typical used markers are: Scutoid (Sco - no bristles on the scutellum), Humeral (Hu - extra hair on the humerus), Stubble (Sb - shortened hair on the back), Serrate (Ser - notched wing tips), ebony (e - black or dark brown body), Tubby (Tb – shortened and tubby larvae and pupae). Markers can be combined if they are not interfering, Humeral for example cannot be observed when Scutoid is present.

1.2.2 Ecto-5'-Nucleotidases

In *Drosophila melanogaster* there are two proteins which possess both cell surface and secreted 5'-nucleotidase activity, NT5E-1 and NT5E-2. These proteins share a similarity of around 40 % amino acid sequence identity to human ecto-5'-nucleotidase CD73. Sequence analysis and detected nucleotidase activity on the cell surface suggest that these proteins are attached to the cell surface by glycosyl phosphatidylinositol (GPI). However, these proteins can also be released from the membrane presumably by phospholipases (Fenckova et al. 2011).

1.2.3 Adenosine Deaminase-Related Growth Factors

Drosophila melanogaster possesses 6 *ADGF* genes (Maier et al. 2001). Significant similarity to ADA can be observed for the predicted ADGF proteins, especially in the amino acid sequences, which are necessary for the catalytic function of ADA. It was possible to proof ADA activity for ADGF-A and ADGF-D. (Zurovec et al. 2001). However, it has to be mentioned that ADGF-A has a higher affinity for adenosine compared to human ADA2 (as cited in Novakova et al. 2011).

1.3 Gene Inactivation

1.3.1 RNA interference

In 2006 the Nobel Prize in Physiology or Medicine 2006 was awarded to Andrew Z. Fire and Craig C. Mello for their discovery of RNA interference (RNAi) - gene silencing by double-stranded RNA in the nematode *Caenorhabditis elegans.* However, in this organism RNAi is systemic (Fire et al. 1998), whereas in *Drosphila melanogaster* RNAi is cell autonomous (Roignant et al. 2002). Dietzl et al. (2007) generated a genome-wide transgenic RNAi library for conditional gene activation in Drosophila. Using a long double stranded hair-pin RNA transcribed from a UAS-GAL 4 system (*see chapter 1.3.2*) driven transgene containing inverted repeats makes it possible to inactivate nearly every desired gene in any cell at any stage of life. The double stranded RNA is cut by dicers into small interfering RNAs (siRNA), which then bind to the target mRNA and cause degradation by the RNA induced silencing complex.

1.3.2 UAS-GAL 4 System

In 1993 Brand and Perrimon have developed a potent system for targeted gene expression. This system is based on the two components; an upstream activation sequence (UAS) and a transcriptional activator from yeast (GAL 4). Although having yeast origin, Fischer et al. (1988) showed that GAL 4 is able to activate transcription in Drosophila. Therefore two transgenic fly lines are used. One of them contains the target gene and the GAL 4 binding sites. The second one is the activator expressing line. Unless these lines are crossed, no binding will occur and therefore the target gene will remain silent. GAL 4 expression itself is regulated by an endogenous promoter, which can make the expression tissue or cell specific, for example the actin promoter, which leads to a global expression of GAL 4.

1.3.3 Enhancement of RNAi

In RNAi several different proteins and enzymes are involved. Dietzl et. al (2007) tested also the effects of the over-expression one of these components for potential enhancement of the gene silencing. Out of the tested factors (dicer-1, dicer-2, argonaute-1, argonaute-2, R2D2 and tudor-SN), dicer-2 turned out to be the only factor capable of consistently increasing the RNAi effect. However, it has to be mentioned that using UAS-Dicer 2 also might enhance off target silencing.

1.3.4 Visualizing Protein Levels

For visualizing protein levels in vivo and in vitro it is one possible method is to fuse the protein of interest with the green fluorescence protein (GFP) of the jellyfish *Aequorea victoria*. For generating a tagged protein, the GFP gene, which was first cloned and sequenced by Prasher (1992), is inserted next to the coding sequence of the target protein. Radoslaw et al. (2009) produced a genomic fosmid library for *Drosopila melanogaster* which can be used together with high throughput recombineering and direct transgenesis to obtain fly lines possessing tagged proteins. When the fused GFP is excited with ultra violet light, it emits green light in the visible range (Shimomura et al. 1962) and therefore making it a marker to monitor protein expression spatially and temporally.

1.4 Aims of the Thesis

The aims of this thesis were to test the possibility of down regulation using RNAi for ADGF-A, NT5E-1 and NT5E-2 proteins fused to GFP by confocal microscopy and to subsequently quantify the results using quantitative real time polymerase chain reaction (qRT-PCR). For ADGF-A and NT5E-1 additionally the effect of UAS-Dicer 2 should be investigated and quantified as well.

For testing the RNAi effect first of all it was necessary to generate flies carrying the required mutations. For ADGF-A and NT5E-2 four new stocks had to be generated (Table 1). Out of the generated and already existing stocks flies were crossed to obtain progenies carrying, sequences for the expression of the tagged protein, double stranded RNA and the GAL 4 protein. For the enhancement experiments the genome of the progeny additionally had to contain a sequence coding for the GAL 4 driven expression of the dicer protein.

Table 1 Genotypes of the stocks generated for the experiments of ADGF-A and NT5E-2

ADGF-A	NT5E-2
pFlyFos-Adgf-A TM6B / CyO GFP ; act-GAL 4	pFlyFos-NT5E-2 TM6B / CyO GFP ; act-GAL 4
CyO GFP TM6B / UAS DCR ; ADGF-A RNAi	NT5E-2 RNAi TM6B / CyOGFP ; UAS DCR

2. Materials and Methods

2.1 Maintaining Drosophila Culture

Flies were raised on a standard cornmeal diet. The food contained of 120 g cornmeal, 75 g sacharose, 60 g instant yeast, 15 g agar and 1.5 L water and was supplemented with 25 mL of 10% methylparaben in ethanol. The flies were kept at room temperature and the diet was changed in regular intervals.

Table 2 Used stocks with corresponding internal stock number, description and genotype

Int. Nr.	Description	Genotype
1278	UAS-DCR2	UAS-DCR/CyO
1279	UAS-DCR2	UAS-DCR/TM3, Sb, e
1045	ADGF-A RNAi	w; P{GD17237}v50426
1315	NT5E-2 RNAi	P{KK107939}VIE-260B
1332	pFF-NT5E-2:GFP	yw; pFlyFos{030888}-NT5E-2:GFP-attP40/CyO
1296	pFF-ADGF-A:GFP	yw; pFlyFos{021854}-Adgf-A:GFP-attP40/CyO
606	Sco/CyO; adgf-a^{karel}	w; Sco/Cyo; adgf-a^{karel}/TM6B, Hu, Tb, e
1295	cactus; act-GAL 4	cactus/CyO, GFP; act-GAL 4/TM3, GFP, Ser, e
1337	pFF-NT5E-1:GFP & NT5E-1 RNAi	w; pFlyFos{027290}-NT5E-1:GFP-attP40/CyO; P{GD5294}v49358/TM6B, Hu, Tb, e
1221	white eye mutant	w
565	UAS-GFP	yw; P{w$^{+mW.hs}$=UAS-GFP}

2.2 Generating Stocks for the Experiments with ADGF-A and NT5E-2

For setting up the stocks which are necessary for the experiments the flies were crossed according to a prepared scheme. Figures 2-5 show the crossing tables (genotype of the second and third chromosome of the crossed flies and all possible genotypic combinations of the progeny) for the generation of the stocks needed for ADGF-A RNAi, ADGF-A RNAi with enhancement using dicer over-expression, NT5E-2 RNAi and NT5E-2 RNAi with enhancement using dicer over-expression. For each cross female virgins were selected to ensure that the female flies only mate with males possessing the required genotype. As flies start to mate approx. 6-8 hours after ecolsion, the stock vials were emptied and every 4 hours all emerged flies which additionally showed the typical light colour and had a meconium (dark spot on the abdomen – waste products which are secreted with the first defecation) were selected and assumed to be virgin. For each cross the ratio of male : female flies was approximately 1:4. In the cases where it was possible, homozygous flies were used, as it reduces the number of possible genotypic combinations.

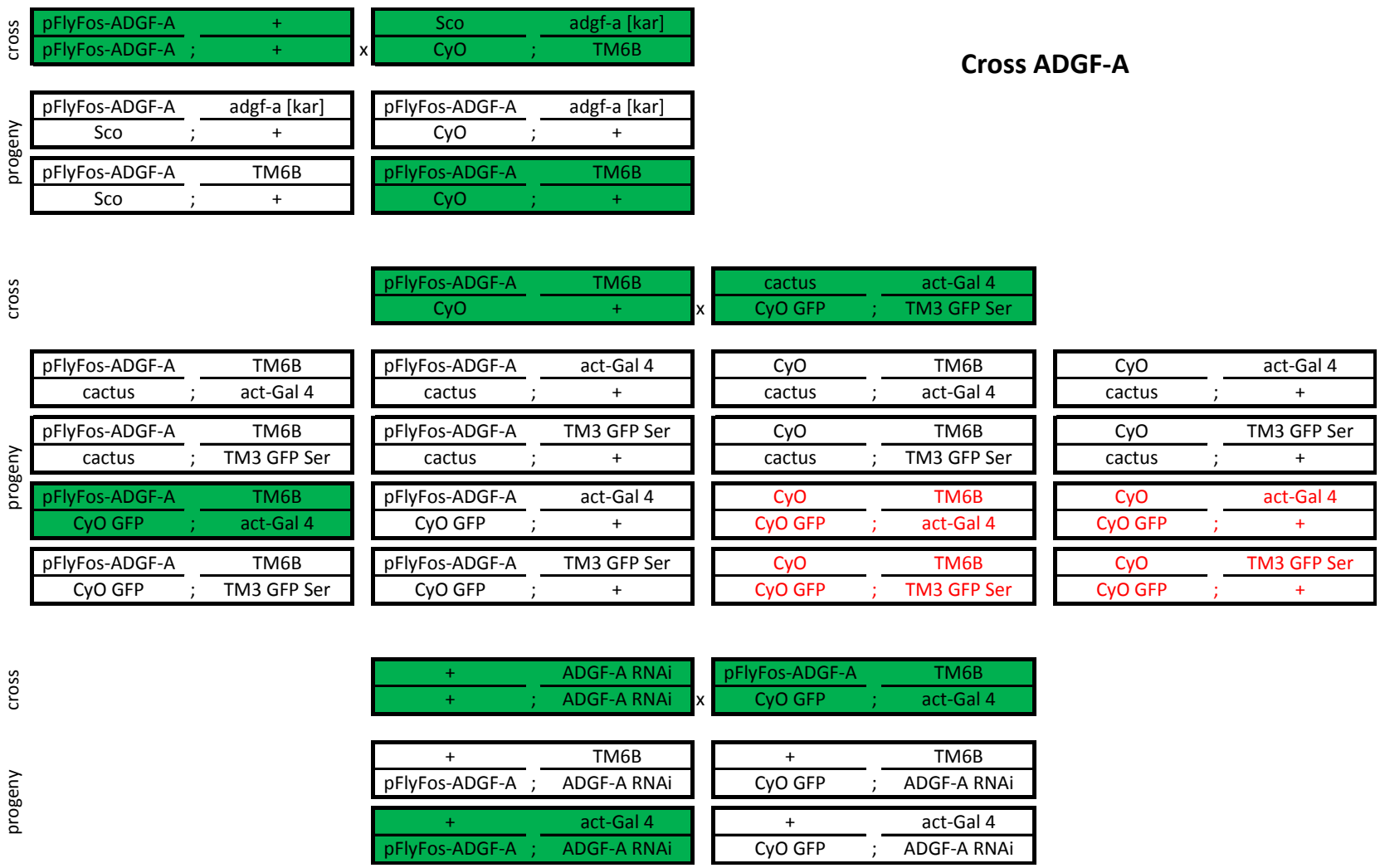


Figure 2 Crossing Scheme for the preparation of the stock containing the sequences for the GFP-tagged ADGF-A protein and the actin GAL 4 driver. These stock flies are then crossed with flies containing the sequence coding for the double stranded RNA, which is silencing the ADGF-A gene. The plus sign stands for the wild type allele. Green shaded boxes show the genotype of the fly of interest. Genotypes written in red are non viable.

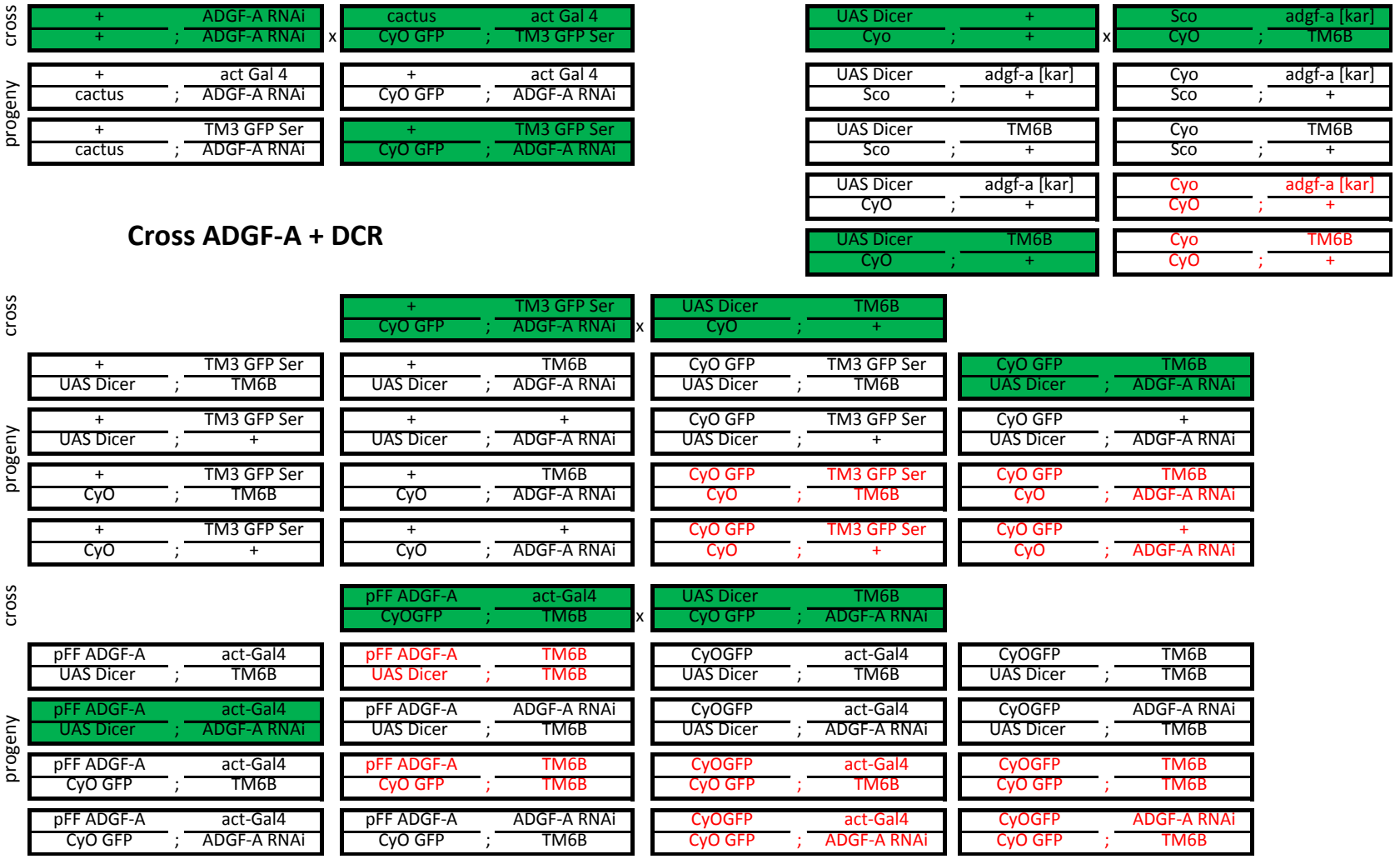


Figure 3 Crossing Scheme for the preparation of the stock containing the sequence coding for the dicer protein and the double stranded RNA, which is silencing the ADGF-A gene. These stock flies are then crossed with the stock flies containing the sequence for the GFP-tagged ADGF-A protein and the actin GAL 4 driver. The plus sign stands for the wild type allele. Green shaded boxes show the genotype of the fly of interest. Genotypes written in red are not viable.

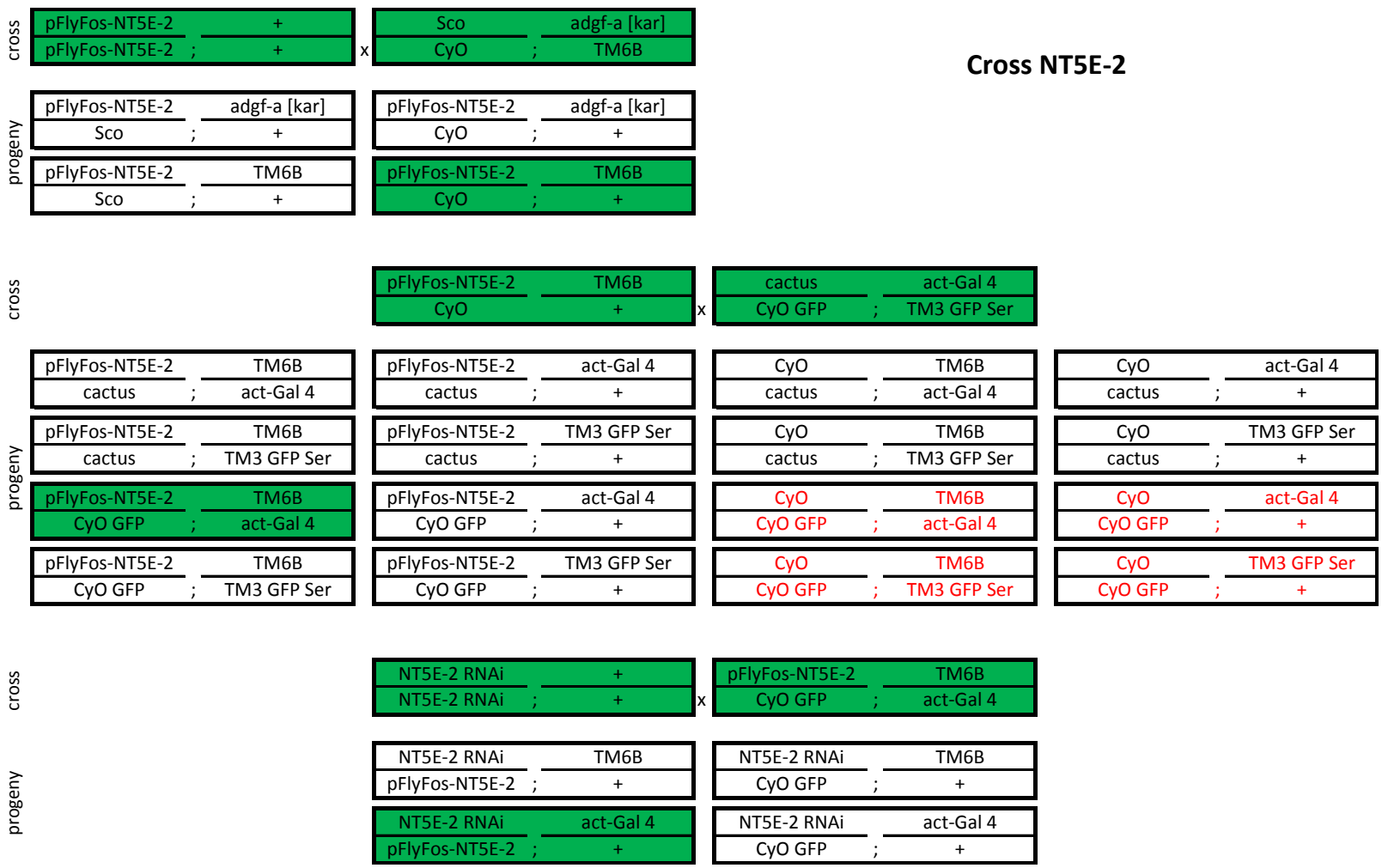


Figure 4 Crossing Scheme for the preparation of the stock containing the sequences for the GFP-tagged NT5E-2 protein and the actin GAL 4 driver. These stock flies are then crossed with flies containing the sequence coding for the double stranded RNA, which is silencing the NT5E-2 gene. The plus sign stands for the wild type allele. Green shaded boxes show the genotype of the fly of interest. Genotypes written in red are non viable.

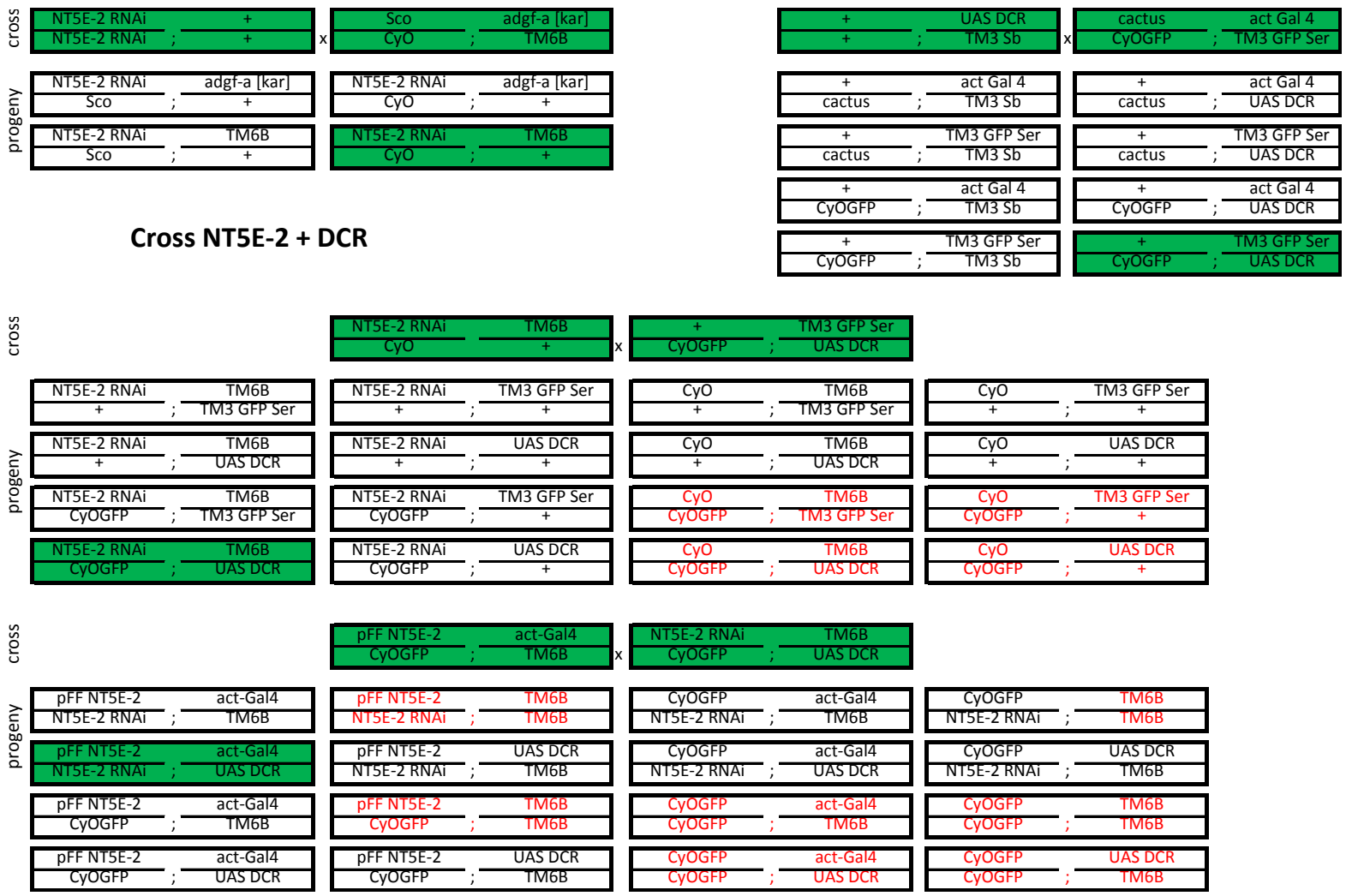


Figure 5 Crossing Scheme for the preparation of the stock containing the sequence coding for the dicer protein and the double stranded RNA, which is silencing the NT5E-2 gene. These stock flies are then crossed with the stock flies containing the sequence for the GFP-tagged NT5E-2 protein and the actin GAL 4 driver. The plus sign stands for the wild type allele. Green shaded boxes show the genotype of the fly of interest. Genotypes written in red are not viable.

2.3 Cross NT5E-1

There existed already a stock which possessing the sequence for the GFP-tagged NT5E-1 protein and the UAS regulated RNA. This stock was then crossed to a fly which contained the gene for the actin GAL 4 driver. As a control, the stock was additionally crossed to a stock containing no actin GAL 4 driver.

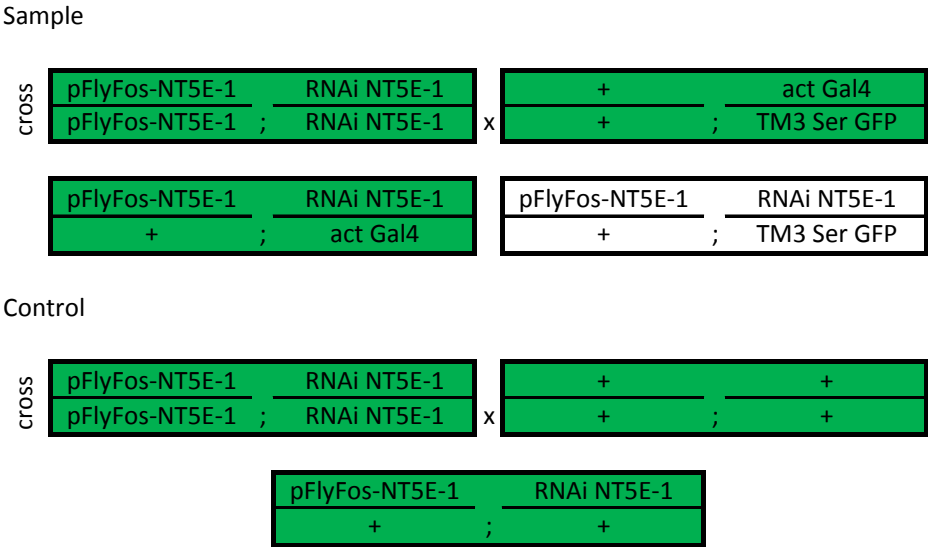


Figure 6 Crossing Scheme to produce the sample and the control fly for the experiment with NT5E-1

2.4 Confocal Microscopy

2.4.1 Dissection and Staining

For the confocal microscopy the salivary gland of third instar larvae were used, because NT5E-1 is highly expressed in these tissues during larval development (Fenckova et al. 2011) and serve as an ideal tissue for observation of RNAi effect. Third instar larvae were collected and dissected under cold ringer solution. Dissected samples were mounted on microscopic glass and mounted with mounting medium containing DAPI (unspecific staining of nuclei) (Aqua polymount, Fisher scientific). Pictures were then made by using confocal microscope (FluoView 1000 confocal microscop, Olympus). All the pictures were taken under the same conditions, therefore we are able to compare the amount of fluorescent signal among samples.

2.5 Quantification with qRT-PCR

2.5.1 RNA isolation

For RNA isolation third instar larvae (approx. 5 days old larvae) were used. In each extraction 5 larvae were taken. The extraction was performed 4 times for the sample and the control. The larvae were put into a microtube and homogenized in 200 µL of TRIzol reagent (vitrogen). Additional 800 µL of Trizol reagent were added and the sample was left for 5 min. at room temperature (RT). 200 µL of chloroform are added and the samples are vortexed for 15 – 30 s. After spinning for 15 min. at 4 °C and a speed of 14,000 rpm, the upper aqueous phase is taken and collected in a new microtube. 500 µL of 100% isopropanol are added and the samples were left for 10 min. on ice. After centrifugation for 10 min. at 4 °C with a speed of 14,000 rpm, the supernatant was discarded and the RNA pellet was washed with 75% ethanol. After further centrifugation for 1 min. under the same conditions like before, the ethanol was discarded. The remaining ethanol was evaporated (5-10 min at RT). According to the size of the RNA pellet an appropriate amount of DEPC water was then used to dissolve it.

2.5.1.1 Purification with DNAse (Ambion)

To the solution of dissolved RNA DNAse Buffer was added to obtain a 1-fold concentration. 1 µL of the Turbo DNAse enzyme was added and the solution was kept for 30 min at 37°C. After that 2µL of the inactivation reagent were added and the solution was kept for 5 min at RT. Finally the solution was spinned for 3 min. at RT with a speed of 14,000 rpm. The quality and concentration of the RNA was determined by Nanodrop (Thermo Scientific).

2.5.2 cDNA Synthesis

For synthesis of the cDNA, a mixture of 2 µL oligo dT primer, 4 µL dNTP, 5 µL of RNA solution (total amount up to 4µg) and 2 µL of DPEC water are put together in a microtube. The solution was heated up to 65°C for 5 min after gentle mixing. The solution was then immediately incubated on ice for 2 min. 4 µL of first strand buffer, 1 µL of DTT and 1 µL of Superscript™ III Reverse Transcriptase (Invitrogen) are added. The solution is gently mixed and incubated at 50°C for 60 minutes. After this time the reaction is stopped by heating up to 70°C and keeping this temperature for 15 min for enzyme denaturation.

2.5.3 qRT-PCR

For quantification of gene expression a certain amount of mRNA was quantified by using qRT-PCR. For this purpose, the gene specific primers (Trab. XX) were designed and iQ Syber-Green supermix was used (Ambion). Specificity of PCR product was tested by melting analysis. All the measurements were made on C1000 Touch Thermal cycler (BioRad) and analyzed with BioRad CFX Manager (BioRad). Amount of transcript were normalized by to expression of ribosomal protein 49 as a housekeeping gene.

Table 3 Primers used in 5‘ to 3‘ direction

NT5E-1	Fwd: GAATCCGTGGCTCCGTCG
	Rev: GCAGGGAAGAGAGCAGAAG
ribosomal protein 49	Fwd: GCTAAGCTGTCGCACAAATG
	Rev: GCGCGCTCGACAATCTCC

2.5.3.1 Program for qRT-PCR

As an initialization step 94 °C were kept for 3 minutes. Afterwards 40 cycles of denaturation of DNA (94 °C for 30 sec.), annealing of primers (56 °C for 35sec.) and elongation (72 °C for 45sec.) were performed. For final elongation a temperature of 72 °C was kept for 5 minutes. The melting analysis was carried out between 65 and 95 °C with steps of 0.5 °C.

3. Results and Discussion

3.1 ADGF-A and NT5E-2

The first milestone for the RNAi experiments of ADGF-A and NT5E-2 was to generate the required stocks. Observations of the set up stocks, which should contain the sequences for the GFP tagged protein and the actin GAL 4 driver, showed that there are larvae and flies which do not possess the Tb and Hu marker, respectively. Losing of the marker indicates that there flies which are homozygous for the actin GAL 4 driver. However, as these homozygous actin GAL 4 driver flies are usually not viable, we concluded that there might have been a problem during the crosses, which lead to a fly with a different genotype than required. Although the work was carefully done and the stock preparation was done in duplicate, it seemed to be the most reasonable explanation, as a single non virgin female or fly containing not the appropriate genotype would be enough to destroy the stock.

3.1.1 Rescue of the Stocks

To rescue the project, flies which are homozygous for the tagged protein and containing the TM6B balancer were again crossed to the stock fly 1295 (cactus/Cyo GFP; act-Gal4/TM3 GFP Ser). Before crossing the flies, the females were kept in vials for 2-3 days to verify their virginity. Additionally all the markers were double checked by other laboratory staff to be sure that only the desired flies are used. The rescue project leaded to the same unwelcome results – flies losing the TM6B balancer, indicated by the absence of the Hu and Tb marker.

3.1.2 Trouble Shooting

As the rescue project was also not successful, we suggested that most likely there is a different reason why the flies are losing the balancer. There are two basic considerations concerning the observed result – either the actin GAL 4 driver is present or not.

Assuming that the actin GAL 4 driver is present, then this outcome is only possible if, either the phenotypes of both markers are suppressed and therefore not visible although the balancer is present or that the combined mutations lead to a viable form of flies homozygous for actin GAL 4.

On the other hand if the GAL 4 driver is not present, there is the possibility that during the cross one of the markers did not show typical phenotype, leading to selection of wrong flies or that the actin GAL 4 driver was already missing in the original stock.

3.1.2.1 Absence of actin GAL 4

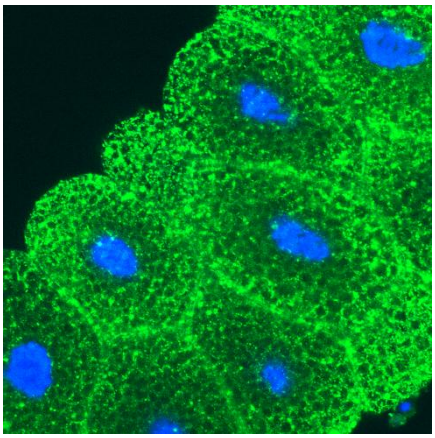
For the determination if the actin GAL 4 driver is present, we crossed flies of the generated stocks and the original stock with a fly line containing a GFP sequence which is under regulation of the UAS. These tests revealed that no actin GAL 4 was present neither in the generated stocks nor in the originals stock.

It seems that there is a different mutation present which is recessive lethal in combination with cactus but only sublethal in the newly generated stocks. The observation that larvae homozygous for this mutation need longer in development compared to larvae carrying the balancer as well, additionally suggest that this is a very damaging mutation as TM6B is already quite harmful itself.

Nevertheless we made no final determination what was the reason why setting up of the stocks was unsuccessful, because it was not the purpose of the thesis. It is advisable to simply use a different stock, which contains the actin GAL 4 driver, for the cross and to repeat the procedure. Unfortunately due to lack of time this could not be done in this thesis as the whole cross takes several weeks. However the generated stocks containing the sequences for the double stranded RNA and the dicer protein can be used as soon as the two other stocks are produced.

3.2 NT5E-1

3.2.1 Confocal Microscopy



From the pictures taken from the control it can be seen that there is a high abundance of NT5E-1 close to the cell surface, this supports the hypothesis of Fenckova et al. (2011) that these proteins are GPI anchored to the membrane. From this observation we can conclude, that anchoring of the protein in cellular membrane is useful for site specific regulation of ATP hydrolysis and generation of adenosine signal.

Figure 7 Expression of NT5E-1 fused to GFP in the control cells

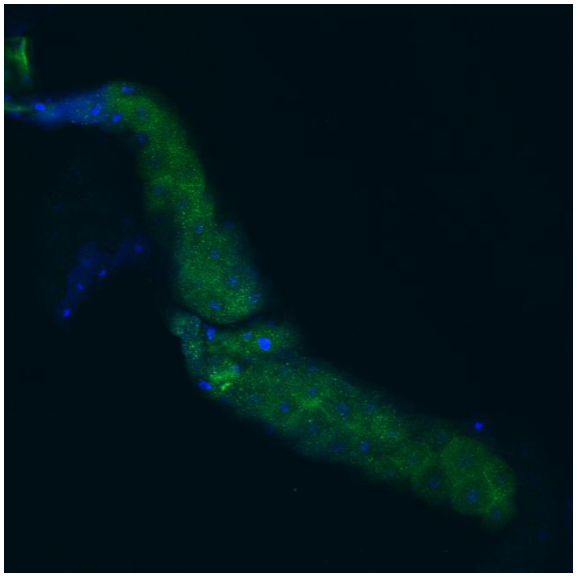


Figure 8 Expression of NT5E-1 fused to GFP in the salivary gland of the control

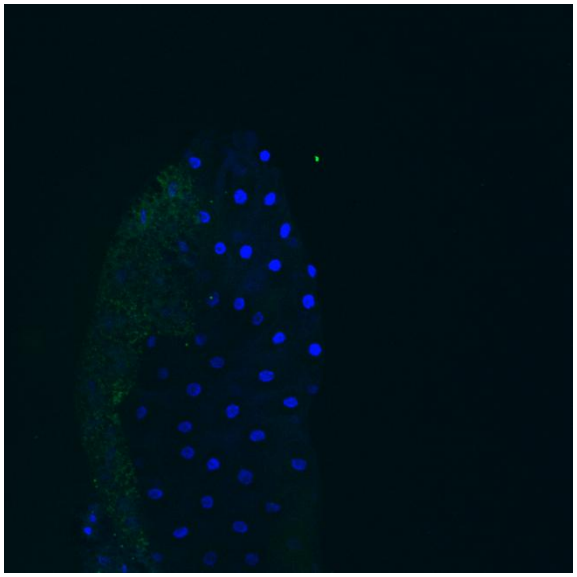


Figure 9 Expression of NT5E-1 fused to GFP in the salivary gland of the sample

In contrast to the picture taken from the sample, the green colour of the GFP can be easily observed in the control. Therefore, comparing the intensity of the GFP of the sample and the control indicates that silencing of the NT5E-1 gene on protein level with tested RNAi construct was highly efficient.

3.2.2 Quantification with qRT-PCR

The obtained data showed that the gene knock down with RNAi was highly efficient. The RNA levels were reduced to an average of 2.49 % compared to the controls. In the study of Dietzl et al. the efficiency of 64 RNAi lines were tested. The best results showed a reduction of 95 % compared to the corresponding controls. However in these studies adult flies were taken. Nevertheless several other studies (Chi and Dimario 2007; Ichimiya et al. 2004; Kavi and Birchler 2009) using third instar larvae showed also a lower efficiency compared to the results of this study. The RNA levels in these studies are approximately 20 – 30 % of the corresponding control, therefore, 10-fold less efficient than in the case of NT5E-1.

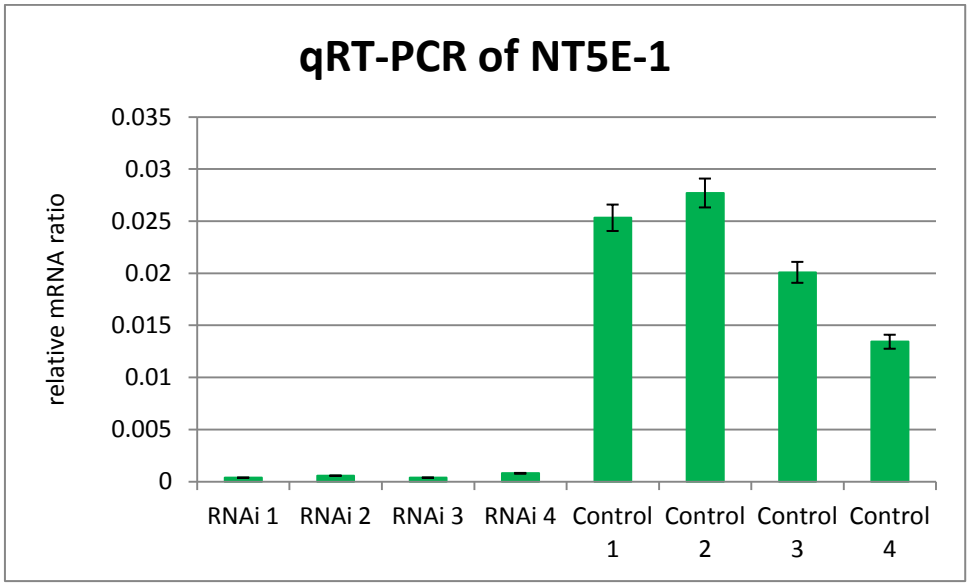


Figure 10 Results of qRT-PCR of gene silencing of NT5E-1

4. Conclusion

E-ATP and e-ado are highly important regulatory molecules modulating immune response. Enzymes like ectonucleotidases and adenosine deaminases are controlling the concentrations of pro inflammatory e-ATP and anti inflammatory e-ado, respectively.

In this study we tried to test the effect of RNAi for 3 different genes (NT5E-1, NT5E-2, ADGF-A in the model organism *Drosophila melanogaster*. However, there were some difficulties, when trying to obtained a fly line carrying the required mutations for the experiments with NT5E-2 and ADGF-A. As it seems one of the used stocks did not possessed the needed mutation. It is necessary to repeat the cross again with a different fly stock and subsequently do the analysis.

Nevertheless, the experiment with NT5E-1 was successful. In this study we show that by RNAi it is possible to reduce the amount of mRNA to 2.5 % compared to the respective control. Compared to different other studies this results show that RNAi is highly efficient. Furthermore we were able to localize the tagged NT5E-1 nucleotidase close to the cell membrane suggesting that it is a GPI anchored protein.

5. Bibliography

1. Atarashi K., Nishimura J., Shima T., Umesaki Y., Yamamoto M., Onoue M., Yagita H., Ishii N., Evans R., Honda K., Takeda1K. 2008. ATP drives lamina propria TH17 cell differentiation. *Nature 455*(7214), 808–812.

2. Bodin P., Burnstock G. 1998. Increased release of ATP from endothelial cells during acute inflammation. *Inflammation Res. 47*(8), 351–354.

3. Bours M.J., Swennen E.L., Di Virgilio F., Cronstein B.N., Dagnelie P.C. 2006. Adenosine 5‘-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol. Ther. 112*(2), 358404.

4. 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.

5. Conway E. J., Cooke R. 1939. The deaminases of adenosine and adenylic acid in blood and tissues. *Biochem. J. 33*(4) 479–92.

6. Cui Z., DiMario P.J. 2007. RNAi knockdown of Nopp140 induces *minute*-like phenotypes in Drosophila. *Mol. Bio. Cell 18*(6), 2179-2191.

7. Deaglio S., Dwyer K.M., Gao W., Friedman D., Usheva A., Erat A., Chen JF., Enjyoji K., Linden J., Oukka M., Kuchroo V.K., Strom T.B., Robson S.C. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med 204*(6), 1257–1265.

8. Drury A.N, Szent-Györgyi A. 1929. The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. *J. Physiol. 68*(3), 213–237.

9. Ejsmont R.K., Sarov M., Winkler S., Lipinski K.A., Tomancak P. 2009. A toolkit for high-throughput, crossspecies gene engineering in Drosophila. *Nature Methods 6*(6), 435 – 437. doi: 10.1038/nmeth.1334

10. Elliott M.R., Chekeni F.B., Trampont P.C., Lazarowski E.R., Kadl A., Walk S.F. Park D., Woodson R.I., Ostankovich M., Sharma P., Lysiak J.J., Harden T.K., Leitinger N., Ravichandran K.S. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature 461*(7261), 282–286.

11. Fire A., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature 391*(6669), 806-811.

12. Fischer J.A., Giniger E., Maniatis T., Ptashne M. 1988. GAL4 activates transcription in Drosophila. *Nature 332*(6167), 853–856.

13. Hirschhorn R., Ratech H. 1980. Isozymes of adenosine deaminase. *Isozymes Curr. Top Biol. Med. Res. 4*, 131–57.

14. Huang S., Apasov S., Koshiba M., Sitkovsky M. 1997. Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood 90*(4), 1600-1610.

15. Idzko M., Dichmann S., Ferrari D., Di Virgilio F., La Sala A., Girolomoni G. Panther E., Norgauer J. 2002. Nucleotides induce chemotaxis and actin polymerization in immature but not mature human dendritic cells via activation of pertussis toxin-sensitive P2y receptors. *Blood 100*(3), 925–932.

16. Ichimiya T., Manya H., Ohmae Y., Yoshida H., Takahashi K., Ueda R., Endo T., Nishihara S. 2004. The twisted Abdomen Phenotype of Drosophila POMT1 and POMT2 mutants coincides with their heterophilic protein O-mannosyltransferase activity. J. Biol. Chem. 279(41), 42638–42647.

17. Kavi H.H., Birchler J.A. 2009. Drosophila KDM2 is a H3K4me3 demethylase regulating nucleolar organization. BMC Res. Notes. 2, 217. doi: 10.1186/1756-0500-2-217

18. La Sala, A. Ferrari D., Corinti S., Cavani A., Di Virgilio F., Girolomoni G. 2001. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. *J. Immunol. 166*(3), 1611–1617.

19. Latini S., Pedata F. 2001. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J. Neurochem. 79*(3). 463–484.

20. Lazarowski E. R., Boucher R. C., Harden T. K. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol 64*(4), 785–795.

21. Maier S.A., Podemski L., Graham S.W., McDermid H.E., Locke J. 2001. Characterization of the adenosine deaminase-related growth factor (ADGF) gene family in Drosophila *Gene 280*(1–2), 27–36.

22. Novakova M., Dolezal T. 2011 Expression of Drosophila Adenosine Deaminase in Immune Cells during Inflammatory Response. PLoS ONE 6(3), e17741.

23. Ohta A., Sitkovsky M. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature 414*(6866), 916–20.

24. Prasher D.C., Eckenrode V.K., Ward W.W., Prendergast F.G., Cormier M.J. 1992. Primary structure of the Aequorea victoria green-fluorescent protein. *Gene 111*(2) 229–233.

25. Raza A., Yousaf W., Giannella R., Shata M.T. 2012. Th17 cells: interactions with predisposing factors in the immunopathogenesis of inflammatory bowel disease. *Expert Rev. Clin. Immunol*. *8*(2), 161–168.

26. Reiter L.T., Potocki L., Chien S., Gribskov M., Bier E. 2001. A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. *Genome Res. 11*(6),1114–1125.

27. Roignant J.Y., Carré C., Mugat B., Szymczak D., Lepesant J.A., Antoniewski C. 2003. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. *RNA 9*(3), 299-308.

28. Shimomura O., Johnson F.H., Saiga Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol*. *59*, 223–239.

29. Van der Weyden M.B., Kelley W.N. 1976. Human adenosine deaminase. Distribution and properties. *J. Biol. Chem. 251*(18), 5448–5456.

30. Wang T.F., Guidotti G. 1996. CD39 is an ecto-(Ca2+,Mg2+)-apyrase. *J. Biol. Chem. 271*(17), 9898–9901.

31. Wilkin F., Stordeur P., Goldman M., Boeynaems J.M., Robaye B. 2002. Extracellular adenine nucleotides modulate cytokine production by human monocyte-derived dendritic cells: dual effect on IL- 12 and stimulation of IL-10 *Eur. J. Immunol. 32*(9), 2409–2417.

32. Zavialov A.V., Engström Å. 2005. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. *Biochem. J. 391*(1), 51–57.

33. Zimmerman H., 1992. 5'-nucleotidase: molecular structure and functional aspects. *Biochem. J. 285*(2), 345–365.

34. Zurovec M., Dolezal T., Gazi M., Bryant P. 2001. ADGFs – growth factors with enzymatic activity. *Ann. Dros. Res. Conf. 42*, 69.

6. List of Tables

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