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Role of extracellular adenosine in *Drosophila*

Ph.D. Thesis

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Annotation

This thesis describes several aspects of the role for extracellular adenosine in *Drosophila*. Reverse genetic, molecular and microscopic methods together with the most forefront *Drosophila* research techniques have been applied to elucidate the role of adenosine signaling in the regulation of development, physiology and metabolism of *Drosophila* larvae. The thesis helps to establish the model for extracellular adenosine as a stress-signal for the release of energy stores. It also describes the elucidation of *Drosophila* extracellular adenosine production pathway by functional characterization of extracellular ATP/adenosine converting enzymes. Further, this thesis describes the attempt to study adenosine signaling by creation of tissue specific knock-down mutation of *Drosophila* adenosine receptor. Finally, it provides results of the utilization of in vivo fluorescent reporters for studying of the localization, function and interaction of enzymes implicated in adenosine signaling pathway.

Declaration [in Czech]

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

Fenckova, M., Hobizalova, R., Fric, Z.F., Dolezal, T. 2011. Functional characterization of ecto-5'-nucleotidases and apyrases in Drosophila melanogaster. Insect Biochem. Mol. Biol. 41(12), 956-967.
Michaela Fencková performed most of the experiments, wrote and reviewed the manuscript.
Contribution in percentage: 70%

Zuberova, M., **Fenckova, M**., Simek, P., Janeckova, L., Dolezal, T. 2010. Increased extracellular adenosine in Drosophila that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. Dis. Model. Mech. 3(11-12), 773-784.
Michaela Fencková participated in adoR/adgf-a interactions, depletion of AKH-producing cells and interaction with adgf-a mutation and detection of AdoR mRNA in fat body cells of wild type Drosophila larvae. Michaela Fencková also participated on writing and reviewing of the manuscript.
Contribution in percentage: 15%

Contents

INTRODUCTION

The function and importance of extracellular adenosine has been reviewed many times and has been extensively studied for many years. A long time passed since the release of first publications referring to extracellular form of this nucleoside (Burnstock et al., 1972) and discovery and cloning of the specific receptors (Linden et al., 1991) to experimental proves of adenosine functioning as a stress hormone. During this time, several diverse roles for adenosine has been described and adenosine is now being understood as an important regulatory molecule in the physiology and homeostasis of the organism. Here, I would like to review the metabolism and different ways of adenosine catabolism and turnover as well as the processes in which extracellular adenosine is implicated in attempt to describe the complex role for this retaliatory metabolite and offer the most recent view on adenosine functions.

1. *Intracellular and extracellular adenosine in mammals*

1.1.Production, transport and metabolism

The amount of extracellular adenosine is controlled by combination of its metabolism, cell release and cell uptake. Production of intracellular adenosine is a result of controlled process of intracellular ATP degradation by a set of dephosphorylating enzymes. ATP represents a high energy state molecule and could be dephosphorylated to AMP which as a part of energy cycle is shortly reconverted to ADP and ATP. When the conditions within the cell switch to high energy demand, AMP cannot be phosphorylated and is metabolized by intracellular 5'-nucleotidases (5'NT; EC.3.1.3.5.). Soluble cytosolic 5'-nucelotidases from the cN-I family represent the main enzymes that are responsible for adenosine production by dephosphorylation of intracellular AMP (Borowiec et al., 2006). They are present in all tissues at different expression levels. The highest expression of cN-I has been detected in skeletal and heart muscle where it functions as a main producer of adenosine during ischemia (Bianchi and Spychala, 2003). Alternatively, adenosine can be generated by hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase (Tabrizchi and Bedi, 2001).

Adenosine depletion is mediated by adenosine kinase (AK) that phosphorylates adenosine back to AMP or by deamination to inosine by adenosine deaminase (ADA). Adenosine metabolism by adenosine kinase (AK) is considered less important than its deamination by ADA. The main reason is that AK has much less affinity to adenosine than ADA. If the concentration of adenosine is 0.5 μM, AK is saturated and therefore its activity is inhibited. There are two forms of adenosine deaminase in humans: ADA1 and ADA2. The originally described ADA1 is the main intracellular adenosine deaminase whereas ADA2 is considered extracellular (Zavialov et al., 2010). But, ADA1 was also described as an extracellular membrane-bound, so-called ecto-ADA (Franco et al., 1998; Franco et al, 1997). Recently, a novel paralogue of ADA enzymes was discovered by comparison of amino acid sequence. Sharing the common protein sequence motif, it is suggested that these enzymes called ADA-like (ADA-L) may share the same catalytic function as ADA (Maier et al., 2005).

Adenosine turnover between intracellular and extracellular space depends on the concentration gradient across the membrane and is mediated by two different types of adenosine transporters: bidirectional equilibrative (ENT) and concentrative unidirectional where the transport of adenosine molecule is coupled to transport of Na^+ iont (CNT). Human ENT1 and ENT2 are predominantly expressed in brain when they are likely to provide extracellular adenosine as a neuromodulatory signal (Jennings et al., 2001). ENT3 was shown to function in mitochondrial and lysosomal transport (Kang et al., 2010) and ENT4 is known to uniquelly transport adenosine in the brain and heart under acidic pH conditions that are connected with ischemia and therefore might represent a source of extracellular adenosine during ischemia (Barnes et al., 2006).

An alternative way of extracellular adenosine production is the dephosphorylation of ATP released from the cells. When under stress, inflammation or damage, cells release a set of proinflammatory molecules known as danger-associated molecular pattern (DAMP). ATP as one of these molecules helps to stimulate the damage response and contributes to development of primary immune response leading to inflammation. To protect the tissues from excessive cell damage resulting by inflammation, mammals and other vertebrates convert extracellular immunostimulatory ATP to immunosuppressive adenosine (Bours et al., 2006). ATP catabolism is mediated by membrane bound ecto-enzymes, members of the alkaline phosphatase, ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), ecto-nucleoside triphosphate/diphosphohydrolases (E-NTPDase, CD39) and ecto-5'-nucleotidase (CD73) families (Yegutkin et al., 2008).

1.2 Signaling - mammalian adenosine receptors

Mammalian adenosine receptors belong to superfamily of G-protein coupled receptors that are ubiquitously expressed in a wide variety of tissues. They are classified into four types: A1, A2a, A2b, A3 (Fredholm et al., 2000). All adenosine receptors contain 7 transmembrane domains, extracellular NH_2 terminal domain and intracellular COOH terminal domain. Histidin residues between VI and VII domain bind adenosine molecule by creating two hydrogen bonds. Formation of disulfide bonds between domains III + IV and VI + VII are also necessary for receptor activation. Transmembrane domains are highly conserved but extracellular residues show certain variability due to ligand binding.

Receptor type	**A1**	**A2a**	**A2b**	**A3**
G protein	Gi/o	Gs	Gs, Gq	Gio/Gq
Signal transduction	cAMP↓, MAPK↑, IP3↑, K+↑, Ca2+↓	cAMP↑, MAPK↑	cAMP↑, MAPK↑, IP3↑	cAMP↓, MAPK↑, IP3↑
Distribution	brain, spinal cord, testis, heart, autonomic nerve terminals	brain, heart, lungs, spleen	Systemic + large intestine, bladder	lung, liver, brain, testis, heart, thymus
Affinity to adenosine	high	high	lower	lower

Tab. 1: Overview of mammalian adenosine receptors.

Existence of different receptors with different affinity to adenosine and opposing downstream pathway activation makes adenosine signaling very diverse. The cell response to extracellular adenosine depends on the level of extracellular adenosine, on the expression of adenosine receptor subtype and on the interaction with other extracellular signals. Therefore, the cell responses to extracellular adenosine cannot be generally classified. This makes adenosine signaling so complex and in many cases often very plastic and dynamic.

An important feature of modulation the extracellular adenosine signaling is dimerization of adenosine receptors. Dimerization of A1 and A2A receptors regulates neurotransmitter release and allows adenosine to modulate glutamate neurotransmission. Low concentration inhibits and high concentration stimulates glutamate release in glutaminergic synapse (**Fig. 3**; Cirulea et al., 2006). Best described are the physical interactions of adenosine receptors with dopamine receptors creating high order receptor mosaics in the brain striatum implicated in pathophysiology of Parkinson's disease, schizophrenia and drug addiction.

Studying of these interactions has now been extensively implicated in treatment strategies using specific antagonists (Fuxe et al., 2010).

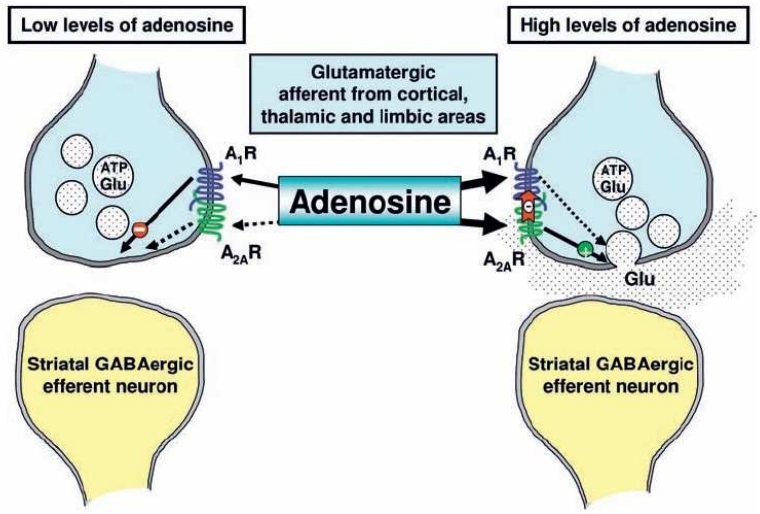

Fig 3. Scheme showing differencies in the effiacacy of adenosine to stimulate A1 and A2A receptors in the A1-A2A heterodimers. Low concentrations of adenosine activate predominantly A1, inhibiting glutamate release. High concentrations of adenosine also activate A2A, which, by means of the A1-A2A intramembrane interaction antagonizes A1 function, therefore stimulating glutamate release. Cirulea et al., 2006.

1.2.1. Diverse roles for adenosine receptors: A1AR

A1 receptor (A1AR) inhibits adenylate cyclase and Ca^{2+} channels which is connected with increase of K^+ ions. A1 receptor is coupled with Gi/o membrane protein which stimulates phospholipase C and IP_3 production (Guieu, 1998). The principal role of A1 receptor is in neural tissues where it acts in modulation of neurotransmission between brain neurons. The A1 receptor activation has been attributed a pronociceptive action (Papagallo et al., 2003; Gaspardone et al., 1995) and vasocontriction stimulation (Castrop, 2007). A1AR is among the first receptors expressed in embryonic brain and heart and is an important modulator of mammalian embryonic development (Rivkees et al, 2001). Faulhaber-Walter et al. (2011) recently described a contribution of A1AR on glucose metabolism. They showed a reduced glucose tolerance, decreased insulin sensitivity and increase in adipose tissue mass in A1AR deficient mice supposing that A1AR signaling significantly contributes to the maintaining of glucose homeostasis and physiological insulin action, yet the cellular mechanisms by which adenosine may enhance glucose uptake remain unknown. They hypothesize

that adenosine, via A1AR, could mediate the uptake of extracellular glucose as a source of energy in oxidative glycolysis for production of ATP. They also consider that central hypothalamic effects of missing Ado/A1AR signaling on the control of food intake may dominate the peripheral adipokine effects leading to fat deposition, impaired glucose tolerance and insulin resistance.

1.2.2 Diverse roles for adenosine receptors: A2AR, A2B

A2 receptors exhibit lower affinity to adenosine than A1 receptors and they activate adenylate cyclase via Gs coupled protein and therefore stimulate production of cAMP. According to their affinity to adenosine, they are classified in two types, A2a and A2b. These receptors modulate several processes such as blood pressure (by induction of vasodilatation; Castrop, 2007), pain enhancing (Sawynok, 1998) or anti-inflammatory action in neutrophiles, monocytes, platelets, T-cells, NK cells (A2a; Sullivan, 2003; Cadieux et al., 2005; Tang et al., 2007, Lappas et al., 2006) and macrophages (A2b; Murphree et al., 2005). Role of A2a recepor in CNS has been extensively studied in these days. It has been shown that A2a which is specifically enriched in the brain striatum (center of the Pavlovian conditioning) forms functional complexes with dopamine receptor D2R and A2a/D2R oligomers are essential for correct striatal function. Blocking of A2a signaling by specific antagonists can reduce the effects of dopamine depletion in striatum, a major cause of motor symptoms of Parkinson's disease (Peterson et al., 2011; Vallano et al., 2011). Functional interaction of A2a and mGlu5 (glutamate type 5 recepor) in mouse striatum has been recently demonstrated to regulate the self-administration of alcohol and conditioned effects of cocaine (Brown et al., 2011).

A2B receptor plays a central role in tissue adaptation to hypoxia and has a protective role in hypoxia-induced tissue inflammation (Koeppen et al., 2011). Van der Hoeven et al. (2011) indicated that A2B receptor suppresses oxidase activity and inhibits superoxide production in murine neutrophils regulating the proinflammatory action of neutrophils. The role of A2B adenosine receptor was also described in sensory synapse of carotid bodies, chemoreceptors that sense changes in arterial PO_2, PCO_2 and pH. A2B was shown to antagonistically interact with dopamine receptor D2R in the control of catecholamin release in hypoxia on the presynaptic (sensory) membrane by the regulation of adenylate cyclase

and possible physical interaction of these two receptors in the plasma membrane is being discussed (Conde et al., 2008).

Hypoxic conditions are known to promote the polymerisation of erythrocyte hemoglobin in sickle cell disease. It has been found recently that adenosine via A2B receptor contributes to mouse erythrocyte sickling in hypoxia. Hypoxia-induced release of ATP from affected cells and tissues is followed by rapid degradation to adenosine that stimulates A2B-mediated intracellular production of 2,3-diphosphoglycerate. 2,3-DPG decreases the oxygen binding affinity of hemoglobin leading to cell sickling, hemolysis and tissue and organ damage (Zhang et al., 2011).

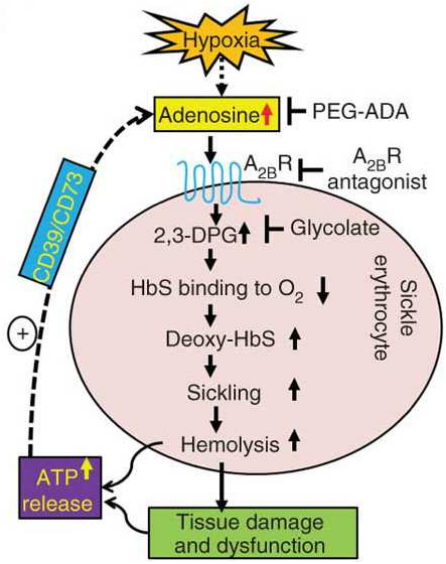

Fig. 2: Model of excessive adenosine signaling in erythrocyte sickling. In hypoxic conditions, increased adenosine-mediated activation leads to production of 2,3-DPG which decreases binding of hemoglobin (HbS) to O_2, resulting in icreased amount of deoxy-HbS, sickling and hemolysis. Erythrocyte hemolysis further promotes tissue damage and more ATP is released in the damage response from these tissues. ATP is converted to adenosine by action of CD39/CD73 enzymes and function as a positive feedback of adenosine stimulated erythrocyte sickling. Treatment with polyethylene glycol-modified adenosine deaminase (PEG-ADA) or application of A2b antagonist could reduce the production of erythrcyte 2,3-DPG and reduce sickling. Modified from Zhang et al., 2011.

1.2.3. Diverse roles for adenosine receptors: A3AR

A3 receptor inhibits adenylate cyclase and stimulates phospholipase C and consequently increases intracellular level of Ca^{2+} by mobilizing G protein subunit, $G\alpha_q$ (Murphee and Linden, 2004). The importance of A3 receptor is in antigene stimulated secretoric reaction and cell cycle regulation (Brambilla et al., 2000). Activation of A3AR was implicated in the inhibition of tumor growth (Fishman et al., 2003, 2004) and Aghaei et al. (2011) suggest possible regulation of p53-mediated G_1 cell cycle arrest and apoptosis in prostate cancer cells by A3AR. The influence of A3AR on proliferation and growth of cancer cells and accumulation of G_0/G_1 arrested cells was described by Merighi et al. (2005). They found that activation of A3AR recepor in human melanoma cells leads

to activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway that in turn reduces phosphorylation of ERK1/2 which is necessary for cell proliferation. This is an example of PI3K/Akt pathway and MAPK pathway crosstalk regulation by A3AR.

The role of A3AR in central nervous system is in promoting the neuropotection. Nice example was recently presented by Dennis et al., (2011) who studied the difference in sensitivity of CA1 and CA3 hippocampal pyramidal neurons to hypoxia. They attribute the low sensitivity of CA3 neurons to hypoxia to metabotropic glutamate receptor 1 (mGluR1) and A3AR-induced depression of synaptic neurotrasmission mediated by glutamate AMPA receptor (AMPAR) and they found that A3AR in cooperation with mGluR1 regulates the internalization and degradation of AMPAR which reduces excitotoxicity and promotes neuroprotection.

Zhu et al. (2011) showed that A3AR physically interacts with antidepressant-sensitive serotonin transporter (SERT) forming receptor/transporter complexes in mice serotonergic neurons to promote the A3AR-mediated modulation of the serotonin transporter. Interestingly, polymorphisms in SERT promoter have been implicated with brain function and neuropsychiatric disorders such as obsessive-compulsive disorder (OCD) and autism (Grados et al., 2007; Prasad et al., 2009; Homberg and Lesch, 2011).

The role of adenosine signaling via A3AR in glucose release from rat liver glycogen subjected to ischemia-reperfusion was recently shown by Cortes et al. (2009). This is the first evidence for adenosine functioning as an anti-insulin hormone to induce early hyperglycemia in ischemic conditions and it assigns adenosine among other important stress-induced anti-insulin hormones, such as adrenaline, arginine-vasopressin, angiotensin, cortisol, dopamine and glucagon. They hypothesize that increased adenosine levels in tissues exposed to ischemia-reperfusion might correspond to lack of ATP synthesis due to decreased O_2 supply at the mitochondrial level. By induction of glycogenolysis in hepatocyte cells of the liver, adenosine stimulates release of glucose in the blood as a quick energy source for ATP-deficient tissues. This is also a unique example of adenosine being carried by blood flow from relatively distant tissues to liver cells where it promotes glucose release and suggests similar mechanism in humans who also exhibit stress-induced hyperglycemia during cardiac surgery, or ischemia-reperfusion injury (reviewed by Hiesmayr et al., 2006).

1.3. Regulation of inflammatory and immune responses by ATP and adenosine – role of purinergic receptors and ecto-enzymes

Interdependent action of ATP and adenosine plays an important role in the regulation of immune response. Depending on the concentration of these metabolites and on cell expression of the specific receptors, the crosstalk between ATP and Ado signals drives the proinflammatory behavior of immune cells to protect the host from infections or immunospresive responses to preserve the minimal damage to healthy tissues (extensively reviewed in Bours et al., 2006).

At the early onset of immune response, in order to induce inflammation, both ATP and Ado can have stimulatory effects on the action of immune cells. Later, to inhibit chronic inflammation and to protect healthy tissues from excessive damage, Ado turns to be immunosuppressive molecule and stimulates the anti-inflammatory responses.

In neutrophils, which create the first line of immune cells and are critical effectors of the early stages of inflammation, both ATP and Ado (via A1) stimulate adhesion and promote neutrophil migration to the damaged tissues. By very fine regulation of ATP and Ado signals, neutrophils are directed to damaged tissues and prevented from excessive damage of surrounding healthy tissues. Adenosine can also induce (via A1) or block (via A2) phagocytosis of neutrophils and production of ROS, inhibit degranulation reactions of microbicidal molecules and modulate apoptosis of neutrophils (A3↑, A2↓).

In macrophages that are responsible for induction of chronic inflammation, adenosine switches from the classical (immunostimulatory, cytotoxic) to alternative (immunosupressive) activation by expressing A2a and A2b receptors (Csóka et al., 2011). The switching between phenotypes coordinates the process of inflammation.

Both ATP and Ado counterbalance and contribute to resolution of inflammation by activating the antigen presenting cells. Naive T-cells are stimulated to migrate from peripheral tissues to T-cell production areas to initiate the cellular and humoral immunity or tolerance.

The central effectors of cellular and humoral immunity are lymphocytes. Both B and T-lymphocytes may subject to autocrine or paracrine regulation by extracellular nucleotides. Extracellular ATP is involved in lymphocyte proliferation but the proliferation is inhibited after ATP breakdown to Ado. Depending on the concentration but also on the cell status, ATP

can stimulate or inhibit effector functions of CD4 T-lymphocytes (cytokine secretion) and promote cell recognition and target cell lysis by CD8 cytotoxic lymphocytes. Extracellular Ado plays an important part in modulation of T-cell function which was described in patients with ADA-SCID syndrome (adenosine deaminase deficiency). Interestingly, both CD4 and CD8 cells express A2a, A2b and A3 adenosine receptors but no or very little of A1 receptor and modulation of A2a receptor has been shown crucial for T cell responses to extracellular Ado in vivo. Ecto-ADA has been demonstrated to localize to the cell surface by binding to T-cell activated antigens. ADA after binding to CD26, colocalizes with A2b receptor and attenuates the immunosuppressive signaling in T cells. CD73 is progressively upregulated in the prolonged stage of inflammation (by IFNα) and produces high levels of extracellular adenosine, suppressing the lymphocyte adhesion and controlling the extent of lymphocyte-mediated inflammation.

ADA deficiency in SCID syndrome is also connected with depletion of B-lymphocytes. Adenosine via activation of A2a receptor increases intracellular cAMP which may influence the developmental fate of B-lymphocytes.

Bours et al. (2006) describe the nature of ATP and Ado driven modulation of immune response as a three-step process where in the first step, the onset of acute inflammation and the initiation of primary immune response take place. In this stage, extracellular ATP functions as an “alarm” molecule after the release from the cytoplasm and induce the primary damage reactions of the cells in the local microenvironment. In the second step, the role of ATP is shifted from immunostimulatory to immunomodulatory and together with the extracellular adenosine, the anti-inflammatory signals predominate the pro-inflammatory signals. Purinergic receptors play a crucial part in this stage, as well as ATP dephosphorylating ecto-enzymes that decrease the concentrations of extracellular ATP and increase the concentrations of extracellular Ado. Together, ATP and Ado cell surface receptors and ecto-enzymes comprise an important purinergic feedback system modulating the immune and inflammatory responses in this stage. In the last step, the main function of extracellular adenosine is in recovery and healing of damaged tissues promoting the tissue regeneration.

2. *Extracellular adenosine in Drosophila*

2.1. Production, transport and metabolism

In *Drosophila*, the turnover of adenosine between intracellular and extracellular space is mediated by equilibrative nucleoside transporters DmENT1, DmENT2 and DmENT3. DmENT1 and DmENT2 are high expressed in all developmental stages. DmENT3 shows low expression (Gelbart, W.M., Emmert, D.B. (2010.10.13). FlyBase High Throughput Expression Pattern Data Beta Version). DmENT2 is the only equilibrative transporter with experimental evidence for nucleoside transporter function (Machado et al., 2007). Based on the sequence similarity, Machado et al. (2007) also describes two possible concentrative nucleoside transporters encoded by the genes CG11778 and CG8083. It has been shown recently that DmENT2 regulates associative learning and synaptic transmission and this may be attributable to altered adenosine receptor activation (Knight et al., 2010).

Production and metabolism of intracellular adenosine in *Drosophila* has not been studied extensively and for many candidate enzymes, experimental evidence is missing. Candidate enzyme for intracellular ATP breakdown is *Drosophila* NTPase that was recently described by Knowles et al. (2009). Regardless, this intracellular enzyme seems to be more specific for other substrates than ATP or ADP. Dephosphorylation of intracellular AMP could be mediated by one of the enzymes from ecto-5'-nucleotidase family (CG11883; CD73-like) with more distant similarity to mammalian CD73 but lacking the glycosyl phosphoinositol anchor and signal peptide (Fenckova et al., 2011). The function of intracellular adenosine deaminase could be attributed to one of the enzymes from ADGF family. It was shown that individual ADGF family members may have different subcellular localizations and the expression pattern of ADGFs is distinctive (Matsushita et al., 2000; Maier et al., 2001; Zurovec et al., 2002). Thanks to this ADGFs can cover the expression and function of *Drosophila* adenosine deaminases that is mediated by enzymes from different family (ADA1) in mammals. *Drosophila* also posses a homolog to classical human ADA but overexpression of this protein did not have significant effect on adenosine depletion and therefore this enzyme is considered to lack adenosine deaminase activity (Zurovec et al., 2002). *Drosophila* homologs of adenosine kinase (AK) have beed also described on the base of the sequence similarity. Out of two candidate genes, CG11255 seems to have very broad and high expression during development and in adult stage and CG3809 is specifically

expressed only in the late larval development, pupal and early adult stages with significant increase of expression during metamorphosis. In adult flies, mRNA for CG3809 was only detected in male testes but surprisingly in quite high level (Gelbart, W.M., Emmert, D.B. (2010.10.13). FlyBase High Throughput Expression Pattern Data Beta Version). Intracellular AMP/ATP ratio that reflects the energy status of the cell is controlled by the action of adenylate cyclase in mammals. This enzyme is capable to transfer phosphates between AMP and ATP to create 2 ADP molecules and vice versa. Because the monitoring and controlling the energetic homeostasis is an important mechanism for the cell survival, there is also a homolog of adenylate kinase in *Drosophila*, named Adk2 with suggested function in neurogenesis (Mummery-Widmer et al., 2009).

Besides the release of intracellular adenosine from the cells, extracellular adenosine can be also produced by dephosphorylation of extracellular ATP by membrane-bound apyrases that belong to family of 5‘-nucleotidases (CG42249-B and CG42249-C) or calcium-activated nucleotidases (CG5276). These enzymes mediate the dephosphorylation of ATP to ADP and AMP. The last step, conversion of AMP to Ado is mediated by ecto-5‘-nucleotidases, NT5E1 and NT5E2 (Fenckova et al., 2011).

Proteins from the family of Adenosine Deaminase-related Growth Factors are responsible for depletion of extracellular adenosine (Zurovec et al., 2002). The family comprises from 6 members: ADGF-A, ADGF-A2, ADGF-B, ADGF-C, ADGF-D and ADGF-E. Mutation in the main *Drosophila* adenosine deaminase: ADGF-A is associated with elevated levels of adenosine in larval hemolymph which results in several developmental defects. *adgf-a* mutation is accompanied by partial larval lethality, fat body disintegration, excessive proliferation and differentiation of hemocytes, melanotic tumor formation and high levels of circulating glucose. (Dolezal et al., 2005, Zuberova et al., 2010). Using in vivo GFP reporter of ADGF-A expression, Novakova and Dolezal (2011) recently showed that ADGF-A is expressed specifically in larval hemocytes during inflammatory response and they suggest that possible function of adenosine deaminase in the site of inflammation could be in depletion of extracellular adenosine to control the excessive release of glucose that might lead to loss of energy reserves and wasting.

2.2. Adenosine signaling – Drosophila adenosine receptor

In contrast to mammals, *Drosophila* possesses only one adenosine receptor (AdoR). This makes the studying of adenosine signaling in this model organism much easier. Activation of AdoR increases intracellular cAMP and induces calcium release. Ubiquitous overexpression of AdoR causes lethality in L1 to L2 transition. Ectopic expression of AdoR in CNS prevents emerging of adults from the puparium and causes lethality at the end of the metamorphosis. (Dolezelova et al, 2007). The phenotypes caused by local AdoR expression resembles those observed in mutations of ADGF family members (ADGF-D and ADGF-C; Dolezal et al., 2003) and can be partially rescued by injection of adenosine deaminase (Dolezelova et al, 2007). Loss-of-function mutation in AdoR can significantly suppress the lethality and other mutant phenotypes of *adgf-a* mutation, mainly the formation of melanotic tumors and excessive hemocyte proliferation and differentiation (Dolezal et al., 2003).

Knight et al. (2010) recently showed impaired associative learning and synaptic function in *adoR* and *ent2* mutants as well as in *AdoR/ent2* double-mutants. They also observed dramatic increase of *ent2* expression in *AdoR* mutants. These results suggest a crosstalk between equilibrative nucleoside transporter 2 (ENT2) and AdoR and outline a possibility for existence of a compensatory mechanism that in response to decrease or changes in Ado signaling via AdoR increases the level of extracellular Ado by increased expression of ENT2. Nevertheless, synaptic defects in *ent2* mutant seem to occur independently of changes in cAMP.

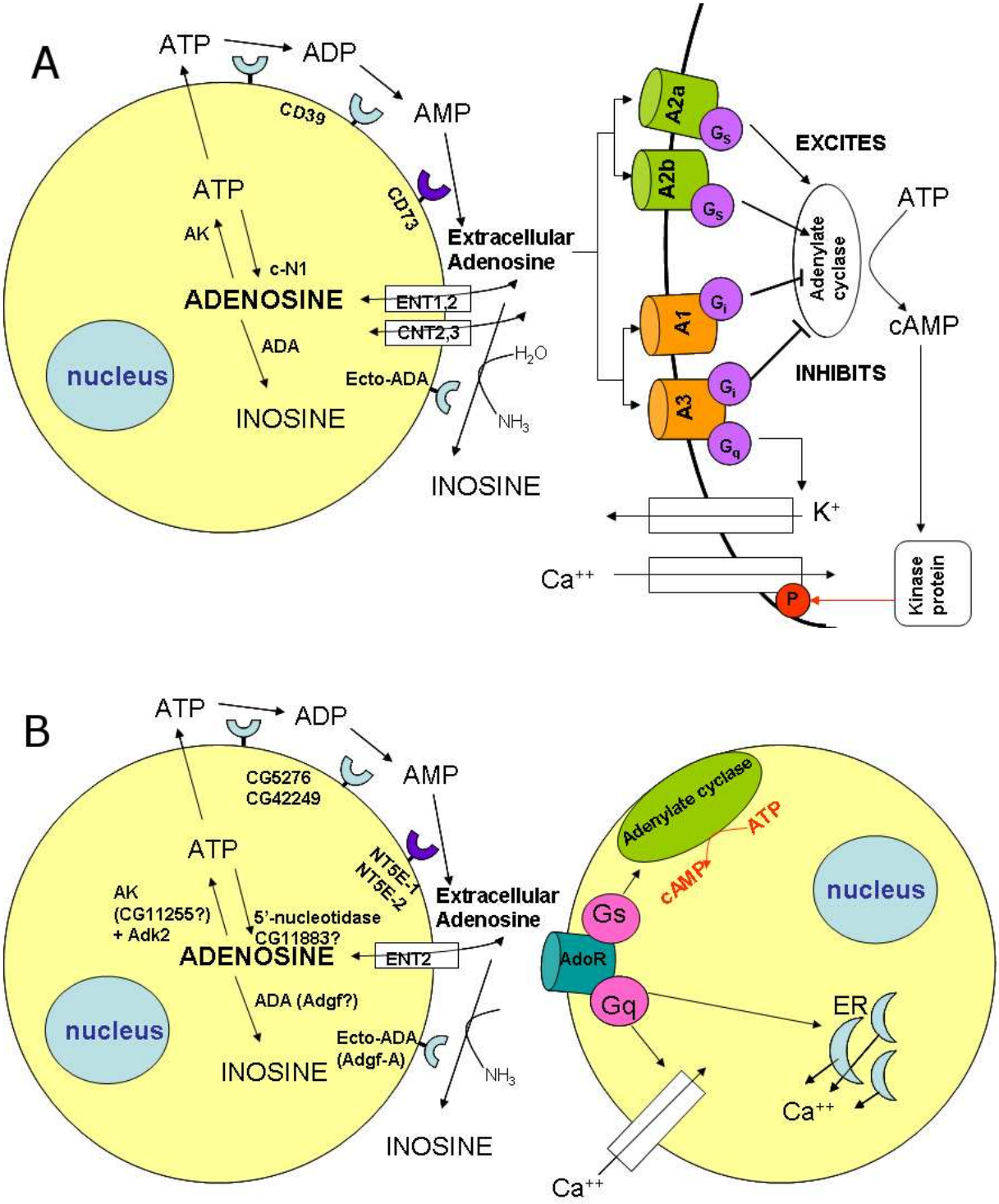

Fig. 3: Simple schematic overview of adenosine turnover and signaling. (A) Production, metabolism, transport and signaling of extracellular adenosine in mammals. ADA: adenosine daminase; AK: adenosine kinase, adenylate kinase; c-N1: 5'-nucleotidase; CD39: NTPDase; CD73: ecto-5'-nucleotidase. (B) Production, metabolism, transport and signaling of extracellular adenosine in *Drosophila*. ADA: adenosine deaminase; AK: adenosine kinase; Adk2: adenylate kinase 2; ER: endoplasmic reticulum.

SUMMARY

Drosophila melanogaster has proved to be a wonderful model organism for studying human diseases. Despite to be an invertebrate species, its genome content encompasses almost 75% gene similarity with humans. Almost every disease gene has a counterpart in *Drosophila*. Thanks to the low redundancy, the complex processes that are controlled by multiple gene products or by large gene families could be easily reduced to a minimum of genes involved in these processes in *Drosophila*. Finally, the forward genetic tools established for this model allow the researchers to perform the most forefront genetic research in a very short time and obtain a striking data about the disease gene function, interaction and possible treatment of the disease that could be applied to a higher mammalian models or directly head for a discovery of new drugs and treatment strategies.

We have chosen *Drosophila* to investigate the role of extracellular adenosine because of the complexity of adenosine functions in mammals. *Drosophila* offers us to study the systemic effects of adenosine signaling within the whole organism as well as the local influence on the cell microenvironment. Despite the fact that adenosine is being considered a local hormone influencing the responses in cell autocrine or paracrine manner, in *Drosophila*, relatively small distances between the tissues open a possibility for these local hormones to be carried with the hemolymph to distant tissues or to trigger a systemic response.

This thesis contributes to development of a model for intensive studying of extracellular adenosine in *Drosophila*. The first part describes a model of increased extracellular adenosine in *Drosophila* larvae carrying mutation in the main *Drosophila* adenosine deaminase, ADGF-A. Using a dominant suppressor screen of the *adgf-a* mutant phenotype, we have discovered that mutation in Phosphorylase Kinase (PhK) significantly suppress *adgf-a* mutant phenotype which is associated with partial larval lethality, pupal defects, fat body disintegration, melanotic tumor formation and excessive hemocyte proliferation. The key function of PhK is in promoting of glycogenolysis which led us to conclusion that *adgf-a* mutant larvae might have impaired carbohydrate metabolism. Following this hypothesis we have shown that *adgf-a* mutant larvae are extremely sensitive to diet restriction, have increased level of carbohydrates in hemolymph and impaired accumulation of glycogen stores. We have also shown that the sensitivity to diet restriction in *adgf-a* larvae can be rescued by the mutation in adenosine receptor (*adoR*) and *adgf-a/adoR* double-mutants have lower level of circulating

glucose in the hemolymph. We have excluded the role of adipokinetic hormone (AKH) on the release of glycogen stores from the fat body by depleting the AKH-producing cells in *adgf-a* mutant larvae with no significant effect on *adgf-a* phenotype. But we have shown that AdoR is expressed in the cells of fat body, main storage tissue in *Drosophila* larvae and suggested a possible mechanism for adenosine to stimulate energy release from fat body in parallel to AKH via AdoR. We created a model in which adenosine (next to AKH) serves as an anti-insulin hormone and is a potential candidate signal for wasting – a progressive loss of energy stores during immune response.

The second part uncovers the mechanism of extracellular adenosine production by subsequent dephosphorylation of ATP and proves that *Drosophila* possess enzymes that are capable to convert extracellular ATP to Ado in the same manner as it is in mammals. We have characterized 5 putative *Drosophila* ecto-5'-nucleotidase genes and using a functional assay, we have shown that two of them are *bona fide* ecto-5'-nucleotidases, and one has cell surface apyrase activity. Furthermore, we have functionally described another *Drosophila* apyrase belonging to different family of calcium-activated nucleotidases.

The third part describes the creation of a conditional knock-down mutation of adenosine receptor that is crucial for the studying of adenosine signaling effects in different tissues. This laborious part brings the results of unsuccessful attempts to create an effective AdoR knock-down by RNAi and microRNA-based gene silencing and suggests using new methods and approach to obtain a functional knock-down.

The last part introduces preliminary and therefore unpublished data describing creation of in vivo gene expression reporters using revolutionary technique of Red/ET recombineering in combination with *D. melanogaster* genomic FlyFos library which allowed us quickly and effectively modify the gene sequence within the intact genomic background by introducing a GFP sequence at the C-terminal of selected proteins. We have created GFP reporters of expression of 10 genes that are implicated in adenosine metabolism and signaling. Here we analyze the expression of three of them, ADGF-A:GFP, NT5E-1:GFP and CG11883:GFP using confocal scanning microscopy and we show that the expression pattern of GFP reporters resembles the mRNA expression of these genes as it was demonstrated in high-throughput mRNA expression profiles. We also discuss future possibilities of using this versatile system for introducing mutations within FlyFos clones or inducible degradation of GFP-fused proteins. The pilot data we obtained so far on GFP-fused

protein expression represent a promising tool for future detailed elucidation of adenosine pathways in *Drosophila*.

RESULTS

Part I:
Increased extracellular adenosine in Drosophila that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death

Monika Zuberova, Michaela Fenckova, Petr Simek, Lucie Janeckova and Tomas Dolezal.
Dis. Model. Mech. 3(11-12), 773-784.

Part II:
Functional characterization of ecto-5‘--nucleotidases and apyrases in Drosophila melanogaster

Michaela Fenckova , Radka Hobizalova , Zdenek Faltynek Fric and Tomas Dolezal.
Insect Biochem. Mol. Biol. 41(12), 956-967.

Part III:
Conditional mutation of adenosine receptor by RNA interference and microRNA-based gene silencing

Unpublished.

Part IV:
Preparing of GFP reporters for live imaging of proteins involved in adenosine metabolism and signaling using FlyFos library and Red/ET recombineering

Unpublished.

Part I:

Increased extracellular adenosine in Drosophila that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death

Monika Žuberová, Michaela Fencková, Petr Šimek, Lucie Janečková and Tomáš Doležal.
Disease Models & Mechanisms 3, 773-784 (2010).

Extracellular adenosine is an important signaling molecule in neuromodulation, immunomodulation and hypoxia. Adenosine dysregulation can cause various pathologies, exemplified by a deficiency in adenosine deaminase in severe combined immunodeficiency. We have established a Drosophila model to study the effects of increased adenosine in vivo by mutating the main Drosophila adenosine deaminase-related growth factor (ADGF-A). Using a genetic screen, we show here that the increased extracellular adenosine in the *adgf-a* mutant is associated with hyperglycemia and impairment in energy storage. The adenosine works in this regard through the adenosine receptor as an anti-insulin hormone in parallel to adipokinetic hormone, a glucagon counterpart in flies. If not regulated properly, this action can lead to a loss of energy reserves (wasting) and death of the organism. Because adenosine signaling is associated with the immune response and the response to stress in general, our results mark extracellular adenosine as a good candidate signal involved in the wasting syndrome that accompanies various human pathologies.

Zvýšená hladina extracelulárního adenozinu u drozofily, nesoucí mutaci v adenozin deamináze, je příčinou odbourávání energetických zásob a vede k postupnému chřadnutí a smrti

Význam extracelulárního adenozinu jako signální molekuly je především v modulaci nervových a imunitních funkcí a v ochraně tkání před nedostatkem kyslíku. Nesprávná regulace hladiny extracelulárního adenozinu je příčinou řady patologií, jako například Těžké Kombinované Imunodeficience. Pro studium účinků extracelulárního adenozinu na organizmus, jsme vytvořily mutaci v adenozin deamináze u drozofily (ADGF-A). Na takto vytvořený model jsme aplikovali genetický skrínink s cílem vyhledat geny, jejichž funkce je ovlivněna zvýšenou hladinou extracelulárního adenozinu. Díky tomuto přístupu se nám podařilo zjistit, že zvýšená hladina extracelulárního adenozinu u larev s deficiencí v adenozin deamináze (*adgf-a*), je spjata s hyperglykémií a neschopností *adgf-a* deficientních larev ukládat energetické zásoby. Adenozin zde funguje jako tzv. anti-inzulínový hormon, který řídí odbourávání energetických zásob v buňkách pomocí signalizace přes adenozinový receptor, podobně jako protějšek lidského glukagonu, adipokinetický hormon. Při nedostatečné regulaci hladiny extracelulárního adenozinu nebo jeho signalizace přes adenozinový receptor, nadměrné odbourávání energie může vést k vážnému chřadnutí a následné smrti organismu. Protože adenozin je celkově spojován s regulací imunitní odpovědi a se stresem obecně, je tímto předurčen pro roli významného stresového regulátoru v tzv. „Wasting“ syndromu, který doprovází řadu lidských onemocnění.

Part II:

Functional characterization of ecto-5‘-nucleotidases and apyrases in Drosophila melanogaster

Michaela Fencková , Radka Hobizalová , Zdeněk Faltýnek Fric, Tomáš Doležal.
Insect Biochemistry and Molecular Biology 41, 956-967 (2011).

Ecto-5’-nucleotidases are glycosyl phosphatidylinositol (GPI)-linked membrane-bound glycoproteins that convert extracellular AMP to adenosine. They play important roles in the inflammatory response where they modulate levels of pro-inflammatory extracellular ATP and anti-inflammatory extracellular adenosine. They are found in the saliva of blood feeding insects and also have a role in male reproduction. Drosophila possesses five genes with eight alternative transcripts encoding proteins with sequence homology to mammalian ecto-5’-nucleotidases. Here we show that two of them - NT5E-1 (CG4827) and NT5E-2 (CG30104) are GPI-linked proteins with ecto-5’-nucleotidase activity but that they can also be released from the GPI anchor and exhibit secreted 5’-nucleotidase activity in growth media. The third locus in the cluster, CG30103, most likely also encodes a GPI-anchored membrane-bound protein but without 5’-nucleotidase activity, possibly due to the numerous substitutions in the amino acid sequence. Together with NT5E-2, CG30103 is also expressed in the testis offering an interesting model to investigate ecto-5’-nucleotidase enzymatic and extra-enzymatic function in male reproduction. CG42249 locus encoding two alternative transcripts is sequentially similar to family of apyrases related to 5’-nucleotidases and we show here that together with CG5276 belonging to another family of calcium-activated nucleotidases function as apyrases converting extracellular ATP to ADP and AMP. The last locus, CG11883, encodes most likely a cytoplasmic/mitochondrial protein.

Funkční charakterizace ekto-5’-nukleotidáz a apyráz u drozofily

Ekto-5’-nukleotidázy jsou membránové glykoproteiny katalyzující přeměnu extracelulárního AMP na adenozin. Charakteristickým znakem ekto-5’-nukleotidáz je přichycení k buněčné membráně pomocí glykosylfosfatidylinositolu (GPI). Jedna z důležitých rolí ekto-5’-nukleotidáz je v regulaci zánětlivých procesů, kde pomáhají snižovat hladinu extracelulárního ATP a chránit zdravé buňky a tkáně před nadměrným poškozením, které je důsledkem stimulace imunitní odpovědi. Vzniklý adenozin pak funguje jako imunomodulátor a tlumí účinky imunostimulujícího ATP. Ekto-5’-nukleotidázy se také vyskytují ve slinách krev sajícího hmyzu a hrají roli při reprodukci. U drozofily bylo nalezeno 5 genů v celkem osmi alternativních formách, příbuzných lidské ekto-5’-nukleotidáze. Tato práce je výsledkem funkční analýzy těchto genů a prokazuje, že produkty dvou z těchto genů jsou funkční ekto-5’-nukleotidázy: NT5E-1 (CG4827) a NT5E-2 (CG30104), které jsou ukotvené v membráně pomocí GPI. Tyto proteiny mohou být odštěpeny od membrány a zároveň fungují jako extracelulární sekretované proteiny. Produktem genu CG30103, který se nachází ve stejném lokusu jako NT5E-1 a NT5E-2, je velmi pravděpodobně také GPI ukotvený membránový protein, ale bez 5’-nukleotidázové aktivity. Exprese NT5E-2 a CG30103 v testes nabízí zajímavé srovnání enzymatické a extraenzymatické aktivity těchto proteinů při reprodukci. Tato práce zároveň ukazuje, že oba transkripty genu CG42249 kódují proteiny s apyrázovou aktivitou, stejně tak jako gen CG5276, patřící evolučně do jiné rodiny, tzv. kalcium-dependentních nukleotidáz. Tyto apyrázy katalyzují přeměnu extracelulárního ATP na ADP a AMP. Sekvenčně nejvíce vzdálený lokus CG11883 kóduje pravděpodobně cytoplazmatický anebo mitochondriální protein.

Part III

Conditional mutation of adenosine receptor by RNA interference and microRNA-based gene silencing

INTRODUCTION

The key role of extracellular adenosine in *Drosophila* is the signal transduction via adenosine receptor (AdoR). In contrast to mammals, *Drosophila* possess only one type of adenosine receptor encoded by a single gene with significant structural similarity to mammalian adenosine receptors. Activation of *Drosophila* AdoR leads to activation of intracellular cAMP and calcium signaling (Dolezelova et al., 2007). We have shown that the mutation in AdoR significantly improves the phenotype of *adgf-a* mutation in developing larvae by blocking the signaling effect of extracellular adenosine (Dolezal et al., 2005). The fact that the increased level of extracellular adenosine in larval hemolymph caused by the *adgf-a* mutation seriously influences the development of *Drosophila* larvae shows that at least under certain conditions the adenosine signaling must play very important role in the regulation of larval development and that it requires precise regulation. Surprisingly, the tissue expression of AdoR mRNA in 3rd instar larvae is very low and is restricted only to certain tissues (brain, ring gland, imaginal discs) as detected by mRNA in situ hybridization (Dolezelova et al., 2007). Elimination of extracellular adenosine by ectopic expression of functional ADGF-A in *adgf-a*$^{-}$ larvae resulted in significant rescue of *adgf-a* mutant phenotype. Interestingly, the rescue could only be observed when expressing ADGF-A in ring gland (the main regulator of hormone release), lymph gland (hematopoetic tissue) and fat body (storage tissue, Dolezal et al., 2005). But the site of extracellular adenosine production can be different from the site of its action. Mammalian studies consider extracellular adenosine as a local hormone (Bowler et al., 2001; Rieg and Vallon, 2009) but proportionally in *Drosophila* the distances between different tissues within the whole body are much shorter and we cannot exclude that adenosine might be released into the hemolymph and carried to the site of action by the hemolymph flow or it can function in triggering of a systemic response.

To find the site of extracellular adenosine action we decided to create a conditional mutation of AdoR by targeted RNA interference (RNAi) and find a tissue-specific interaction between *adoR* mutation and *adgf-a*. By utilization of UAS-Gal4 system (Brand and Perimon, 1993) we ectopically expressed dsRNA against AdoR from 496bp long inverted repeats of AdoR cDNA sequence. We have also tried to utilize recently established method based on gene silencing by micro-RNA (Haley et al., 2008). The advantage of using micro-RNA (miRNA) based method is in the specificity of RNAi. Long dsRNA produced from more than 400bp inverted repetition undergoes cleavage and results in dozens of short (21

nucleotides) exogenous siRNAs. miRNA-based gene silencing mimics the naturally occurring hairpin-loop of endogenous miRNAs and its cleavage results in single highly specific 21 nucleotides long siRNA that is effectively loaded into RISC complex and induce the endonucleolytic cleavage of the complementary mRNA. Here we summarize the results of both approaches in attempt to produce targeted loss-of-function mutation of *Drosophila* AdoR.

MATERIAL AND METHODS

Construction of transgenic vector for RNAi

In order to produce *Drosophila* lines with RNAi against adenosine receptor, we constructed P-element vector containing 496bp sequence of exon III in sense and antisense orientation. Both sequences are spaced with intron II with the original splice site on 3' end. On 5' end of exon III AG/GT splice site was added (**fig.1**). The sequence of intron II and exon III was amplified from the complete genomic sequence using primers: AdoREcof (5'-GAATTCACTCACCCACATAGAGGC-3') and AdoRXhoR (5'-CTCGAGGTCCGTCAGATTGTTACGATG- 3') and first cloned to pGEMT easy (Promega). The sequence of exon III was amplified using primers: AdoRXhof (5'-CTCGAGGTGAGTGGTAGATAGATTTTCG-3') and AdoRXbaR (5'-TCTAGAACTCACCCACATAGAGGCGC-3') and cloned to pGEMT easy. The inserts were then subcloned in the pUAST vector (Brand and Perrimon, 1993) using EcoRI, XhoI and Xba I cloning sites.

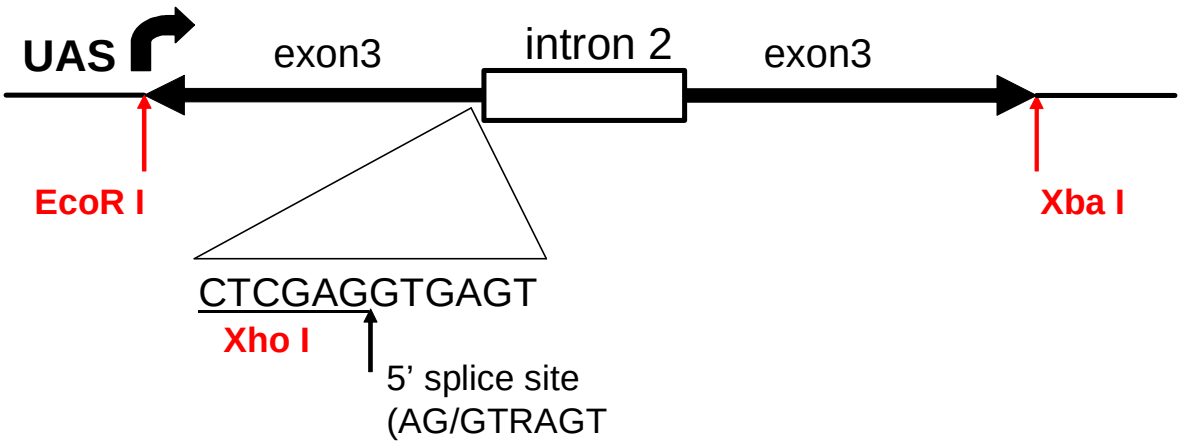

Fig.1: AdoR-RNAi construct tail to tail

Construction of miRNA-based transgenic vectors

siRNAs directed against adoR were designed using the Dharmacon "siDesign" center (Reynolds et al., 2004) and Ambion "siRNA target finder" algorithm. To minimize systematic "off-target" phenotypes resulting from sequence similarity of designed 21-mers, each input was cross-referenced against the database of all annotated D. melanogaster miRNA sequences (http://www.targetscan.org/fly_12/seedmatch.html). The stem loop hairpin was designed using an interactive shmiR web design page (http://flybuzz.berkeley.edu/cgi-bin/constructHairpin.cgi) which incorporates specific mismatched bases (at nts 2 and 11) to mimic the native D. melanogaster pre-miR-1 structure (**fig.2**). 71-nt oligos for each shmiR (shmiR1 and shmiR2) were obtained from Generi-Biotech and annealed at a final concentration of 50μM in 1X annealing buffer (75 mM KCl, 20 mM Tris [pH 8.0]), boiled for ~2 minutes, and then cooled to room temperature for ~30 minutes. Annealed oligos were diluted 1:100 in 1X annealing buffer, and then ligated to ~500 ng of the vector pNE3 (Haley et al., 2008; previously cut with NheI and EcoRI restriction enzymes) for ~15minutes at room temperature using T4 DNA ligase (Promega).

shmiR1 oligos:

5'-ctagcagtAAATCCGGTCGTGTATGCCAAtagttatattcaagcataTAGGCATACAGGACCGGATTTgcg 3'

5'-aattcgcAAATCCGGTCCTGTATGCCTAtatgcttgaatataactaTTGGCATACACGACCGGATTTactg-3'

shmiR2 oligos:

5'-ctagcagtTACTTAGGCTAGTGCCCATATtagttatattcaagcataAAATGGGCACAAGCCTAAGTAgcg-3'

5'-aattcgcTACTTAGGCTTGTGCCCATTTtatgcttgaatataactaATATGGGCACTAGCCTAAGTAactg-3'

Tandem shmiR was constructed by excision of shmiR1 with KpnI and SpeI from shmiR1-pNE3 and subcloning of this fragment into KpnI and XbaI sites in shmiR2-pNE3. The sequences cut with SpeI and XbaI share the same overhang and can be ligated together (**fig.3**). shmiR1, shmiR2 and tandem shmiR (shmiR1+2) were finally subcloned into pattB-ftz^{IN}-mCherry (Haley et al., 2010) that allows synchronic expression of the hairpin loop and a reporter (mCherry) from UAS promoter and site-specific integration of transgenic construct. shmiRs were subcloned into KpnI and XhoI restriction sites designed in the intron that is placed upstream of the reporter gene (**fig.3**).

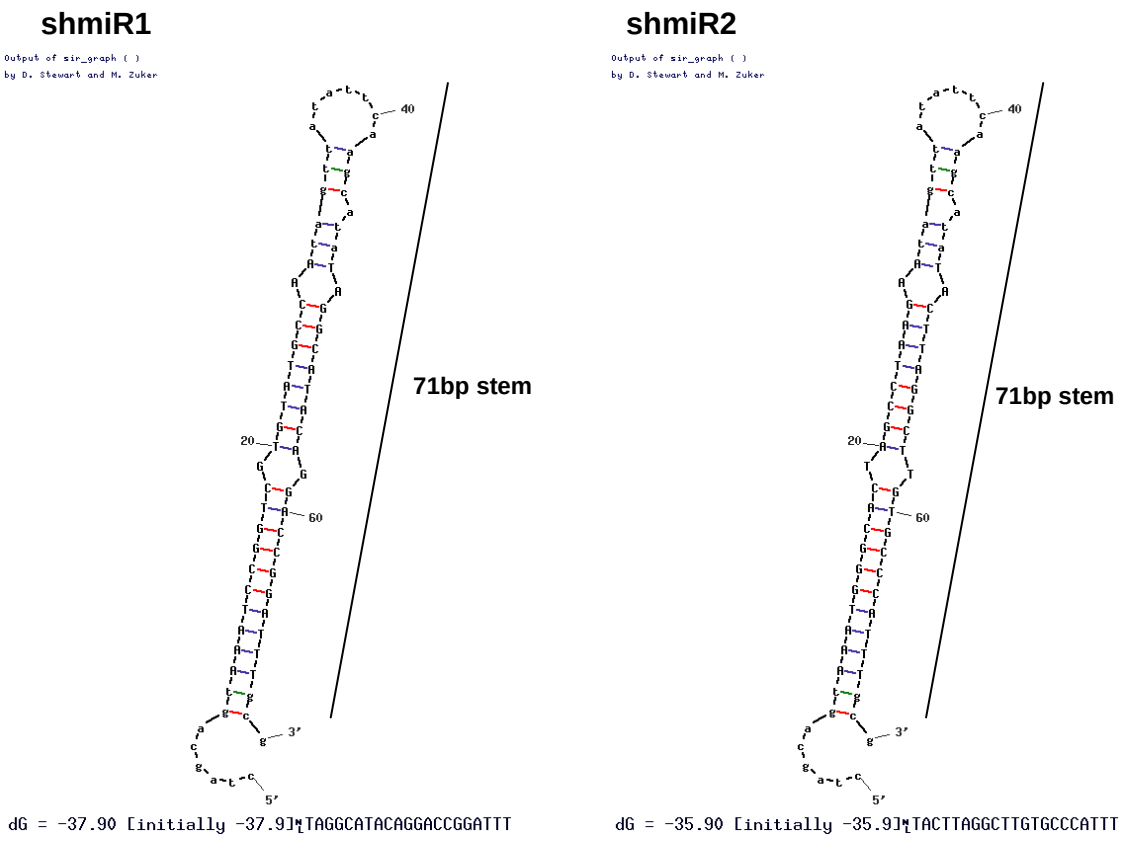

Fig.2 : stem hairpin loop containing 21-nt siRNA on 3'end with essential mismatches. Release from shmiR web design page (http://flybuzz.berkeley.edu/cgi-bin/constructHairpin.cgi)

Fly transgenesis

AdoR-RNAi construct was injected in *Drosophila* embryos (yw strain) and 5 independent insertions were obtained - 3 on chromosome II (AdoR-RNAi^{1}, AdoR-RNAi^{1b}, AdoR-RNAi^{3}) and 2 on chromosome III (AdoR-RNAi^{2}, AdoR-RNAi^{4}). Except insertion 4 all insertions were homozygous-viable. Another two AdoR-RNAi lines were obtained from Vienna Drosophila RNAi center (VDRC; www.vdrc.at, stock IDs 1385 and 1386). adoR-shmiR constructs were send for injection into 51D attP landing line (Bloomington stock number: 24483) to Fly Facility (www.fly-facility.com). Only transgenic line containing adoR-shmiR1 was obtained so far.

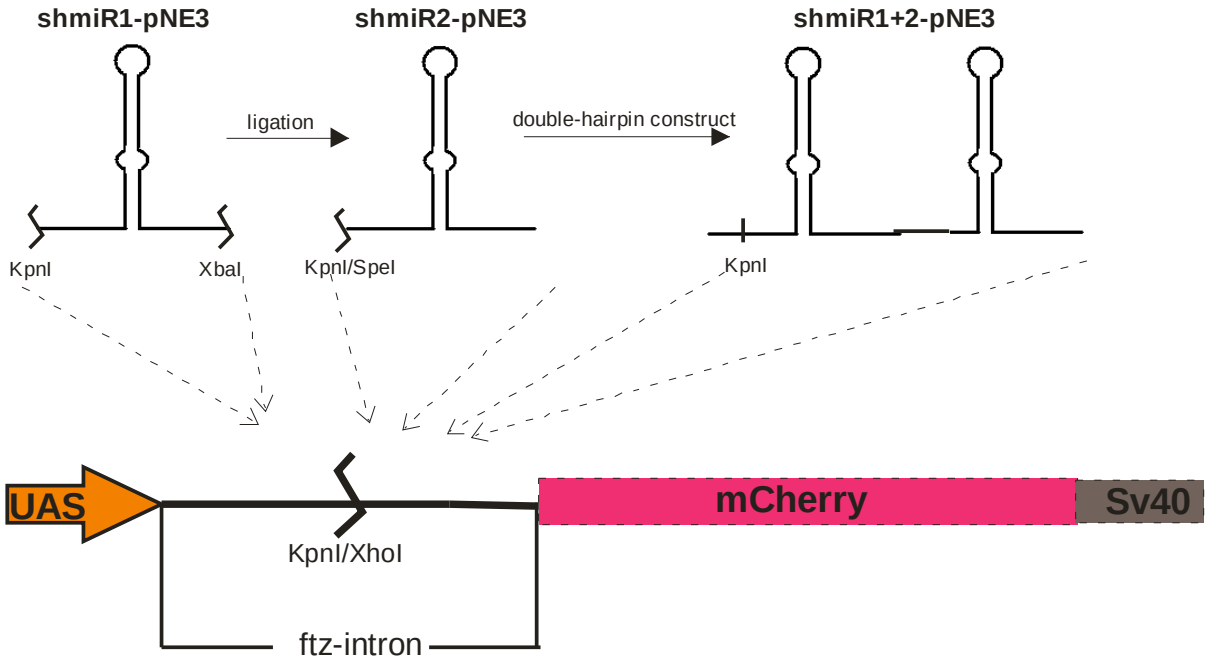

Fig. 3: Tandem shmiRs and cloning into the pattB-ftz^{IN}-mCherry

***adgf-a* rescue experiments**

adoR-RNAi lines and adoR-shmiR line were crossed in *adgf-a* mutant background and observed for genetic interaction with *adgf-a* upon induction with several Gal4 drivers. Flies were kept in egg-laying chambers and allowed to lay eggs for 4 hours on agar juice plates supplemented with yeast paste. 20 hour old embryos were collected and washed first with tap water and then with ethanol. The ethanol treated embryos were transferred to sterile agar plates with diet containing either 5%sucrose and 8% yeast (sucrose supplemented diet) or 0% sucrose and 8% yeast (pure yeast diet). First instar larvae of desired genotype were collected from the plates with a sterile needle and transferred into vials containing diet of the same composition supplemented with 80µl of Penicillin/Strepomycin solution (Sigma; 10000 U Penicillin /10mg Streptomycin per ml). The development from larvae into adults was observed for each individual vial. All experiments were done in triplicates. Gal4 driver lines used in the rescue experiments are listed in **tab.1**.

Gal4 driver line	**reference**	**expression in 3rd instar**
Act-Gal4 (Chromosome III)	Provided by Marek Jindra	Strong ubiquitous
Arm-Gal4 (Chromosome II)	Provided by Marek Jindra	Ubiquitous
AKH-Gal4 (Chromosome III)	Guillaume et al., 2005	Corpora cardiaca
Aug21-Gal4 (Chromosome II)	Bloomington (BL-30137)	Corpora allata
Feb36-Gal4 (Chromosome II)	Bloomington (BL-29968)	Corpora cardiaca + protoracic gland
Lg-Gal4 (Chromosome II)	Bloomington (BL-2721)	Ring gland
Hml-Gal4 (Chromosome II)	Bloomington (BL-6397)	Circulating hemocytes + lymph gland

C7-Gal4 (Chromosome II)	Provided by Marek Jindra	Fat Body, conjugated with UAS-mc08-GFP
Cg-Gal4 (Chromosome II)	Bloomington (BL-7011)	Fat Body + hemocytes

Tab. 1: List of Gal4 expressing lines used in *adgf-a* rescue experiments

RNA isolation and Real-Time PCR of adoR-shmiR

Larvae containing adoR-shmiR with or without induction of shmiR expression by act-Gal4 and control larvae (yw strain) were synchronized and total RNA was isolated from 2nd, 3rd and late 3rd (wandering) larval instars using TriReagent® (Molecular Research Center) and treated with DNase (TURBO DNA-*free*™ Kit, Ambion). cDNA synthesis was performed using Superscript® III Reverse Transcriptase (Invitrogen).). Gene expression was analyzed by Real-Time PCR (StepOne Real-Time PCR System, Applied Biosystems). PCR reactions were performed in a volume of 20 µl containing 250 nM primers and FastStart Universal SYBR Green Master Mix (Roche). Primers used for Real-Time PCR amplification of AdoR: AdoR-RealFOR (5'-CCCATCTGAACTCGGCGGTAAATC-3'), AdoR-RealREV (5'-GCCTCCTGCTGCTGCCTCAAC-3'). Actin 5C primer set used for amplification of internal standard: Actin5CF (5'-TACCCCATTGAGCACGGTAT-3'), Actin5CR (5'-GGTCATCTTCTCACGGTTGG-3').

RESULTS

Interaction of the *adgf-a* mutant phenotype with adoR-RNAi

All insertions obtained from the P-element mediated fly transgenesis of adoR-RNAi construct were tested for genetic interaction with *adgf-a* mutation upon induction of broad expressed armadillo Gal4 driver (arm-Gal4). Insertions adoR-RNAi^1 and adoR-RNAi^2 proved to be the most viable after the induction of dsRNA expression and did not have a negative impact on *adgf-a* mutation. Other insertions negatively influenced the larval development (data not shown). Therefore, these two insertions (further named adoR-RNAi-1, adoR-RNAi-2) were tested for interaction with *adgf-a* upon induction with strong actin Gal4 driver (act-Gal4) or tissue specific Gal4 drivers (all Gal4 drivers used are listed in **tab.1**.). The knock-down effect was also improved by addition of UAS-Dicer2 in 2 RNAi lines (adoR-RNAi-1 and adoR-RNAi-2) and by recombination of adoR-RNAi-1 and adoR-RNAi-3 on chromosome II creating adoR-RNAi-1/3.

We have previously shown that the *adoR* mutation is able to significantly improve the development of *adgf-a* larvae on sucrose supplemented diet. And even on the pure yeast

diet where *adgf-a* larvae rarely reach the 3rd instar or form pupae, the *adoR* mutation can significantly improve the larval development and enables larvae to overcome the lack of sugars in diet (Zuberova et al., 2010). Therefore the main emphasis was given on interaction of *adgf-a* and adoR-RNAi on the pure yeast diet. **Fig.4** resumes the data from genetic interaction of *adgf-a* mutation and adoR-RNAi upon induction with strong act-Gal4 on sucrose supplemented diet and on pure yeast diet. Except about 30% of pupae in adgf-a; adoR-RNAi-2; act-Gal4 on pure yeast diet there was no significant improvement of larval development and even in adgf-a; adoR-RNAi-2; act-Gal4 the pupae occurred very late (approx. 8-10 days after egg laying) and did not overcome the pupal stage and finally turned black. **Fig.5** shows *adgf-a* rescue upon induction of adoR-RNAi with Gal4 drivers expressing in different parts of ring gland (AKH-Gal4, Aug21-Gal4, Feb36-Gal4 and lg-Gal4). There was a slight improvement with Aug21-Gal4 (expression in corpora allata) and lg-Gal4 (strong expression within the whole ring gland) on sucrose supplemented diet but no effect on pure yeast diet. Finally, we tested the influence of adoR-RNAi in circulatig hemocytes (Hml-Gal4) and in fat body cells (C7-Gal4) on *adgf-a* larvae but again we did not find any improvement of larval development on pure yeast diet (**Fig.6**).

Transgenic flies expressing adoR-RNAi obtained from VDRC proved to be lethal after induction of act-Gal4 and were not tested for further interaction with *adgf-a*.

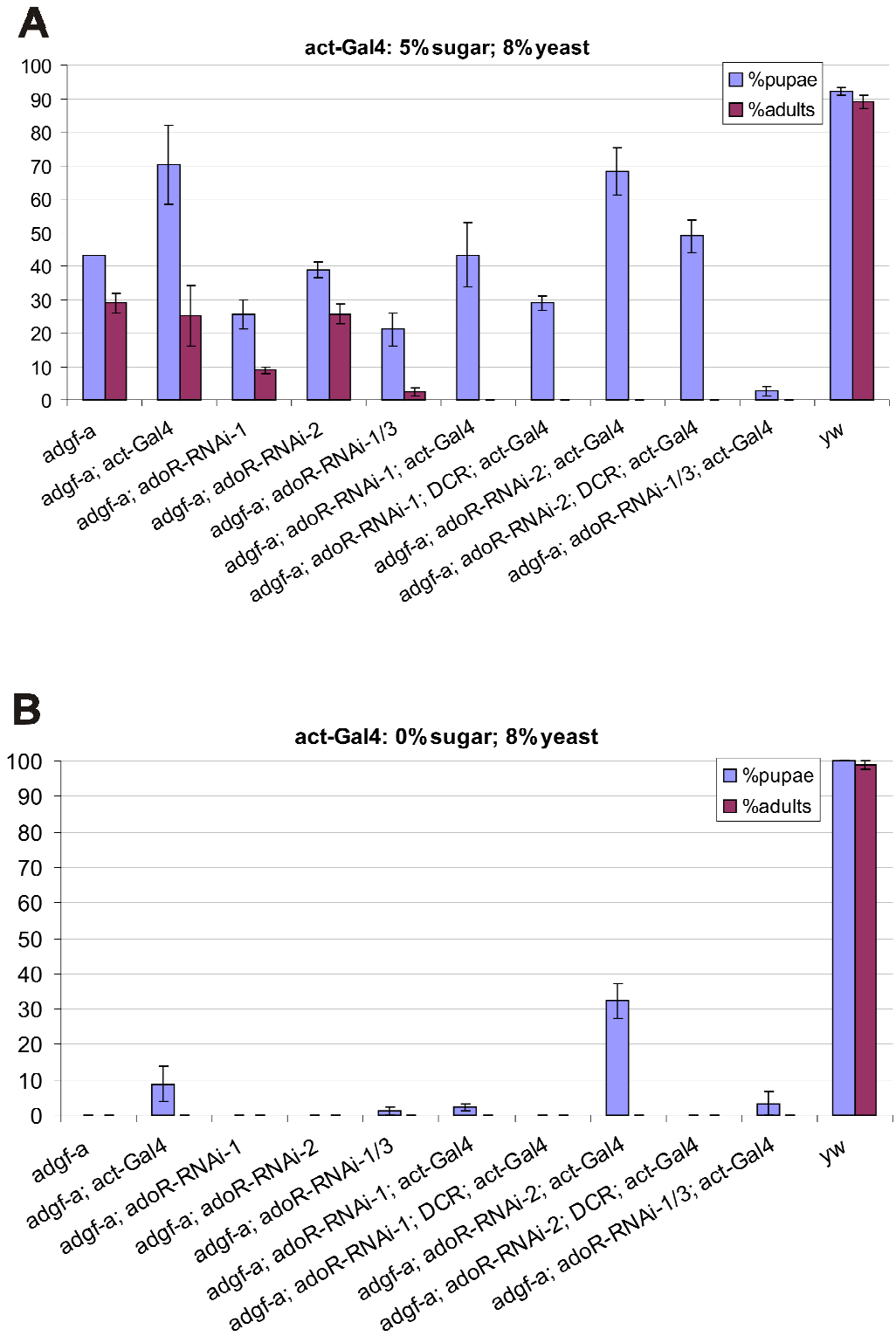

Fig. 4: Interaction of adoR-RNAi and *adgf-a*. Pupae and adult counts of *adgf-a* mutants on diet supplemeted with 5% sucrose (A). *adgf-a* mutant forms about 40% pupae and 30% adults on sucrose supplemented diet. Non-induced adoR-RNAi does not significantly change total number of pupae and adults. Addition of act-Gal4 itself improves the larvae survival up to 70% pupae but does not influence the adult number on sucrose supplemented diet. None of act-Gal4 induced adoR-RNAi is able to produce adult flies from pupae of *adgf-a* mutants. On pure yeast diet (B) *adgf-a* mutant does not pupate. Only the addition of act-Gal4 forms about 9% of pupae and 1 or 2 pupae occurs in non-induced adoR-RNAi-1/3;adgf-a and act-Gal4 induced adoR-RNAi-1. Induction of adoR-RNAi-2 with act-Gal4 forms about 30% pupae but no adults on pure yeast diet. yw strain was used as a control on both diets. yw larvae developed normally on both types of diet forming almost 100% pupae and adults. Data are presented as mean percentage ± s.e.m.

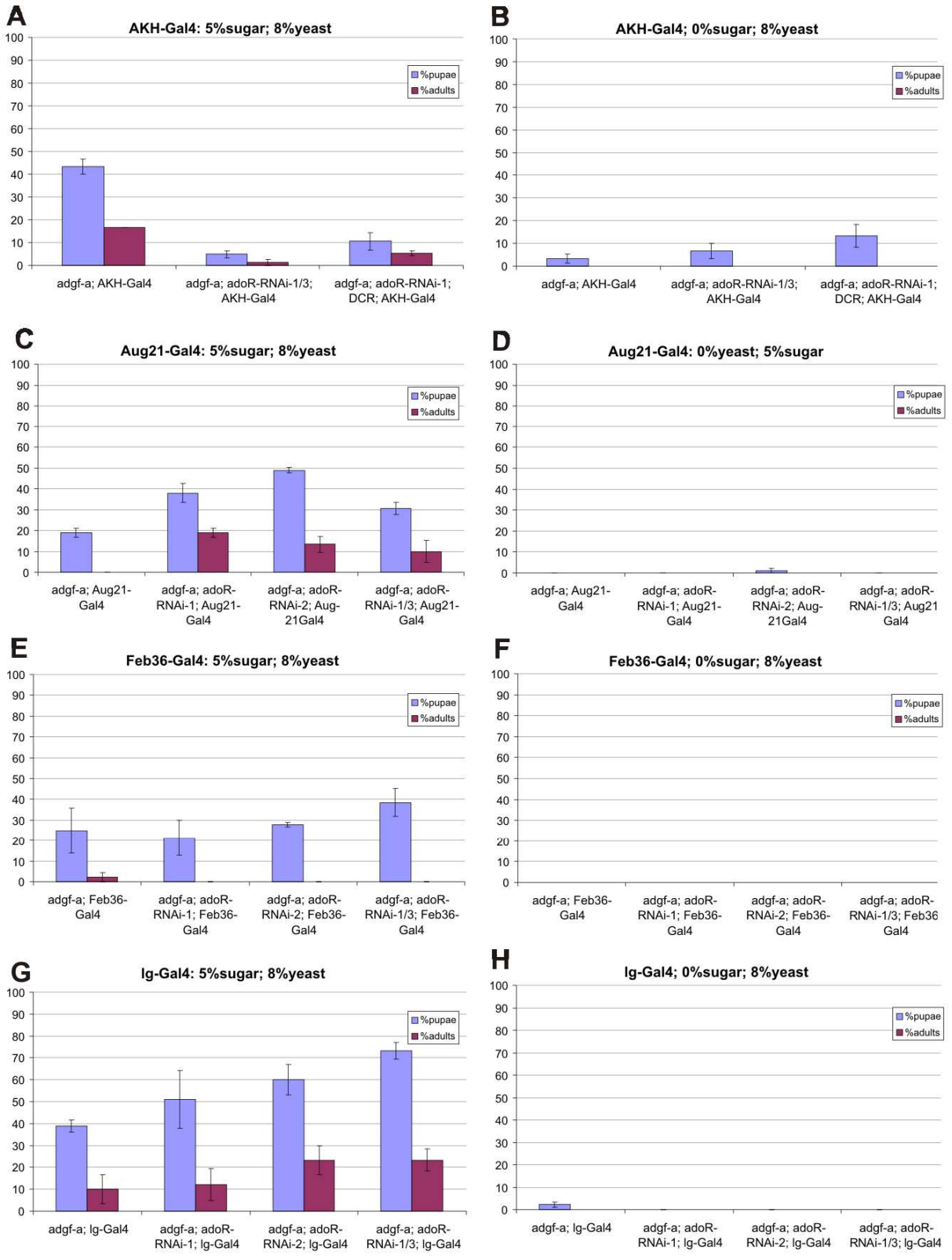

Fig. 5: Interaction of adoR-RNAi and *adgf-a* upon induction with ring gland Gal4 drivers. Viability of *adgf-a* on sucrose supplemented diet does not change significantly in adoR-RNAi larvae induced with Feb36-Gal4 (E) and is even worse in adoR-RNAi larvae induced with AKH-Gal4 (A). Induction of adoR-RNAi with Aug21-Gal4 and lg-Gal4 slightly improves the pupation rate and adult hatching on sucrose supplemented diet (C,G) but none of the adoR-RNAi lines tested is able to significantly improve the viability of *adgf-a* on pure yeast diet with any of the tested ring gland Gal4 drivers. Combination of *adgf-a* and Gal4 was used as a reference of *adgf-a* phenotype in each rescue. Data are presented as mean percentage ± s.e.m.

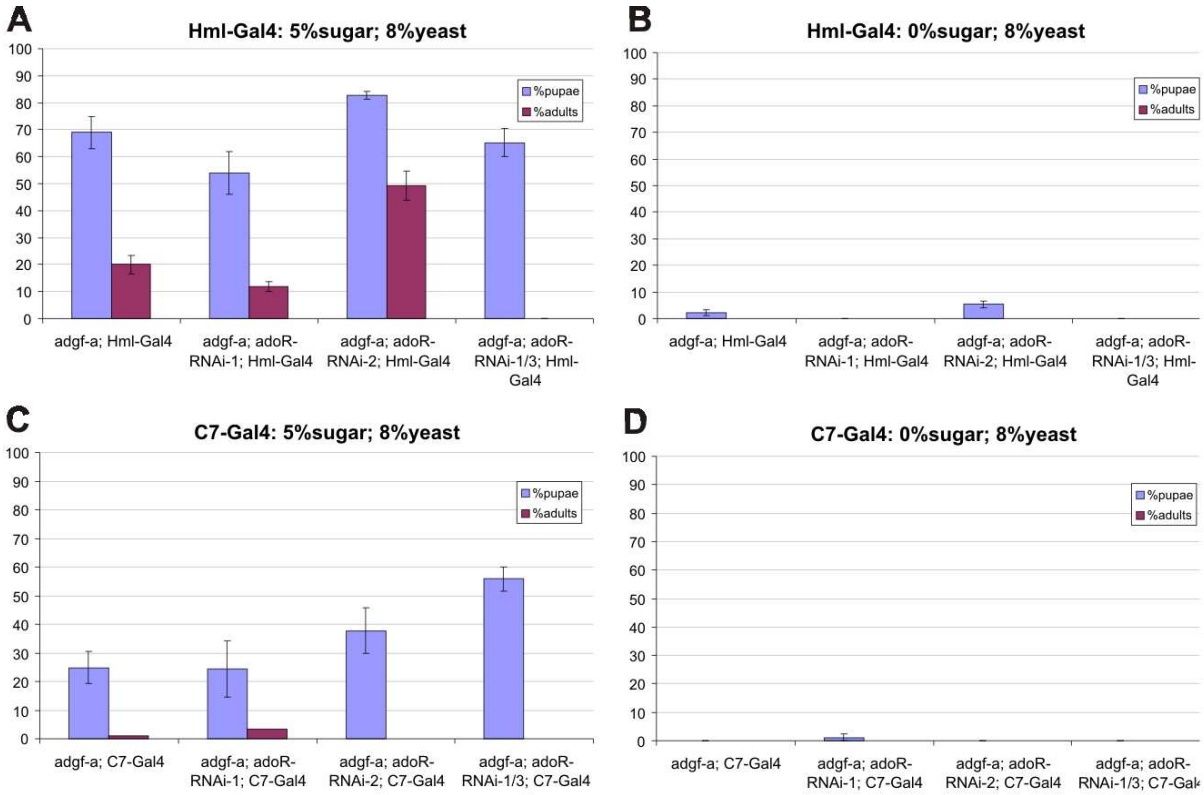

Fig. 6: Interaction of adoR-RNAi and *adgf-a* upon induction in hemocytes (A,B) and fat body (C,D). Induction of adoR-RNAi in *adgf-a* larvae with Hml-Gal4 leads to slight improvement of adult number in adoR-RNAi-2 line (A) but no significant survival can be observed on pure yeast diet (B). Induction of adoR-RNAi-2 and adoR-RNAi-1/3 in *adgf-a* larvae with C7-Gal4 leads to slight higher number of pupae but no adult flies hatch from the pupae (C) and no significant survival can be observed on pure yeast diet (D). Combination of *adgf-a* and Gal4 was used as a reference of *adgf-a* phenotype in each rescue. Data are presented as mean percentage ± s.e.m.

Rescue of *adgf-a* mutant phenotype with adoR-shmiR

We observed that the knock-down of adenosine receptor by adoR-RNAi often resulted in more severe phenotype of *adgf-a* larvae and it negatively influenced the larval development even without induction with specific Gal4 driver (**Fig. 4**). We explained these effects by either possible non-specific targeting of other mRNAs by adoR-RNAi and downregulating unintended genes (off-target effects) or in case of negative phenotype without RNAi induction we suggested that insertion of the P-element carrying transgenic AdoR-RNAi might have disrupted or influenced gene expression in the genomic locus where the insertion occurred (position effect). To eliminate the possible off-targets or the position effect of AdoR-RNAi, we decided to use a novel micro-RNA based gene silencing method that should bring improvement to the RNAi process. We subcloned only short 21-nt long siRNA sequence into a hairpin loop mimicking the original *D. melanogaster* micro-

RNA precursor. This hairpin loop is easily recognized and loaded into RISC and the specificity of this short siRNA is greater than loads of siRNAs resulting from digestion of long dsRNA by Dicer. We have prepared two independent constructs expressing adoR-shmiR (adoR-shmiR-1 with target sequence in exon III and adoR-shmiR-2 with target sequence in exon IV). To empower the effect of miRNA silencing we also prepared a tandem adoR-shmiR1+2 construct by subcloning both shmiRs into one construct. Expression of all shmiRs is coupled to expression of mCherry reporter. The constructs allows ΦC31 mediated insertion into attP landing sites that eliminates the position effect of random integration of the P-element (Bischof et al., 2007).

All constructs were sent for injection to Fly Facility (www.fly-facility.com). Out of these constructs only the injection of adoR-shmiR-1 led to a production of transgenic fly. We have tested the effect of adoR-shmiR by comparing the expression of AdoR mRNA in control (yw and adoR-shmiR heterozygous) and in induced (adoR-shmiR; act-Gal4) larvae of three different developmental stages. **Fig.8b** shows that the level of adoR-shmiR; act-Gal4 is 4 times lower than both controls in 2nd instar larvae, and 5 times lower than yw control in wandering 3rd instar larvae. Non-induced control (adoR-shmiR heterozygous larvae) shows reduction in expression of AdoR mRNA by 1/3 in wandering 3rd instar. After induction of shmiR expression the level of AdoR mRNA is about 0.05 (normalized to actin expression) in all tested stages.

adoR-shmiR was then crossed in *adgf-a* background and tested for genetic interaction with *adgf-a* upon induction with several Gal4 drivers on diet supplemented with 5% sucrose and on pure yeast diet. We did not find any significant rescue phenotype but the induction of adoR-shmiR with strong act-Gal4 negatively influenced the larval development in the wild type background (without *adgf-a*) compared to adoR-shmiR control larvae (**Fig. 8**). This might be due to effect of possible "off-targets" that could be targeted by shmiR. Interaction of *adgf-a* with adoR-shmiR induced with tissue specific Gal4 drivers is depicted in **Fig. 9**.

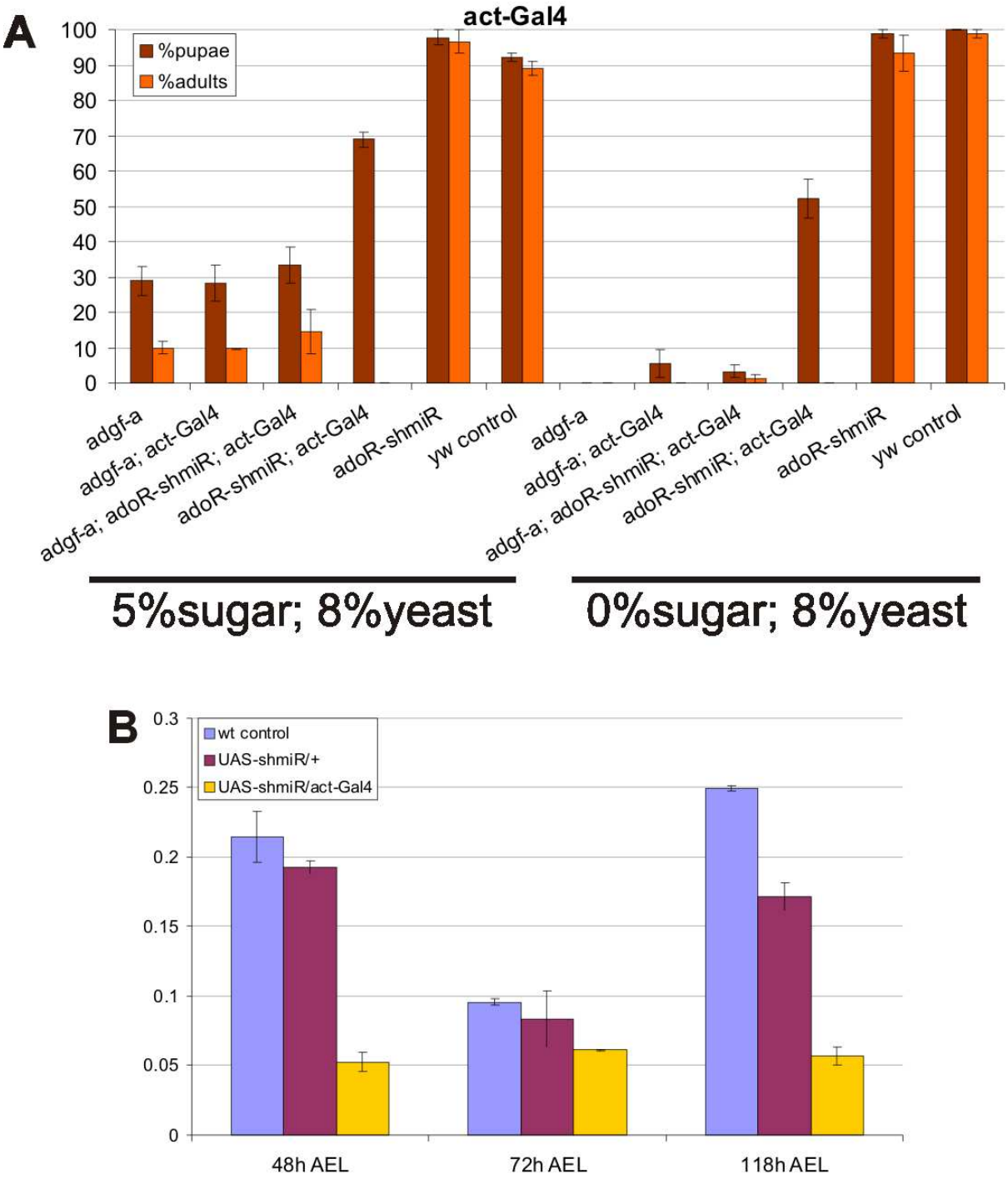

Fig. 8: Interaction of adoR-shmiR and *adgf-a*. (A) Viability of *adgf-a* and adoR-shmiR on sucrose supplemented diet and pure yeast diet. Addition of act-Gal4 does not significantly change the survival of *adgf-a* larvae and even induction of adoR-shmiR in *adgf-a* larvae with act-Gal4 does not significantly influence larval development. Induction of adoR-shmiR with act-Gal4 in wild type background decreases the number of pupae into ½ compared to adoR-shmiR heterozygous or yw control. The same effect can be observed on pure yeast diet. Data are presented as mean percentage ± s.e.m. (B) Relative levels of AdoR mRNA in 2nd instar larvae (48 hours after egg laying, AEL), 3rd instar larvae (72 hours AEL) and wandering 3rd instar larvae (118 hours AEL). AdoR mRNA in act-Gal4 adoR-shmiR induced larvae is significantly lower then in non-induced control larvae or yw larvae of 2nd instar and 3rd instar wandering but in middle 3rd instar (72h AEL) AdoR mRNA level is comparable to both controls. Interestingly AdoR mRNA level of both controls is lower in middle 3rd instar then in other two instars. Data are presented as mean percentage ± s.e.m.

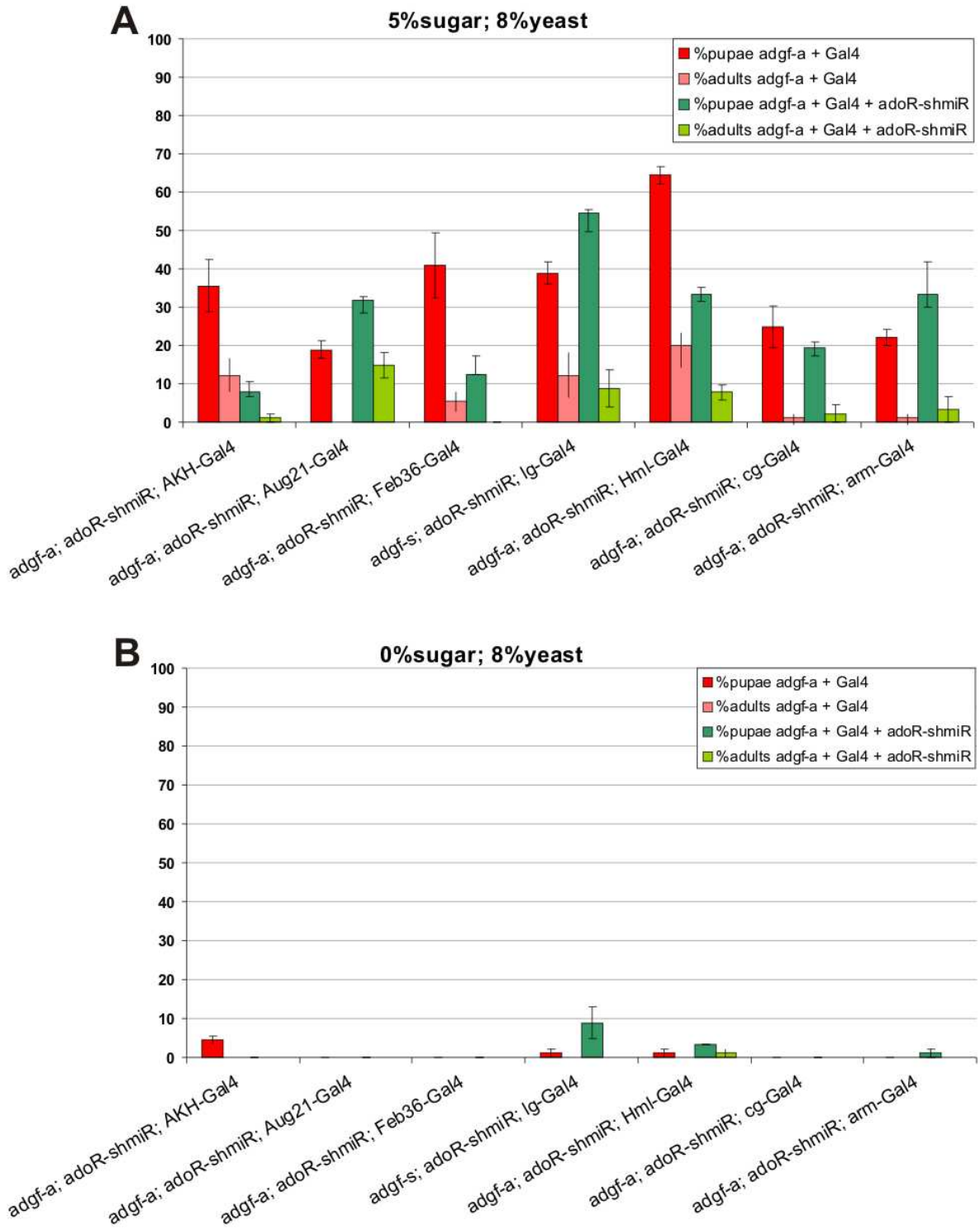

Fig. 9: Rescue of *adgf-a* with adoR-shmiR induced with tissue specific Gal4 drivers on sucrose supplemented diet (A) and pure yeast diet (B). No significant rescue of *adgf-a* can be observed in adoR-shmiR induced larvae on both types of diet. Data are presented as mean percentage ± s.e.m.

DISCUSSION

We attempted to perform a tissue specific knock-down of adenosine receptor (AdoR) by using two different approaches of RNAi-mediated gene silencing. First we constructed a P-element vector containing UAS promoter sequence followed by >400bp of AdoR mRNA sequence in sense and antisense orientation spaced by an intron sequence (AdoR-RNAi). Second we utilized a recently established method of miRNA-mediated gene silencing and designed 21nt microRNA sequence against AdoR mRNA sequence. We inserted the sequence into a hairpin loop pre-miRNA precursor sequence and cloned the single hairpin miRNA (shmiR) into transgenic vector compatible with site-directed mutagenesis (pattB-ftz^{IN}-mCherry; Haley et al., 2010). We induced the tissue specific knock-down of AdoR mRNA by crossing the transgenic lines containing either of two constructs with several Gal4 expressing lines in *adgf-a* mutated background. We did not observe any significant interaction between *adgf-a* and RNAi-mediated AdoR knock-down as we had shown previously using complete *adoR* mutation (Dolezal et al., 2005, Zuberova et al., 2010). Any significant improvement was not obtained even when supporting the RNAi with the parallel expression of Dicer2 or combining several insertions of AdoR-RNAi construct. Induction of some of the AdoR-RNAi lines in wild type background with strong actin Gal4 driver even lead to serious developmental defects that should not be the result of AdoR mutation (compared to complete viability of *adoR* mutants). Significant negative effects on larval development were also observed after strong induction of AdoR-shmiR1. Induction of AdoR-RNAi in transgenic lines obtained from VDRC resulted in complete embryonic lethality.

Impaired development of many induced RNAi lines led us to the conclusion that AdoR-RNAi constructs could also target other mRNAs that have impact on larval development. Notably, the stronger the induction is the more serious is the effect on development. Overcoming the "off-target" effect of AdoR-RNAi is a crucial step in successful targeted knock-down of AdoR and the subsequent interaction with *adgf-a* that would lead to the important discovery of extracellular adenosine responsive tissues. Since the strong AdoR knock-down is accompanied with the effects on other mRNAs we cannot exclude that the positive effect on *adgf-a* mutation could be superposed by these negative effects even in the case of induction with weaker or tissue specific Gal4 drivers. To overcome the "off-target" effect of RNAi we plan to test another microRNAi-based construct (AdoR-shmiR2) which we have now obtained as a transgenic line.

After utilization of the site-directed mutagenesis by RNAi stock centers, there have been several novel RNAi lines produced. VDRC has released VDRC-2 RNAi library of 'New Generation' transgenic lines that were created using the ΦC31 integrase system to integrate RNAi transgenes to specific landing site (P{attP,y[+],w[3']}VIE-260B) which has been chosen for its very low basal expression without Gal4 driver induction. The RNAi transgenes consist of inverted repeats to reduce the risk of off-target effects (Keleman et al., 2009.8.5). Unfortunately, for AdoR there are no 'New Generation' lines available. Drosophila RNAi screening center at Harvard University (DRSC) has created PhiC31 integrase-mediated RNAi construct against AdoR on the base of long dsRNA (P{TRiP.JF02687}attP2) and continues in production of shmiR based transgenes that introduce the second generation of the TRiP integrated constructs (Perkins et al., 2009).

We also suggest the utilization of other tissue-specific mutagenesis such as introduction of FRT sites within the genomic sequence of AdoR in its natural locus by homologous recombination that have previously been proven targetable when producing a loss-of-function *adoR* mutation by "ends-out" homologous recombination (Dolezal et al., 2005). Transgenic expression of UAS driven flipase enzyme that specifically recognizes the FRT sites will result in a deletion of a part of the gene leading to a truncated protein producing effective and specific knock-out. To avoid the laborious homologous recombination in flies, FRT sites could also be introduced into large genomic clone containing AdoR sequence in its natural surroundings by cheap and effective method of Red/ET recombination (Muyrers et al., 1999) followed by site-directed insertion of the modified clone into *adoR* mutant background (for details see part IV).

The data we have obtained so far about the influence of extracellular adenosine signaling on glycogen metabolism and fat body disintegration strongly suggest an important role of AdoR in the fat body (Zuberova et al., 2010). To investigate the direct role of AdoR in larval fat body without the possibility of using inducible knock-out we have prepared *adgf-a* mutant larvae with Gal4 driver induced production of *adoR* mitotic clones in the developing fat body. Since the fat body is polyploid tissue and the polyploidization starts in late embryonic/early larval development we had to choose the fat body Gal4 drivers with expression in early developmental stages to achieve the complete recombination in all chromosomes. For this purpose we prepared lines expressing C7-Gal4 and Cg-Gal4 in combination with UAS-FLP and FRT spaced *adgf-a* and *adoR* alleles (see fig. 10). Clonal analysis of glycogen content in *adgf-a* fat body cells possessing and lacking functional copy

of AdoR on pure yeast diet could verify the hypothesis for the role of AdoR on glycogen breakdown and glucose release directly on the cells of the fat body.

With the help of these proposed experiments we hope to be able to unravel the role of adenosine signaling on energetic status of *Drosophila* larvae and to distinguish between the tissue specific and systemic role of extracellular adenosine in the larval metabolism.

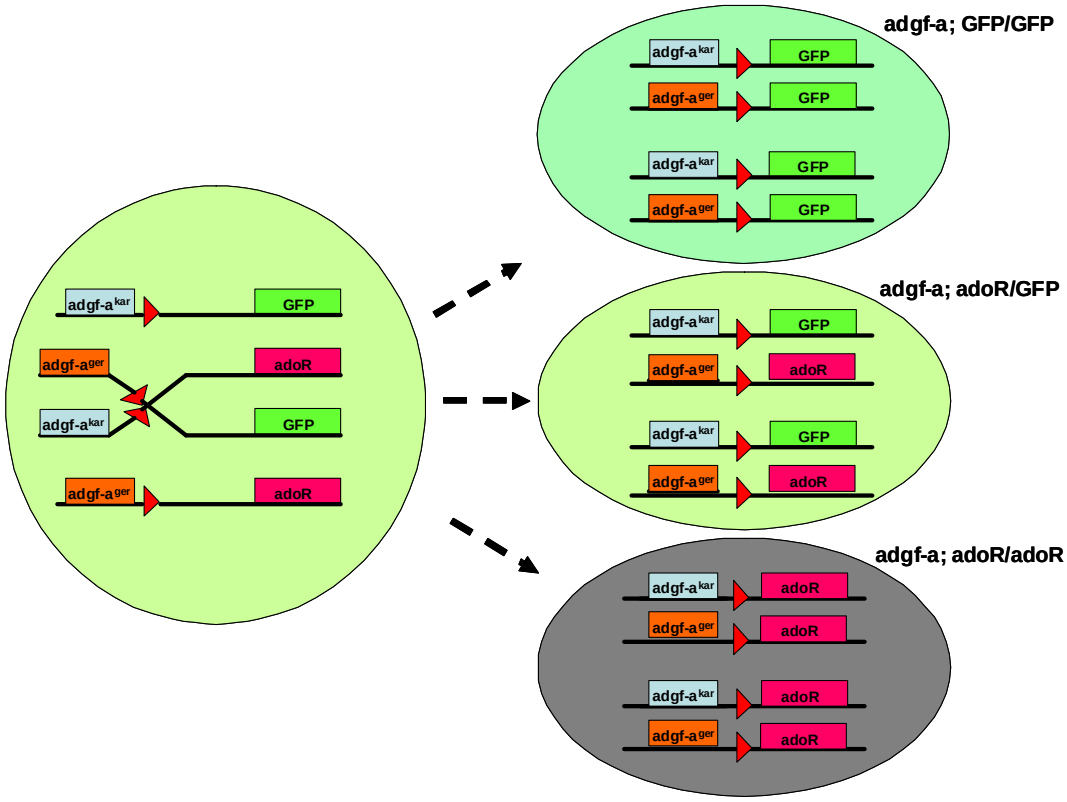

Fig. 10: Scheme of clonal analysis of adoR mutation in *adgf-a* background in fat body cells. Flipase-mediated mitotic recombination between the FRT sites on chromosomes of the same pair is induced in fat body precursor cells which results in exchange of chromatids bearing GFP and *adoR* in the background of *adgf-a* trans-heterozygous phenotype formed by *adgf-a*^{kar}/*adgf-a*^{ger}.

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Part IV:

Preparing of GFP reporters for live imaging of proteins involved in adenosine metabolism and signaling using FlyFos library and Red/ET recombineering

INTRODUCTION

Action of extracellular adenosine on tissues and cells is a very complex process and formation, degradation and signaling of extracellular adenosine needs precise and dynamic regulation. In *Drosophila*, these processes are orchestrated by a subset of different enzymes and proteins. (1) Adenosine producing enzymes that convert extracellular ATP to adenosine by sequential dephosphorylation of ATP to ADP, AMP and adenosine. These enzymes are well studied in mammals but we were first to report the characterization of *Drosophila* extracellular ATP dephosphorylation machinery (Fenckova et al., 2011). Adenosine production by ATP dephosphorylation is a two step process in which enzymes with ATP/ADP dephosphorylating activity are employed (apyrases, E-NTPDases; Zimmerman, 1992) followed by the action of AMP dephosphorylating enzymes (5'-nucleotidases; Zimmerman, 1992). We have found that *Drosophila* posseses homologs of three apyrase subfamilies: vertebrate CD39-like NTPase (encoded by CG3059, Knowles et al., 2009), 5'-nucleotidase family (CG42249, Fenckova et al., 2011) and Ca^{2+} dependent apyrase family (CG5276, Fenckova et al., 2011). Dephosphorylation of AMP is catalyzed by two members of 5'-ectonucleotidase family – NT5E-1 and NT5E-2 (Fenckova et al., 2011). (2) Degradation of extracellular adenosine is mediated by adenosine deaminases that catalyze the deamination of adenosine into inosine. In *Drosophila* adenosine deamination is mediated by proteins of Adgf family (homologs to human ADA2, Zurovec et al., 2002). The main adenosine deaminase during larval development is Adgf-A (Dolezal et al., 2005) but the other members of the family (Adgf-B, Adgf-C, Adgf-D) might play role in other developmental stages or are specific only for certain tissues. (3) Signal transduction of extracellular adenosine is mediated by adenosine receptor (AdoR, Dolezelova et al., 2007).

In order to study the dynamics of extracellular adenosine action, we decided to use a recently published method for generating of in vivo reporters of gene expression (Ejsmont et al., 2009) for the genes that are concerned in extracellular adenosine production, metabolism and signaling in *Drosophila*. This method combines three important approaches that make the transgenesis easier than ever before. (1) Library of large *D. melanogaster* genomic clones covering almost 90% of the annotated genes (FlyFos library). The average size of genomic DNA fragment subcloned in each fosmid clone is 36 kilobases (kb). Thanks to this size an integration of the FlyFos clone in *Drosophila* genome enables expression of almost any gene in the context of its intact cis-regulatory neighborhood. (2) FlyFos vector is compatible with the ΦC31 integrase-mediated site-specific transgenesis for introducing of large bacterial

plasmids or artificial chromosomes into *Drosophila* genome (Bischoff et al., 2007). (3) To introduce modifications such as addition of fluorescent tags FlyFos clones are adapted to liquid culture recombineering of DNA – Red/ET recombineering (Muyrers et al., 1999).

Using liquid culture Red/ET recombineering, we tagged 5 genes in FlyFos clones that have been already found to be active in extracellular adenosine production or have a high probability to be employed in the process (NT5E-1, NT5E-2, CG42249 – both CG42249-B and CG42249-C and CG11883). We have also tagged 4 adenosine deaminase genes (Adgf-A, Adgf-B, Adgf-C, Adgf-D) and *Drosophila* adenosine receptor (AdoR). All genes were tagged with C-terminal GFP allowing expression of tagged genes as fusion proteins for in vivo imaging of the protein localization and dynamics. We have sent the tagged FlyFos clones for site-specific injection into *Drosophila* attP lines and so far we obtained 5 transgenic lines: Adgf-A:GFP, Adgf-C:GFP, NT5E-1:GFP, CG42249-B:GFP, CG11883:GFP. Here we describe a pilot characterization of Adgf-A:GFP, NT5E-1:GFP and CG11883:GFP expression by observing GFP fluorescence.

MATERIAL AND METHODS

Transgenic vectors

We have modified large genomic clones from the FlyFos genomic library of *D. melanogaster* (http://transgeneome.mpi-cbg.de/index.php?id=42) by attachment of GFP sequence at the C-terminal of selected genes. We have selected clones based on modENCODE database of possible enhancer and insulator sequences of gene expression (www.modencode.org) to have highest probability of clones carrying the full regulatory sequences. Selected clones are listed in **table 1**.

PCR amplification of GFP tagging cassette

We have used S0062-R6K-2xTY1-GFP-FNF-3xFLAG plasmid (kind gift from Mihail Sarov, MPI-CBG, Dresden) for PCR amplification of C-terminal GFP tagging cassette. The cassette possess EGFP cDNA sequence without stop codon allowing attachment of GFP tag either on N-terminal or C-terminal end, Kanamycin resistance gene for selection of recombinant clones (neo), FRT sites for flip-out of Kanamycin resistance and rpsL for counterselection on Streptomycin. Complete scheme of the GFP tagging cassette is on **Fig. 1**. R6K plasmid does not replicate in T1-resistant EPI300 cells in which the FlyFos library is transformed because R6K needs binding of Pi protein encoded by the pir gene

to start replication and this is achieved only in pir+ E.coli strains (EPI300 is pir-). Therefore subcloning the tagging cassette in R6K plasmids eliminates possible "false-positives" that could result from transformation of the template plasmid DNA together with the PCR product and replication of the plasmid DNA without recombination of the PCR product.

HPLC purified oligonucleotides containing 25bp homology with 5' and 3' arm of the cassette and 50bp homology with the targeted genomic location were supplemented from biomers (www.biomers.net). The reverse oligonucleotide was designed to include the original stop codon at its 5' end allowing the expression of phusion GFP in frame with the tagged gene. In NT5E-1 and NT5E-2, GFP tag was integrated upstream from predicted GPI-anchor cleavage site. Oligonucleotide sequences are listed in **table 1**. PCR reactions were set up in 50µl and tagging cassette was amplified using Phusion High-Fidelity DNA Polymerase (Finnzymes). Annealing temperature was 55°C and the amplification step was repeated 25 times. 5µl of the PCR reaction was run on agarose gel and the rest 45µl was purified using Invisorb® Fragment CleanUp (Invitek) and electroporated directly without longer storing.

Liquid culture recombineering

Recombineering of FlyFos clones with the C-terminal GFP cassette was performed according to Ejsmont et al., 2009. Briefly, FlyFos clones were grown overnight in 1ml of LS-LB medium containing 15µg/ml Chloramphenicol. In the morning 1ml of TB medium was inoculated with 30µl of the overnight cultures. Bacteria were quickly grown to the exponential phase and transformed with 25ng of pRedFlp4 plasmid (Sarov et al., 2006) by electroporation (Day 1). Transformed bacteria were selected for presence of pRedFlp4 by growing in liquid culture containing 15µg/ml Chloramphenicol and 100µg/ml Hygromycin. On day 2 fresh cultures were set up in TB medium using 30µl of the overnight culture containing the FlyFos clone and pRedFlp4. After growing Red/ET recombination was induced from Red operon of pRedFlp4 by adding 25µl of 25% L-Rhamnose and PCR product was electroporated. Bacteria were selected for the recombination event in liquid culture containing 15µg/ml Chloramphenicol, 100µg/ml Hygromycin and 15µg/ml Kanamycin. After 2 days Kanamycin resistance gene was flipped-out by induction of Flp operon in pRedFlp4 with 200nM Anhydrotetracyclin. After flip-out bacteria were spread on agar plates containing 15µg/ml Chloramphenicol and single colonies were picked out and tested for loss of Kanamycin resistance by spreading on Kanamycin and Chloramphenicol agar plates

simultaneously. Clones growing only on Chloramphenicol but not on Kanamycin were tested for C-terminal GFP by sequencing.

Fosmid isolation and fly transgenesis

Bacterial stocks with modified FlyFos clones were sent for injection into attP2 or attP40 landing lines (see **table 1**). Both lines (Bloomington IDs: 25709 and 25710) express ΦC31 from nanos promoter and were used previously for FlyFos injection (Ejsmont et al., 2009). All transgenic constructs were injected by Genetic Services (www.geneticservices.com) with the following recommendation for fosmid isolation: Isolate the fosmid DNA from 100ml grown liquid culture after induction of fosmid replication with CopyControl™ Fosmid Autoinduction Solution (Epicentre) and inject the fosmid DNA directly without storing longer than 1 day in 4°C.

GFP fluorescence analysis

GFP fluorescence was analyzed in dissected 3rd instar larva using fluorescent stereomicroscopy and inverted microscopy. Hemocytes, melanotic capsules and parasitic wasp eggs were obtained by careful dissection of third-instar larvae in a drop of Ringer solution on a microscopic slide and immediately examined by one of fluorescent microscopic techniques. Samples were analyzed using differential interference contrast (DIC) and the Olympus U-MWG2 GFP filter settings. Micrographs were obtained using a color CCD, Olympus DP70 camera.

Immunohistology

3rd instar larvae were dissected in Ringer by turning the larva inside out leaving all larval organs attached to the cuticle and fixed for 1 hour at room temperature in 3,7% formaldehyde in PBS. Larval organs were then washed with several changes of PBST and blocked for 1 hour with 5% NGS. After blocking the samples were incubated overnight at 4°C with primary antibody and 1% NGS. 4F3 anti-discs large (from Developmental Studies Hybridoma Bank; dilution 1:1000) was used as primary antibody for staining of cell membrane surface (antigen: Discs-large PDZ2 domain). Primary antibody was washed out and the samples were incubated with secondary antibody (Goat anti-mouse conjugated with Cy3,5; Rockland; dilution 1:1500) for 2 hours at room temperature. Nuclear DNA was labeled by addition of DAPI during washing. Stained organs were dissected from the cuticle and mounted in VECTASHIELD® Mounting Medium (Vector Labs) on the microscope

slides. Samples were analyzed with Olympus FLUOVIEW FV1000 confocal laser scanning microscope.

Parasitic wasp infection

Second instar heterozygous ADGF-A>GFP larvae and control Oregon-R larvae were challenged by immunization with parasitic wasp *Leptopolina boulardi* for two hours. After the immunization the wasps were discarded and the larvae were allowed to develop for another 24-48 hours on the plate with cornmeal diet (8% cornmeal, 5% sugar, 4% yeast, 1% agar, 0.16% methylparaben) at 25°C. GFP fluorescence was analyzed in encapsulated wasp eggs and aggregating lamellocytes isolated from the 3rd instar larvae using differential interference contrast (DIC) and the Olympus U-MWG2 GFP filter settings. Micrographs were obtained using a color CCD, Olympus DP70 camera.

FlyFos clone ID	**Gene ID**	**Oligos sequence (5'>3')**	***D.melanogaster* landing line**
FF021854	Adgf-A	Fw: ATGTCAATGATCGTTCCTCCAACCGGGGATCCCC CAGTCAACGGATCGATgaagtgcataccaatcaggacccgc Rev: GTCATCAGGGAGAACGCAACCAGCAACGCTGCC ACCAACCGTGCTAGTCActtgtcgtcgtcatccttgtagtca	attP40
FF021854	Adgf-B	Fw: AGGAGAAGTGGGACAAGTGGATCGACGACGTGG TGGAGCACAAGTACTGTgaagtgcataccaatcaggacccgc Rev: AATGCGAAAATAAAAGGCAGCATGAGTTATTTA CTAAAAGGGGGTCATTActtgtcgtcgtcatccttgtagtca	attP40
FF019027	Adgf-C	Fw: AACTCAGCTGGTCGCGATTCATAGACGACGTATT GGAGGGCAGTGTATTTgaagtgcataccaatcaggacccgc Rev: AATGATTTATAAACTCTTTGGCTGTTTATTTTCAC TTATAAGTTCATTTActtgtcgtcgtcatccttgtagtca	attP2
FF019027	Adgf-D	Fw: TCCAGCGCAAGTGGCAGGAGTTTATTGCGAATG TCTTGAATCCAAAATTCgaagtgcataccaatcaggacccgc Rev: TACGTTTAGATTTAGACTAGATTTAGACTAGCAT TTAATCTTAGAACTTActtgtcgtcgtcatccttgtagtca	attP2
FF025050	AdoR	Fw: TGGGTCTGACCACCTCCTCGCCGTCCCTTCTGGC GACCAGCGCGGAGAGTgaagtgcataccaatcaggacccgc Rev: TCCAGTGGACTTTCCTGCTGATCCCGCTTCTTTTC CGACCGCGAAGGTCActtgtcgtcgtcatccttgtagtca	attP40
FF027290	NT5E-1 (CG4827)	Fw: TCGAGGAGTCGGATCCCTTCACCGAGAGCATGC AGCGAAACGATCTGAATgaagtgcataccaatcaggacccgc Rev:	attP40

		TCGATCTCGGGATACACGAAATGGCGCTGCTTCA GGTACTCCATGGTAGCcttgtcgtcgtcatccttgtagtca	
FF030888	NT5E-2 (CG30104)	Fw: TCCTGCTAGATGGTGGCGATGGACATGTAATGA GGGACTCAGCCCACCAAgaagtgcataccaatcaggacccgc Rev: TTCAGATATTGGGATACCGCCTCGAGATCGTTAT TTTGCAGGCGCTGTGGcttgtcgtcgtcatccttgtagtca	attP40
FF017908	CG42249-RB	Fw: ACATCAATCCCATTTACCAGGGCCTCGAGGGAC GCATCACAGTGCTCAACgaagtgcataccaatcaggacccgc Rev: AAATGCAATTATTGCACATTATGGATATAATACA TATGTATGTATAATCActtgtcgtcgtcatccttgtagtca	attP2
FF017908	CG42249-RC	Fw: CCGGGCCAATTACGACTGCAATCGAGCAAAGGA TTCAGTTTGTAACTACTgaagtgcataccaatcaggacccgc Rev: TATTTTATTATATAGGTTTACTACATTTTTCGACC ATACTATAAAGTCTActtgtcgtcgtcatccttgtagtca	attP2
FF022811	CG11883	Fw: TTTCCCGGAGGCATAGTCTGGTGCAGTGCCTGGA TAGTATGGATCTGGATgaagtgcataccaatcaggacccgc Rev: ATCGACTTGTTGTGATGGCCCACGGACAATTTGC GAATGGGCGATGGTCCcttgtcgtcgtcatccttgtagtca	attP2

Table 1: List of FlyFos clones used for tagging. Flybase ID's or synonyms are listed for each tagged gene within the clone. Oligonucleotides used for amplification of PCR cassette are listed in 5'>3' orientation. The part of the sequence written in capitals corresponds to 50bp homology with the clone. Part of the sequence common for all oligonucleotides corresponds to the tagging cassette and is written in lower-case. *D. melanogaster* landing lines used for injection of the clones are also listed here.

Fig. 1: Structure of the tagging cassette used for recombineering of FlyFos clones. The cassette alows recombination of EGFP either or N-terminal or C-terminal. The amplified region contains 2xTY1 and 3xFLAG sequences as internal tag for immunoprecipitation, EGFP sequence without stop codon. Kanamycin resistance and rpsL flanked with FRT for flip-out of the selection markers. Modified from Ejsmont et al., 2009.

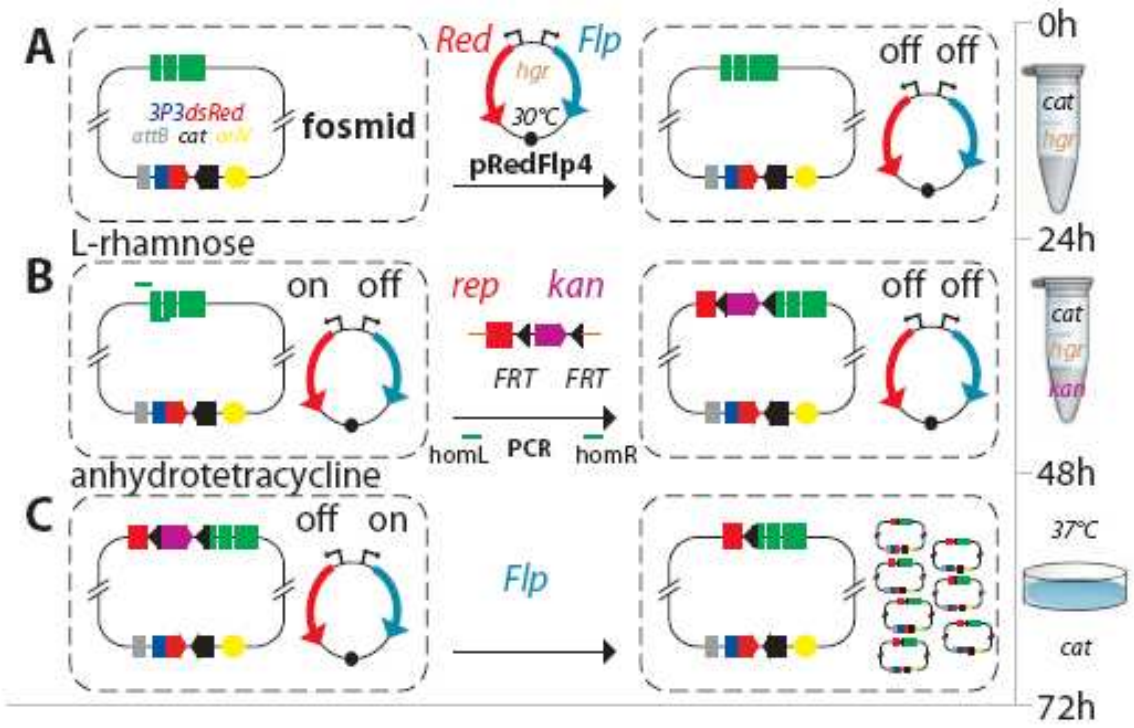

Fig. 2: Fosmid recombineering in liquid culture. *E. coli* cultures containing a fosmid clone are transformed with pRedFlp4 plasmid carrying an inducible *recA* operon making them competent for homologous recombination (A). Next, a PCR product carrying 50 bp homology arms surrounding the tagging cassette and *FRT* flanked resistance gene, Kanamycin (*Kan*), is electroporated into the cells. Only recombinant fosmids are able to grow efficiently in the presence of Kanamycin (B). The *Kan* gene is removed by inducing a flippase operon on the pRedFlp4 plasmid leaving the tagged transgene with a residual *FRT* sequence on the gene-tag boundary (C). Modified from Ejsmont et al., 2009.

RESULTS

Expression of Adgf-A>GFP

We have analyzed expression of Adgf-A:GFP by observing the fluorescence of GFP in Adgf-A:GFP/CyO 3rd instar larvae. We detected fluorescence in larval brain, midgut (as it is in agreement with FlyAtlas Anatomical Expression Data (Gelbart, W.M., Emmert, D.B. (2010.10.13): FlyBase High Throughput Expression Pattern Data Beta Version) and lymph gland recapitulating the tissue expression pattern of Adgf-A mRNA (Zurovec et al., 2002). To observe the cellular localization of Adgf-A:GFP, we analyzed the fluorescence of GFP in fixed larval tissues using laser scanning confocal microscopy. **Fig. 3 (A,B,C)** shows GFP fluorescence in midgut, lymph gland and in neuronal cells of the larval brain optical lobe. The background red colour is a result of dsRed expression driven by Pax promoter, a selective marker for fosmid transgenesis. We have also used DAPI staining and anti-discs large staining to mark the nuclei and cell membranes. GFP fluorescence of stained larval brain detected by laser scanning confocal microscopy is shown in **Fig. 3D**. The expression of Adgf-A:GFP seems to be localized to the surface of cell membranes or secreted in the vicinity of cells.

AGFP reporter of Adgf-A expression that has been recently generated in our laboratory shows strong and specific fluorescence in the melanization reaction of the parasitic wasp egg in accumulated plasmatocytes and lamellocytes (Novakova et al., 2011). Therefore, we challenged Adgf-A:GFP heterozygous larvae with infection of parasitic wasp *Leptopilina boulardi* under the same conditions as AGFP larvae. We observed that Adgf-A:GFP larvae express GFP specifically in the surrounding of the encapsulated egg and in melanizing tissues under immune challenge with the living parasite in the same manner as AGFP reporter (**Fig. 4**)

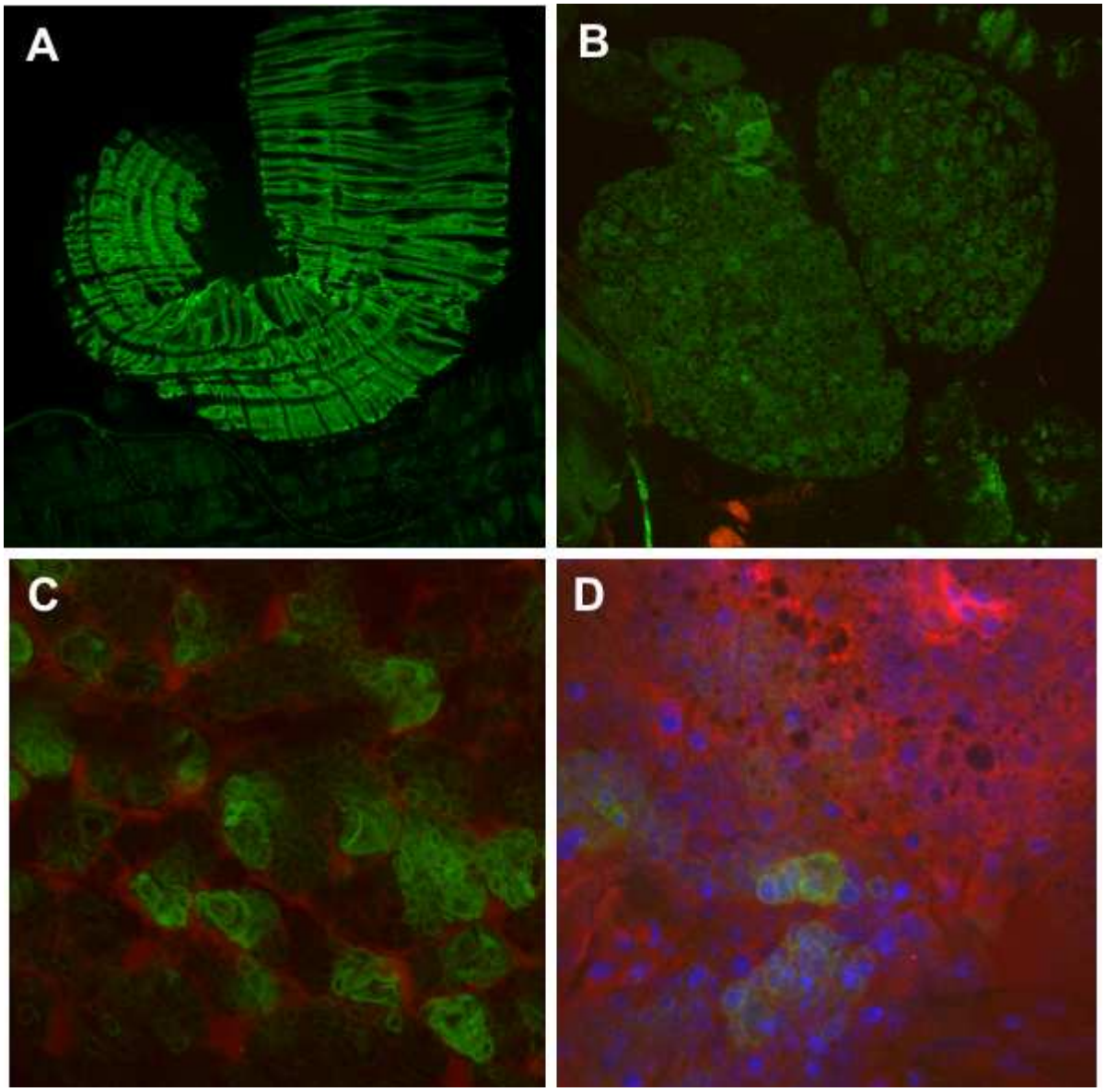

Fig. 3: Adgf-A:GFP expression detected by confocal scanning microscopy. (A) EGFP fluorescence in midgut (B) lymph gland and (C) cells of the brain optical lobe. (D) EGFP fluorescence in brain neural cells after staining of cell membranes with anti-discs large antibody (red) and staining of nuclei with DAPI (blue). All pictures were obtained at magnitude of 40x.

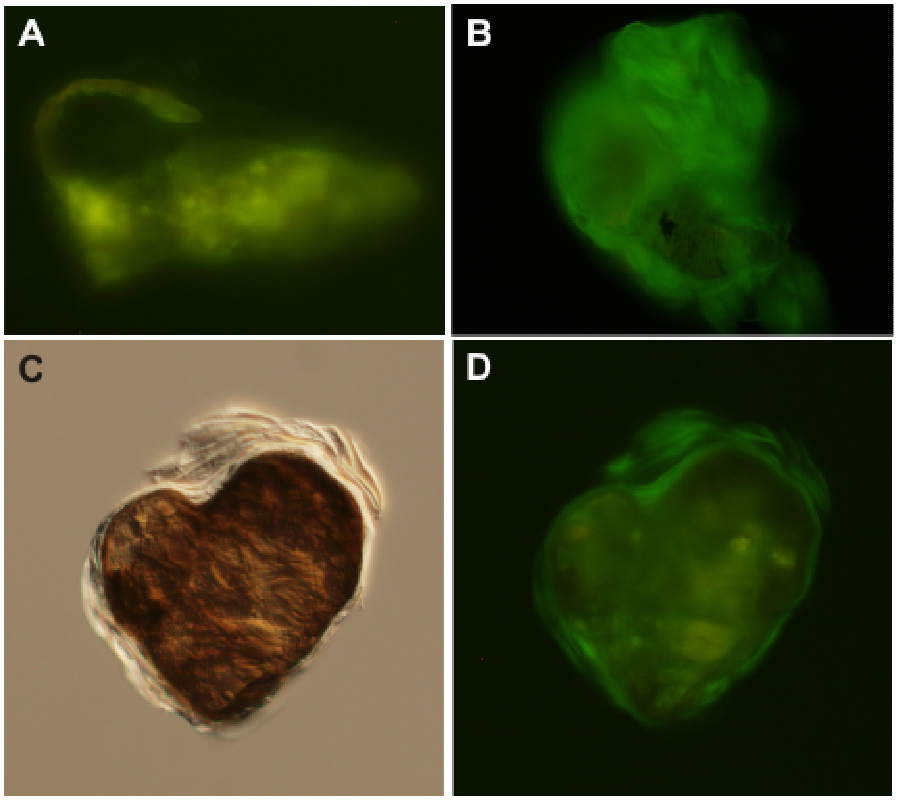

Fig. 4: Adgf-A:GFP expression during encapsulation of wasp egg. (A) Partially encapsulated wasp egg from Oregon-R larva displays only yellow background fluorescence of the wasp egg. (B) Partially encapsulated wasp egg from Adgf-A>GFP/+ larva exhibits strong expression of GFP in the encapsulating mass. (D) Other Adgf-A>GFP tissues that undergo melanization exhibit cleraly visible GFP fluorescence in the surrounding hemocytes. (C) Corresponding DIC image is shown in parallel.

Expression of NT5E-1:GFP

We have analyzed expression of NT5E1:GFP by observing the fluorescence of EGFP in NT5E-1:GFP homozygous 3rd instar larvae. We detected fluorescence in midgut and in the lumen of salivary glands. These observations are in agreement with FlyAtlas Anatomical Expression Data. We also analyzed GFP fluorescence of fixed tissues with laser scanning confocal microscopy (**Fig. 5A,B**) and expression in salivary glands after staining of cell membranes with anti-discs large antibody and DAPI staining of cell nuclei (**Fig. 5C**).

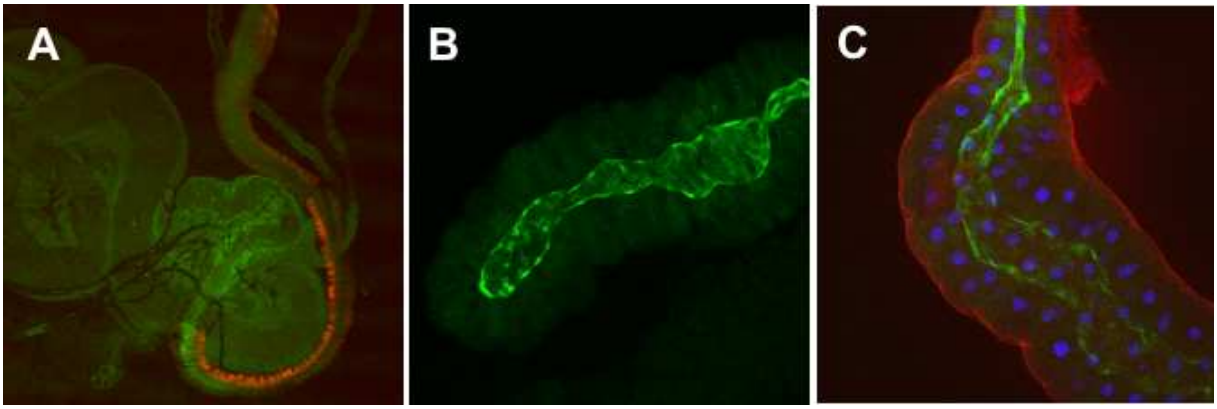

Fig. 4: NT5E-1:GFP expression detected by laser scanning confocal microscopy. (A) EGFP fluorescence in midgut. Red colour indicates expression of dsRed marker used for transgene selection. (B) EGFP fluorescence in the lumen of salivary glands after fixation. (C) Salivary glands expressing NT5E-1:GFP in the lumen after staining with DAPI (blue) and anti-discs large (red). All pictures were obtained at magnitude of 40x.

Expression of CG11883:GFP

We have analyzed expression of CG11883:GFP by observing the fluorescence of GFP in CG11883:GFP homozygous 3rd instar larvae. We detected fluorescence in CNS and eye/antenna imaginal discs. This observation reproduces data obtained from high-troughput FlyAtlas Anatomical Expression Data. FlyAtlas data also show moderate expression of CG11883 mRNA in fat body that was not detected by analysis of GFP fluorescence of CG11883:GFP. **Fig. 6** shows GFP expression in ommatidial and antennal part of eye disc analyzed with laser scanning confocal microscopy. In samples stained with DAPI and anti-discs large, GFP fluorescence surrounding the nuclei indicates possible localization of CG11883:GFP in the cytoplasm.

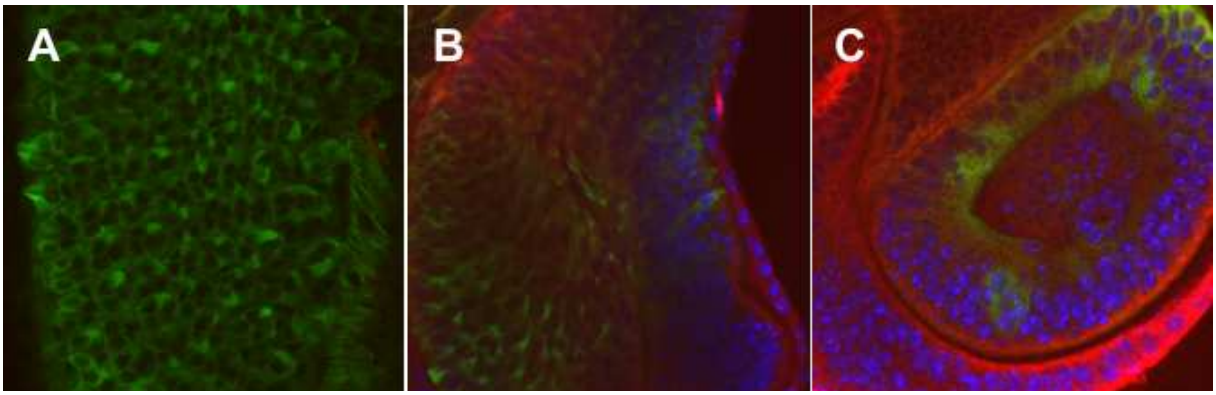

Fig. 5: CG11883:GFP expression detected by laser scanning confocal microscopy. (A) EGFP fluorescence in ommatidia precursor cells of eye disc from 3rd instar larva. (B) EGFP fluorescence in the cytoplasm of ommatidial precursors after DAPI (blue) and anti-disc large (red) staining. (C) EGFP fluorescence in the cells of antennal imaginal disc of 3rd instar larva after DAPI and anti-disc large staining. All pictures were obtained at magnitude of 40x.

DISCUSSION

Adenosine signaling is important in many physiological processes controlling the homeostasis, development and physiology either of the whole organism or on tissue/cell level. Our previous work shows that under certain conditions extracellular adenosine functions as an important signaling molecule stimulating the release of energy stores in order to overcome processes that are energetically costly such as immune response or stress (Zuberova et al., 2010). Utilization of in vivo GFP reporter of Adgf-A expression by "ends-in" based method of homologous recombination (Rong and Golic, 2011) allowed us to observe the dynamic changes in expression of main *Drosophila* adenosine deaminase, Adgf-A. Thanks to the AGFP reporter, Novakova and Dolezal (2001) showed that the level of Adgf-A expression dramatically raises in the sites of infection and hemocyte aggregation. Moreover, the changes in expression of AGFP reporter are very dynamic. This suggests that the regulation of extracellular adenosine level in the vicinity of immune cells is very dynamic. In mammals, modulation of inflammatory processes and immune responses is controlled by the ratio of pro-inflammatory signals (ATP/ADP) and anti-inflammatory signals (Adenosine) and requires the action of purinergic enzyme cascade by which the extracellular levels of purinergic signaling molecules and their signaling can be dynamically controlled. To reveal the dynamics of this purinergic cascade in *Drosophila*, we constructed a reporter system for in vivo expression of all genes regulating the extracellular adenosine production, signaling and degradation. With this system, we could be able to precisely study the action of purines on the immune cells into the detail and in the context of whole organism which has been incredibly missing in all mammalian studies.

Utilization of AGFP reporter using previously published method of "ends-in" homologous recombination showed to be very laborious and time demanding. Therefore, we decided to use a recently published method based on expression of GFP-tagged proteins from large genomic clones inserted in the wild type background. This work presents the results of pilot observations of GFP expression. Although the expression of reporters will require further verification, the data we have so far are very promising and predict the utilization of FlyFos reporters for dynamic in vivo expression studies of genes involved in adenosine signaling. The pilot observations of Adgf-A:GFP, NT5E-1:GFP and CG11883:GFP expression in 3rd instar larval tissues recapitulates the expression pattern of endogenous Adgf-A, NT5E-1 and CG11883. Moreover GFP fluorescence of Adgf-A:GFP precisely recapitulates the fluorescence of previously published AGFP reporter in aggregating

hemocytes during encapsulation of parasitic wasp egg showing the dynamics of Adgf-A:GFP reporter expression in the site of inflammation. Interestingly, all pilot studies were performed in heterozygous Adgf-A:GFP larvae because the homozygous individuals could be hardly found in the vial. This might be a result of incorrect dimerization of two Adgf-A:GFP molecules or one wild type Adgf-A with Adgf-A:GFP and therefore insufficient function of wild type Adgf-A mimicking the phenotype of *adgf-a* mutation (Dolezal et al., 2005). Although dimerization of *Drosophila* Adgf-A has not been proved, Zavialov et al. (2010) has described functional homodimer of human ADA2 (homolog to Adgf-A) suggesting this enzyme may function as a homodimer.

In mammals, the extracellular adenosine is produced by subsequent dephosphorylation of extracellular ATP released from the cells after damage or inflammation by membrane-bound ecto-nucleoside-triphophate/diphosphohydrolases and ecto-5'-nucleotidases. Whereas the main role of extracellular ATP is to induce the damage response and inflammation, the role of adenosine is to protect the tissues from a damage that would result from an excessive immune response. After activation of specific receptors, adenosine is depleted by adenosine deaminase. The ratio of extracellular ATP and adenosine requires dynamic regulation and adenosine producing and adenosine degrading enzymes are located in close proximity to each other (Yegutkin, 2008). Our previous studies indicated that NT5E-1 might function as the main *Drosophila* ecto-5'-nucleotidase and we suggested that similar mechanism of ATP/Ado ratio might drive the immune response in process of encapsulation of the parasite by larval hemocytes and that the expression pattern of Adgf-A:GFP and NT5E1:GFP may overlap during the encapsulation of the parasitic wasp egg. We found that NT5E-1:GFP is expressed exclusively in the lumen of salivary glands but GFP fluorescence was not detected in aggregating hemocytes after wasp parazitation. This suggests that the production of extracellular adenosine in activated hemocytes might be the results of the other *Drosophila* ecto-5'-nucleotidase (NT5E-2) or it might be produced as intracellular metabolite and released from the cells. Therefore we constructed NT5E-2:GFP reporter which is now being injected. Expression of other extracellular adenosine producing enzyme reporters (apyrases: CG42249, CG5276) can tell us more about the dynamics of extracellular adenosine production/degradation on the surface of activated hemocytes.

Not less important is the observation of in vivo expression of adenosine receptor (AdoR). We have shown that extracellular adenosine via AdoR increases circulating glucose in hemolymph and therefore it is the AdoR signaling that is responsible for the cell response to increased extracellular adenosine (Zuberova et al., 2010). We have come across several

difficulties when trying to uncover the role of AdoR in certain tissues (see part III). Utilization of AdoR:GFP reporter could help us to reveal the expression of AdoR in vivo and allow us to observe the fluorescence in living cells under inflammation and several types of stress. Modified FlyFos clone containing GFP-tagged AdoR can be further adapted to produce a conditional and tissue specific knock-out of AdoR. Using the technique of liquid culture recombineering in bacteria we should be able to modify the reporter construct by addition of one FRT site within the intron sequence of AdoR. Together with the flanked FRT sequence that resulted from the GFP tagging, we can flip-out a large piece of the gene directly within the integrated clone in the transgenic fly. When crossed in *adoR*$^{-}$ background, AdoR:GFP would be the only copy of functional AdoR in the fly. A conditional mutation can be induced by Gal4 driver activation of UAS-Flipase. The cells that loose the fluorescence will clearly indicate the loss of AdoR:GFP expression. Complete scheme of generation of conditional AdoR mutation is on **Fig. 6**. Unfortunately we have not obtained AdoR:GFP transgenic line yet and have not verified the expression of AdoR:GFP from the inserted FlyFos clone. There is a possibility that GFP tag can influence the localization and function of AdoR. To keep the proper expression of modified AdoR we could insert two FRT sites by a two-step recombination within the intron sequences of AdoR in original FlyFos clone and integrate such modified clone in the fly genome without the GFP tag.

Expression of AdoR:GFP from the integrated modified fosmid clone in *adoR*$^{-}$ genetic background can be also used in combination with the newly established method of GFP-tagged protein degradation by anti-GFP nanobody produced from the inducible transgenic construct (deGradFP). GFP nanobody is fused to F-Box protein that targets the GFP with its fusion protein to the proteasome and mediates proteasomal degradation of the whole complex. GFP nanobody combines the advantage of high affinity with high specificity and guarantee a complete degradation of GFP fused proteins without additional toxicity. The simplicity of this method is in basic cross of GFP-tagged line with F-Box-deGradFP line (Emmanuel Caussinus, EDRC, Lisbon, 2011, unpublished). Once the AdoR:GFP line is available, it could be utilized also for creation of conditional induced mutation by proteolytic degradation. This can be applied also to all the other adenosine cascade GFP-tagged proteins.

We hope that FlyFos-derived in vivo GFP reporters will represent a powerful tool in future research of adenosine signaling and will help to elucidate the plasticity of extracellular adenosine function in *Drosophila.*

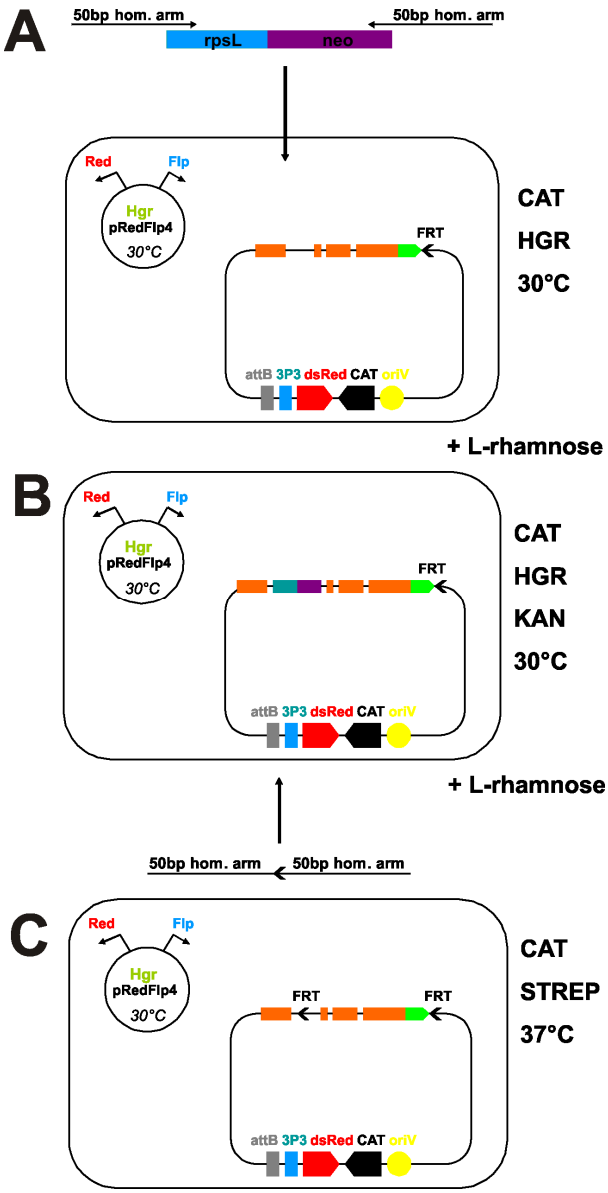

Fig. 7: Introducing an FRT site into intron I of AdoR:GFP modified fosmid using rpsL-based counter-selection. E.coli cells with AdoR>GFP are Strep^{R}. (A) In the first step rpsL-neo recombination cassette via 50bp homology arms is inserted by Red/ET recombination after induction of Red operon from pRedFlp4 plasmid. (B) Positive clones are selected on Kanamycin. Due to the additional wild type allele of rpsL, the clones become Strep^{S}. In the second step of recombination rpsL-neo cassette is replaced by Red/ET recombination against double-stranded or single-stranded fragment containing FRT sequence flanked with 50bp homology arms. (C) Positive clones become Strep^{R} and are selected on Streptomycin. By increasing the incubation temperature pRedFlp4 stops replicating and becomes diluted.

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CONCLUSIONS

This PhD thesis has contributed to elucidate some aspects of adenosine signaling in *Drosophila*. It is composed of two publications and unpublished results. One of the publications is a first author publication and the other is a co-author publication.

The main conclusions are as follows:

1. The identification of extracellular adenosine as a stimulator of glucose release in *Drosophila* and establishing a model for studying the effects of extracellular adenosine on energy metabolism. Identification of extracellular adenosine as an anti-insulin hormone and implication of adenosine signaling in wasting.
2. Functional characterization of *Drosophila* ecto-5'-nucleotidases and apyrases. Identification of two functional ecto-5'-nucleotidases (NT5E-1 and NT5E-2) and two *Drosophila* apyrases (CG42249 and CG5276). Discovery of extracellular ATP/Ado converting enzymes in *Drosophila* that allows us to study the opposite effects of ATP and Ado on the cells as well as the plasticity of ATP/Ado signaling.
3. Creation of in vivo GFP reporters of expression of the genes implicated in adenosine production, degradation and signaling that will hopefully become a powerful tool in studying the dynamics of extracellular adenosine functions.

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