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Regulation of extracellular adenosine level by ADGF-A in *Drosophila melanogaster*

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Annotation

Regulation of adenosine deaminase-related growth factor A (ADGF-A) expression was studied by preparing a vital GFP reporter system. Ends-in homologous recombination method was adapted to establish a functional reporter system used for localization of ADGF-A protein signal, as well as for clarification a conditions and regulation level of enzyme expression. This thesis uncovers a piece of puzzle on our understanding of adenosine signaling and its regulation during immune response in *Drosophila melanogaster*.

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Declaration

I hereby declare that I did all the work, presented in this thesis, by myself or in collaboration with the co-author of published article, and using only the cited literature.

České Budějovice, March 31, 2011 Milena Nováková

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Author contributions

Milena Novakova, as the first author of the presented paper, conceived and designed the experiments, performed the experiments, analyzed the data and wrote the paper.

Mgr. Tomas Dolezal Ph.D., as the co-author, conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

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Mgr. Tomáš Doležal Ph.D.

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Contents

Abstract

Adenosine is a key cellular metabolite but its extracellular form also serves as an evolutionary ancient signaling molecule. Improper regulation of adenosine levels leads to significant pathological consequences as severe combined immunodeficiency in humans. Therefore, we decided to use Drosophila model to investigate the regulation of adenosine levels through the adenosine deaminase activity by generating a reporter of the *ADGF-A* gene expression. We used ends-in homologous recombination method to create an expression reporter system by gene replacement. We precisely replaced the *ADGF-A* coding sequence with the coding sequence of a reporter gene, destabilized version of *GFP*, leaving all the surrounding endogenous regulatory sequences intact.

Using the reporter system, we showed that ADGF-A protein is most likely expressed at very low level under normal conditions. However, its expression is strongly increased in adhering hemocytes during immune response. Various mutants inducing melanotic capsules formation and also a real immune challenge by parasitic wasp indicate that the acute expression of the ADGF-A protein is not driven by one specific signaling cascade but is rather associated with the behavior of immune cells during the inflammatory response. We also showed that the ADGF-A expression is most likely regulated at post-transcriptional level by unknown mechanism.

General Introduction

Adenosine

Adenosine (Ado) is an endogenous purine nucleoside composed of a molecule of adenine attached through β-N_9-glycosidic bond to ribose sugar molecule (Figure 1). Adenosine is present in all living cells and its intracellular form is well-known in many biological processes as a metabolic precursor for nucleic acids, in energy transfer as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) as well as in intracellular signaling as cyclic adenosine monophosphate (cAMP). In addition to this intracellular role, adenosine in the extracellular compartment serve as an evolutionary ancient signaling molecule and contribute to the regulation of various biological processes, including hormone action, neural function, platelet aggregation, lymphocyte differentiation (NEWBY 1984), regulation of cardiovascular and ion channel activity and modulation of immune response (HASKO and CRONSTEIN 2004).

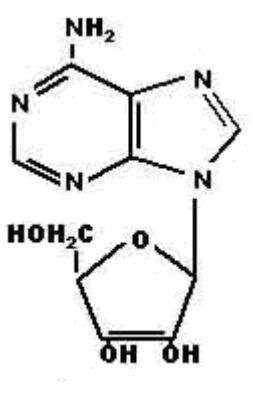

FIGURE 1. —Adenosine molecule.

Adenosine receptors

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

In humans, four adenosine receptor (ADR) subtypes were described and denoted as A_1, A_{2A}, A_{2B} and A_3. Adenosine receptors are expressed in a variety of cell types and their activity is determined by concentration of extracellular adenosine. A_1, A_{2A} and A_3 receptors are activated by low adenosine concentration between 0.01μM and 1 μM that correspond to physiological level, whereas A_{2B} receptor activation requires pathophysiological conditions where adenosine levels exceed 10 μM concentration (FREDHOLM *et al*. 2001; FREDHOLM 2007). Although cellular responses to adenosine are highly dependent on the extracellular adenosine concentration, several other factors, such as receptor density and localization or the functionality of the intracellular signaling pathways are also crucial (HASKO *et al*. 2008). Adenosine receptor signaling occurs through the inhibition (A_1, A_3) or stimulation (A_{2A}, A_{2B}) of adenylate cyclase (AC) with subsequent decrease or increase in intracellular cAMP concentrations, however it is known to be also linked to various other pathways including phospholipase C (PLC), protein kinase C (PKC), MAP kinase or phosphoinositide 3 kinase (PI3) (HASKO *et al*. 2008) (Table1).

TABLE 1. — Human adenosine receptors and effects on associated signaling pathways

Adenosine receptor	**A_1**	**A_{2A}**	**A_{2B}**	**A_3**
Associated signaling pathways	↓ AC	↑ AC	↑ AC	↓ AC
	↑ PLC		↑ PLC	↑ PLC
	↑ PKC			
	↑ PI3 kinase			↓ PI3 kinase
	↑ MAP kinase	↑ MAP kinase		
	↑ K^+ channels			
	↓ Ca^{2+} channels			

Drosophila and probably insect in general contains only a single adenosine receptor homolog (AdoR). The protein sequence of Drosophila AdoR is quite divergent from the mammalian and other insect species, except adenosine binding domain. It consists of 774 amino acids including a long (~350 amino acids), highly divergent C-terminal part of unknown function (DOLEZELOVA *et al*. 2007). DOLEZELOVA *et al*. (2007) also showed that AdoR is functional adenosine receptor that mediates activation of the c AMP/Protein

kinase A pathway in vivo. Although *AdoR* mRNA is present during whole fly development, with strongest expression in head part of Drosophila body, particularly in brain, ring gland, imaginal discs and salivary glands, precise localization of AdoR protein remain unknown (DOLEZELOVA *et al*. 2007).

Nucleoside transporters

Nucleoside transporters (NTs) are evolutionary conserved membrane proteins facilitating nucleoside transport across cell membranes (Figure 2) (MACHADO *et al*. 2007) and thus ensure cellular homeostasis. NTs are present in a diverse array of organisms and can be divided into two evolutionary distinct gene families based on mechanism of translocation substrate. The equilibrative nucleoside transporters (ENTs) family facilitates the bidirectional movement of nucleosides down their concentration gradients, while the concentrative nucleoside transporters (CNTs) actively transport nucleosides against their concentration gradient by unidirectional cotransport of cation (MACHADO *et al*. 2007). The human ENTs (hENTs) are represented by four members. hENT1, hENT2 and hENT4 are reported to show plasma membrane localization and they differ in type of transported molecules. hENT1 and hENT2 possess similar broad permeant selectivities for purine and pyrimidine nucleosides, but hENT2 also efficiently transports nucleobases. hENT4 is uniquely selective for adenosine, and also transports a variety of organic cations. hENT3 has permeant selectivity for nucleosides and nucleobases and appears to function in intracellular membranes, including lysosomes (YOUNG *et al.* 2008). The CNTs family has three members, hCNT1, hCNT2 and hCNT3. Na+-coupled hCNT1 and hCNT2 transport pyrimidine and purine nucleosides respectively, whereas hCNT3 mediates transport of both pyrimidine and purine nucleosides utilizing Na+ and H+ electrochemical gradients (SLUGOSKI *et al*. 2009)**.** Except the previously mentioned molecules, NTs transport also pharmacologically nucleoside analogues used as chemotherapeutic agents, and thus significantly affect the treatment of cancers and viral diseases (SLUGOSKI *et al*. 2009; GRIFFITH and JARVIS 1996).

Based on sequence similarity to known NTs, three ENTs and two CNTs have been identified in Drosophila (SANKAR *et al*. 2002). While we have poor information about Drosophila CNTs, ENTs have been partially characterized. Drosophila *ENTs (DmENTs)*

genes share with human orthologs only between 16-27% amino acid identity, however predicted structural motifs are remarkably conserved and thus implying similar function. *DmENTs* are located on chromosomes 2 and 3 and are simpler than human *ENT* genes with fewer exons and relatively small or nonexistent introns (REYES and COE 2005). *DmENT1* and *DmENT2* are expressed at differing RNA level at every stage of development with the exception of early embryogenesis, whereas *DmENT3* is nearly unexpressed. Analysis of all three DmENTs identified DmENT2 to be only one active nucleoside transporter. DmENT2 exhibit broad substrate specificity for nucleosides and nucleobases, suggesting that may be derived from an ancestral NT that was able to transport many substrates. Mutagenesis studies on Drosophila showed that null mutants of *ents2* are lethal during larval/early pupal stages, indicating that DmENT2 is essential for normal development. Hyphomorphic mutants in *ent2* are viable, but exhibit reduced associative learning caused probably by improper adenosine receptor activation (KNIGHT *et al*. 2010).

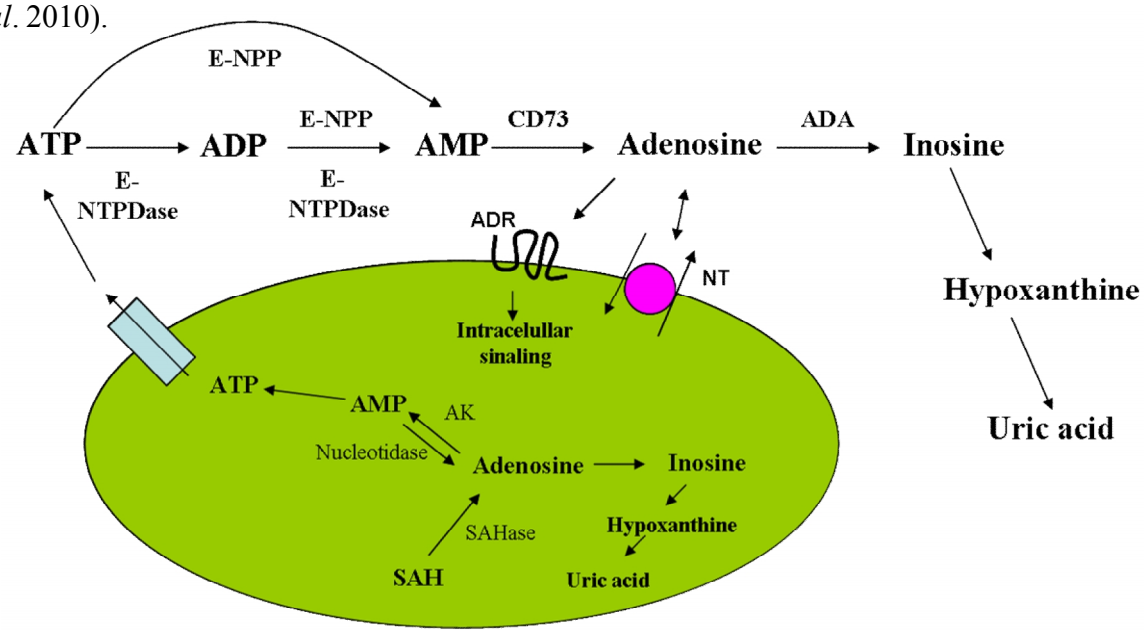

FIGURE 2. —Mechanisms underlying formation and degradation of adenosine. Extracellular adenosine can be derived either from adenine nucleotides sequentially broken down by ecto enzymes including CD 39 (E-NTPDase) and CD 73 (ecto 5-nucleotidase), or from intracellular adenosine exported by nucleoside transporters (NT). A transporter for ATP is also indicated. In addition, ATP can be exported from cells by exocytosis. ATP is obviously also appearing in the extracellular space when cells are damaged and die by necrosis. Adenosine is formed intracellularly from AMP by the action of an intracellular 5 - nucleotidase or from S-adenosyl homocysteine (SAH) by means of Sadenosyl homocysteine hydrolase (SAHase). The reaction between Adenosine and AMP is reversible through the action of adenosine kinase (AK). Adenosine is degradated by adenosine deaminase (ADA) to inosine and next to hypoxanthine and uric acid.

Ectonucleotidases

Concentration of extracellular adenosine can be increased indirectly by the action of purine inactivating enzymes including ecto-nucleoside triphosphate diphosphohydrolase CD39 (E-NTPDase) hydrolyzing nucleotide tri or diphosphates into nucleotide monophosphates, ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) digesting ATP and ADP to monophosphates and ecto-5´-nucleotidase converting nucleotide monophosphates to adenosine (Fig. 2).

5´-nucleotidases are terminal members in list of enzymes participating in conversion of ATP to adenosine. Seven humans 5´-nucleotidases have been characterize to date, five are located in cytosol, one in the mitochondrial matrix and one ecto-5´- nucleotidase is localized in outer plasma membrane. Ecto-5´-nucleotidase, otherwise know as CD73 consists of two non-covalently tethered glycoprotein subunits binding zinc ions on the N-terminal ends and with C-terminus anchored to the plasma membrane by glycosyl-phosphatidylinositol. CD73 is expressed in different tissues, with strongest expression in the colon, kidney, brain, liver heart and lung (YEGUTKIN 2008). Ecto-5´-nucleotidase is also expressed in circulating B and T lymphocytes in a dependence on cell maturity, while neutrophils, erythrocytes, platelets and other blood cells shows little or no expression (YEGUTKIN 2008). During the development of both T and B lymphocytes CD73 expression is increased and has been described as a lymphocyte maturation marker (THOMPSON *et al*. 1986). Abnormally elevated CD73 level serves as an indicator of variety of lymphomas and leukemias (GUTENSOHN and THIEL 1990) while low activity has been found on lymphocytes of patients suffering with a variety of immunodeficiency diseases characterized by a block in lymphocyte maturation (SALAZAR-GONZALES *et al*. 1985). In addition to B and T lymphocytes, CD73 was detected also on follicular dendritic cells, epithelial cells and endothelial cells (THOMPSON *et al*. 1990).

Preliminary analysis of the Drosophila genome revealed putative gene family including five 5´-nucleotidase genes *CG42249, CG4827, CG30104, CG30103* and *CG11883*. Ectonucleotidases RNAs are expressed during larval and adult stages with strongest expression in fat body, gut, tubules and brain. All nucleotidases were characterized as protein coding genes, some possess predicted signal peptide on the N- terminus, indicating different localization in cell. Two of these genes – *CG4827* (*NT5E-1*) and

CG30104 (*NT5E-2*) - encode bona-fide ecto-5'-nucleotidases (DOLEZAL pers. communication).

ADAs/ADGFs

Adenosine deaminases (ADAs) decrease Ado levels by irreversible deamination of adenosine and 2´-deoxyadenosine to inosine and deoxyinosine (Fig. 2). Two different ADAs, ADA1 and ADA2, are found in humans. *ADA1* gene is mapped on chromosome 20 and encodes 35-kDa protein with monomeric Zn-binding single domain. ADA1 is primarily cytosolic and is present in most body cells, particularly in lymphocytes and macrophages, and in erythrocytes. Although ADA1 does not express any signal sequence normally required for protein secretion, the enzyme has been also found in extracellular environment (ZAVIALOV *et al*. 2010a). The primary role of ADA1 embraces adaptive immune system development and T-cells proliferation. ADA1 reduce the intracellular level of adenosine, which is toxic for lymphocytes and thus protect cells from apoptosis (FRANCO *et al*. 2007). On the cell surface, ADA1 function via its interaction with dipetidyl peptidase IV/CD26 complex, which results in costimulation of T-cell proliferation (FRANCO *et al*. 1998; PACHECO *et al*. 2005). In lymphocytes, ecto ADA1 is associated with adenosine receptors A_1 and A_{2B} by means of them regulate actions of adenosine inside the cells (CIRUELA *et al*. 1996; HERRERA *et al*. 2001). Genetic absence of ADA1 causes dramatic increase of adenosine level, thereby triggers an apoptotic death of lymphocytes and inhibits their proliferation. ADA1 deficiency is generally known as a severe combined immunodeficiency disease (SCID) (HERSHFIELD 2005) characterized by dysfunction of B and T lymphocytes with impaired cellular immunity and decreased production of immunoglobulins.

The second, less investigated human adenosine deaminase is known as ADA2 (ZAVIALOV and ENGSTRÖM 2005). *ADA2* is encoded on chromosome 22 by *CECR1* (cat eye syndrome critical region 1) gene and belongs to novel family of Adenosine deaminase-related growth factors (ADGFs) (RIAZI *et al*. 2000). ADGFs were first described as growth factors in insects (MATSUSHITA *et al*. 2000) and later were found to possess the ADA activity (ZUROVEC *et al*. 2002). ADA2 has homodimeric structure with catalytic domain and two specific domains that mediate the protein dimerization and

binding to the cell surface receptors. Furthermore, ADA2 possesses several features like disulfide bonds, glycosylation sites and secretion signal sequences, strongly suggesting that enzyme is designed specifically to act in the extracellular environment (ZAVIALOV *et al*. 2010b). ADA2 is secreted by antigen presenting cells and induce differentiation of monocytes to macrophages and subsequent macrophages proliferation. This cytokine-like growth factor activity is mediated through binding to cell surface receptors. Ecto-ADA2 binds to different type of cells via proteoglycans and more specifically to T cells via ADRs or ADR proteoglycan complexes. Human ADA2 and ADA1 have different catalytic and biochemical properties. ADA2 has much lower capacity to catalyze adenosine deamination than ADA1 because of 100 fold lower substrate affinity and acidic pH requirement (ZAVIALOV and ENGSTRÖM 2005). This suggests that ADA2 functions only at high levels of adenosine and low pH, conditions associated with tumor growth, hypoxia and inflammation (ZAVIALOV and ENGSTRÖM 2005). However, at physiological conditions, ADA2 has enzymatic activity close to zero. Over production of ADA2 caused by duplication of region on chromosome 22 involving *CECR1* gene disturb the homeostasis of adenosine and result in cat eye syndrome (CES), a developmental disorder with multiple organ involvement (RIAZI *et al*. 2000).

Drosophila genome contains seven genes encoding adenosine deaminases divided into two evolutionary distinct subfamilies. ADA subfamily embraces only one member, an *ADA* gene-human ortholog of adenosine deaminase-like gene (*ADL*). During evolution, ADA lost its enzymatic activity due to accumulated mutations and it seems likely that the members of ADGFs family replaced its function (ZUROVEC *et al*. 2002). Six genes belong to ADGFs subfamily, known among many distantly related species. The founding member of the ADGFs family is insect derived growth factor (IDGF). The name of Drosophila ADGFs originated from its sequence similarity to ADA and to the IDGF growth factor family (ZUROVEC *et al*. 2002). ADGF members are found in three different chromosomal locations (Figure 3). *ADGF-E* gene maps to chromosome 2R and is composed of two exons. Two multiple exon genes, *ADGF-C* and *ADGF-D* are located on chromosome 3R and probably arise by chromosomal duplication followed by gene divergence. Three other genes are organized in tight cluster on chromosome 3L. *ADGF-A2* gene is included within the 5´ intron of *ADGF-A* and both genes share together first

exon. *ADGF-A2* and *ADGF-A* are transcribed in the same direction and are subject to alternative splicing. *ADGF-B* expression is regulated independently to *ADGF-A* and *ADGF-A2*, from own promoter sequence (MAIER 2001).

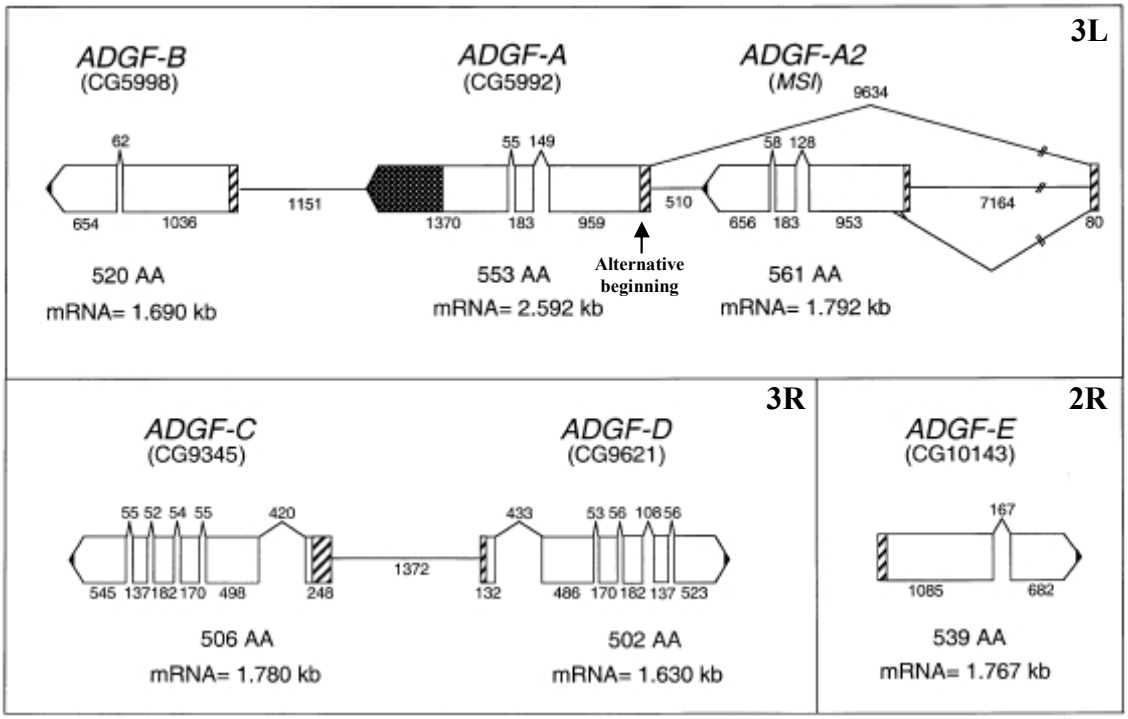

FIGURE 3. — Genetic structures an chromosomal localization of the six Drosophila ADGF genes (modified from MAIER *et al.* 2001). Open boxes represent coding exons, hatched boxes indicate 5´ UTR, and stippled boxes denote 3´ UTR. Introns are represented by chevron-shaped lines, intergenic distances appear as horizontal lines, numbers indicate base pairs of sequence. Exon and intron lengths are indicated below boxes and above chevrons, respectively. Numbers in the right corners represent chromosomal localization.

Developmental profile of ADGF genes showed that only *ADGF-A* is expressed through all development. *ADGF-D* gene is expressed only in postembryonic stages, with higher level in adult males. *ADGF-B*, *ADGF-E* and probably *ADGF-A2* are predominantly male specific. *ADGF-A* site-specific expression was found in gut and lymph glands, analogous to sites of human ADA enzyme activity. *ADGF-D* is expressed mainly in the larval brain and in the fat body, whereas *ADGF-A2* mRNA has testis specific expression and is restricted exclusively to mature primary spermatocytes (MATSUSHITA *et al*. 2000; ZUROVEC *et al*. 2002). No Specific expression of *ADGF-C* and *ADGF-E* was detected in

larval tissue and in adults, *ADGF-B* and *ADGF-E* are expressed only in male abdomen (ZUROVEC *et al*. 2002). ADGF proteins show 30-56% identity among themselves. Amino-terminus contains two conserved regions that have no similarity to other proteins, carboxy-terminus, on the other hand, show significant similarity to the sequence of ADA, especially in the conserved amino acid residues necessary for ADA catalytic activity (MAIER *et al*. 2001; WILSON *et al*. 1991). ADGF-A, ADGF-C and ADGF-D proteins have signal peptide indicating their function in extracellular space. ADGF-B and ADGF-E are predicted to possess mitochondrial targeting peptides, while ADGF-A2 is a membrane-bound protein with transmembrane domain (MATSUSHITA *et al*. 2000; MAIER *et al*. 2001). It was found that, three of six ADGF genes exhibit growth factor activity: ADGF-A, ADGF-A2 and ADGF-D. ADGF-A and ADGF-D are in addition classified as active ADA with ability to catalyze the hydrolytic deamination of adenosine or 2´-deoxyadenosine to inosine or 2´-deoxyinosine (MATSUSHITA *et al*. 2000; ZUROVEC *et al*. 2002). Deaminase activity is required for ADGFs mitogenic function, making them unique among growth factors. Functional analysis showed ADGF-A to be a main source of ADA activity during Drosophila larval development (DOLEZAL *et al*. 2005). ADGF-A is a homolog of human ADA2, nevertheless the capacity to catalyze adenosine deamination is much closer to ADA1. Null ADGF-A mutation causes extremely elevated levels of adenosine and deoxyadenosine in the larval hemolymph leading to larval death and developmental delay associated with the disintegration of fat body and the development of melanotic tumors. The *adgf-a* third-instar larvae also show seven-fold more hemocytes and altered hemocytes morphology than the wild type. Most of the mutant's cells are adhesive with filamentous and membranous extensions. In circulation appear also lamellocytes, large flat cells undetectable under normal conditions, and the crystal cells in elevated concentration. Larval hematopetic organ, lymph gland, is remarkably augmented during early larval stage and disperses earlier, in the late third instar. Homozygous pupae are also affected by mutant genotype. They usually die soon after puparization and most of them show abnormal curvature of the pupal abdomen (DOLEZAL *et al*. 2005).

The role of ATP and adenosine in immunity and inflammation

Purinergic signaling is crucial for modulation of inflammatory and immune response. Extracellular ATP and Ado are endogenous regulatory molecules that exert their immunological roles in cooperation with purinergic receptors, transporters and ectoenzymes expressed by immune and non-immune cells (Figure 2). Both ATP and Ado are so- called damage-associated molecular patterns (DAMPs), constitutively present at high intracellular and negligible extracellular concentrations under physiological conditions. Moreover, they are easily released in response to injury, infection or other inflammatory stimuli and consequently quickly degraded (BOURS *et al*. 2006).

ATP has predominant role at the onset of acute inflammation and at the initiation of primary immune responses upon encounter with infectious agents. Within cellular stress, ATP is released in high concentration from cell cytoplasm into extracellular space, where functions as a proinflammatory and immunostimulatory signal in the close proximity of damaged cells. During early inflammation, rising extracellular ATP levels as well as physiological Ado levels facilitate initiation of primary immune responses by stimulating chemotaxis, antigen uptake and maturation of dendritic cells, cytokines production, activation of neutrophils and macrophages, and proliferation and activation of lymphocytes. Mediators and oxidative stress inhibit expression of CD73 and preserve ADA activity, thereby sustain immunostimulatory nucleotide and nucleoside levels.

Upon progression of acute phase of inflammation, nucleotide-mediated signaling switch from being predominantly pro-inflammatory to being most immunomodulatory. In addition, extracellular Ado concentrations begin to rise by breakdown of excessive extracellular ATP and function as an anti-inflammatory mediator at this stage (BOURS *et al*. 2006). Extracellular ATP and Ado levels provide negative feedback mechanism to prevent cell damage and destruction of healthy tissue. Crucial components of purinergic feedback system are purinergic receptors as well as the ectoenzymes mediating progressive decrease in nucleotide concentrations and an increase in nucleoside concentrations. The expression of receptors and ectoenzymes by immune cells changes under inflammatory conditions. Tissue hypoxia arising during ongoing inflammatory responses decreases nucleotide transporter expression and increases the activity of both

CD73 and CD39, thereby promoting and maintain high Ado levels (LEDOUX *et al*. 2003). Activity of both CD73 and CD39 may also be enhanced by Ado itself. On the other hand, ADA activity is inhibited upon sustained inflammation (BOURS *et al*. 2006). Activation of purinergic receptors attenuates pro-inflammatory cytokine production by monocytes and macrophages, diminishes stimulatory effect of dendritic cells to T-lymphocytes, inhibits lymphocyte effector function and contributes to alternative activation of macrophages.

At the tissue-healing phase of immune response, extracellular Ado has a crucial immunosuppressive role. Inflammatory mediators progressively up-regulate the expression of specific adenosine receptors through which Ado mediates its immunosuppressive effects (CEPECCHI *et al*. 2005). Ado mediated signaling deactivates macrophages, suppresses proliferation and effector functions of lymphocytes, and promotes angiogenesis and tissue regeneration. Moreover, extracellular Ado influences secretion of non- immune cells, especially hepatocytes.

Stress conditions and whole process of immune response is extremely energetically costly and the process of energy supply restoration must be strictly regulated. High pool of extracellular Ado triggers liver gluconeogenesis and glycogenolysis via A3 receptor signaling into hepatocytes and thus increases blood glucose levels (GUINZBERG *et al*. 2006). Released glucose than serve as an excellent substrate to generate ATP as a source of energy.

A similar role of adenosine signaling through AdoR stimulating the glucose release was described for Drosophila fat body- a homolog of mammalian liver and adipose tissue (ZUBEROVA *et al*. 2010). Drosophila mutant in adenosine deaminase shows excessive Ado signaling leading to progressive lost of energy stores observed during certain chronic infections in both humans and flies (ZUBEROVA *et al*. 2010; DIONNE *et al*. 2006).

The role of Drosophila ADGF-A in immune response and its connection with fly metabolism is discussed in the second part of my thesis. The First part is focuses on the design and preparation of GFP reporter system, a tool for monitoring of ADGF-A expression.

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Part I

Development of GFP reporter system for ADGF-A expression monitoring using homologous recombination

Introduction to part I

Analysis of protein expression provides important information, relevant to cellular physiology, function and regulation. Genetic reporter systems represent extensive toolboxes to perform such analysis *in vivo*.

In Drosophila, several approaches to prepare expression reporters have been developed to date. The most frequently used approach is based on P-element-mediated germ-line transformation of reporter sequence under the control of the target gene promoter. Alternatively, fusion protein can be prepared and inserted into the genome in the same way. The one characteristic of P-elements is their random integration behavior that could lead to position effect and subsequently influence the expression analysis of transgene. A further shortcoming of this system is that several gene regulatory sequences can be too extensive to be successfully inserted into the genome by this way.

Two years ago, a new system for imaging of gene product in living flies was invented (EJSMONT *et al*. 2009). The base is a fosmid vector (pFlyFos) containing several features for efficient fly transgenesis. The attB sequence recognized by φC31 integrase is used for site-specific integration of the fosmid into the Drosophila genome. Integrase is capable of introducing large, bacterial artificial chromosome-sized construct into Drosophila genome, enabling functional analysis of any gene in the context of its intact *cis*-regulatory neighbor. Insulatory elements can be also placed into the construct to protect inserted region from possible position effect (MARKSTEIN *et al*. 2008).

At the beginning of project focused on generation of GFP reporter for monitoring ADGF-A, FlyFos system was not available and other approaches were unsuitable due to inability of inserting whole regulatory sequences together with GFP into the genome. Therefore, we decided to use homologous recombination (HR) system that brings some advantage contrary to previously used methods. HR technique permits precise targeting to homologous genomic sites and thus allows expression from original regulatory sequences. Consequently, it can be applicable to any genes irrespective to size of regulatory sequences.

Homologous recombination is a process found in all organisms from bacteria to the higher eukaryote as well as in viruses. The principle is nucleotide sequence exchange

between two similar or identical DNA (RNA) molecules, triggered by (double) strand breaks. The eukaryotic cells usually use this method for DNA breaks repair and thus to protect themselves against genome reconstitution. HR also produces new genetic combinations during meiosis and thus keeps genetic variability. For bacteria and viruses, homologous recombination represents a possibility to exchange genetic material between different strains and species through horizontal gene transfer.

Yeast and mouse were first and for a long time sole eukaryotic organisms where HR has been used as a technology of modifying endogenous genes. The reason why this method has not been promptly extended to other species was difficulty of introducing a linear DNA molecule, which is much more effective substrates for gene targeting than a circular form, into the germ line cells. Yeast transformation with linear DNA is very easy and works with high efficiency (ORR-WEAVER *et al*. 1981). To generate transgenic mice a linear DNA with desired target gene modification is transferred into the pluripotent, mouse embryo-derived stem (ES) cells. ES cells containing the altered genetic locus are injected into mouse blastocysts, which are in turn brought to term in surrogate mother (MÜLLER 1999).

In Drosophila, the problem of introduction linear DNA into the embryo was figured out by RONG and GOLIC (2000) 20 years later. They used yeast-derived site-specific endonuclease to perform donor linearization directly inside cells whereby they extended HR as a gene targeting method into Drosophila genetics.

HR method is based on replacement of chromosomal target gene by an extra chromosomal donor molecule. Generally, donor DNA is designed to have at least 2Kb long homologous sequence (altered by introduced modification-mutation or deletion) with targeted DNA, P-element ends, two FRT sites in the same direction, recognized by FLP recombinase, unique I-*Sce*I recognition site for rare-cutting endonuclease and genetic marker for transgene identification (BI and RONG 2003) (Figure1).

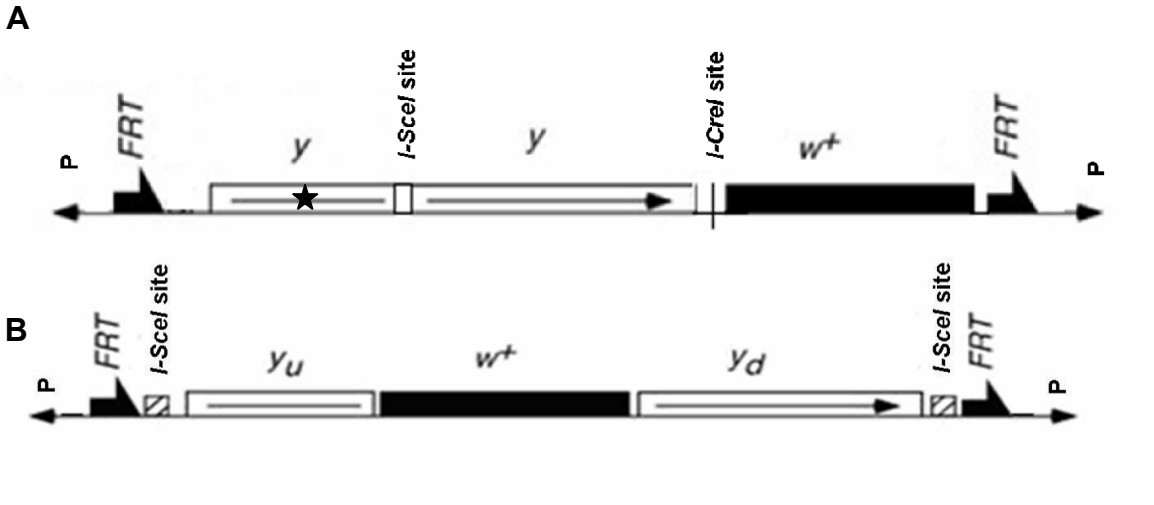

FIGURE 1.— Schematic constructs for (A) ends-in and (B) ends-out targeting. Locations of target (*y)* and marker (*w)* genes are indicated, along with the *FRT*s, the *I-CreI* and the *I-SceI* recognition sequences. The small arrowheads at the left and right ends of each construct indicate the *P* element inverted repeat termini. Asterisk indicates inserted mutation. Modified from GONG and GOLIC 2003.

The principle of HR in Drosophila is as follows:

Specially designed P-element construct (Figure 1) is injected into Drosophila embryos and randomly incorporated into the genome. Transgenic line carrying the construct is then crossed to flies with two, heat-inducible yeast enzymes (Figure 2A): FLP site-specific recombinase and SceI endonuclease. Three days old progeny of this cross are heat-shocked. FLP excises donor molecule from the chromosome as a circle that is then linearized by SceI enzyme. Linearized donor DNA search for homologous sequence in the genome to induce homologous recombination.

Two HR strategies can be used to modify a gene of interest, ends-in and ends-out. As the term refer, the differences between both approaches are done mainly by placement of I-*Sce*I site in a donor construct (inside (ends-in) or outside (ends-out) the homologous sequence), but number of crossing, marker position, presence of I-*Cre*I site and accuracy of recombination are also characteristic. In spite of the differences, both methods are generally used for the same purpose: to introduce a mutation or deletion into selected region or to rescue the mutant allele (DOLEZAL *et al*. 2003; XIE and GOLIC 2004; GONG

and GOLIC 2003; RONG and GOLIC 2000). The selection of appropriate method depends on a character of targeted region, localization of targeted gene or time investment.

Ends-in strategy was accomplished by Rong and Golic (2000) as the first of both methods. This approach is more complicated and laborious than the ends-out variant because it requires two steps of crossing (Figure 2; 4). First step results in integration of the entire donor molecule into the target, creating a tandem duplication of the target gene. Each of the duplicates consists partially of the original target and partially of the introduced fragment (RYDER and RUSSELL 2003). In a second step, the flies with target duplication are crossed with one expressing heat inducible endonuclease CreI. This site-specific nuclease induce a double strand breaks between the target gene copies at its cut site, previously inserted in the donor construct. This break induces recombination between the target copies, leading to reduction of the duplication to a single copy on the chromosome. This reduction event is recovered based on loss of the marker gene in offspring of heat-shocked flies. Two-steps design ensures that all exogenous DNA is eliminated from the altered locus. Therefore, ends-in strategy is preferred in several cases where precise replacement with no other remnants of the targeting procedure is required. This method of gene targeting can be used to introduce a mutation into alternatively spliced transcript without disrupting normal transcriptional regulation, to introduce a nonsynonymous mutation encoding amino acid replacement leading to changes of protein function or introduce a mutation into gene localized within the intron of another gene without impinging on the regulation of the second gene.

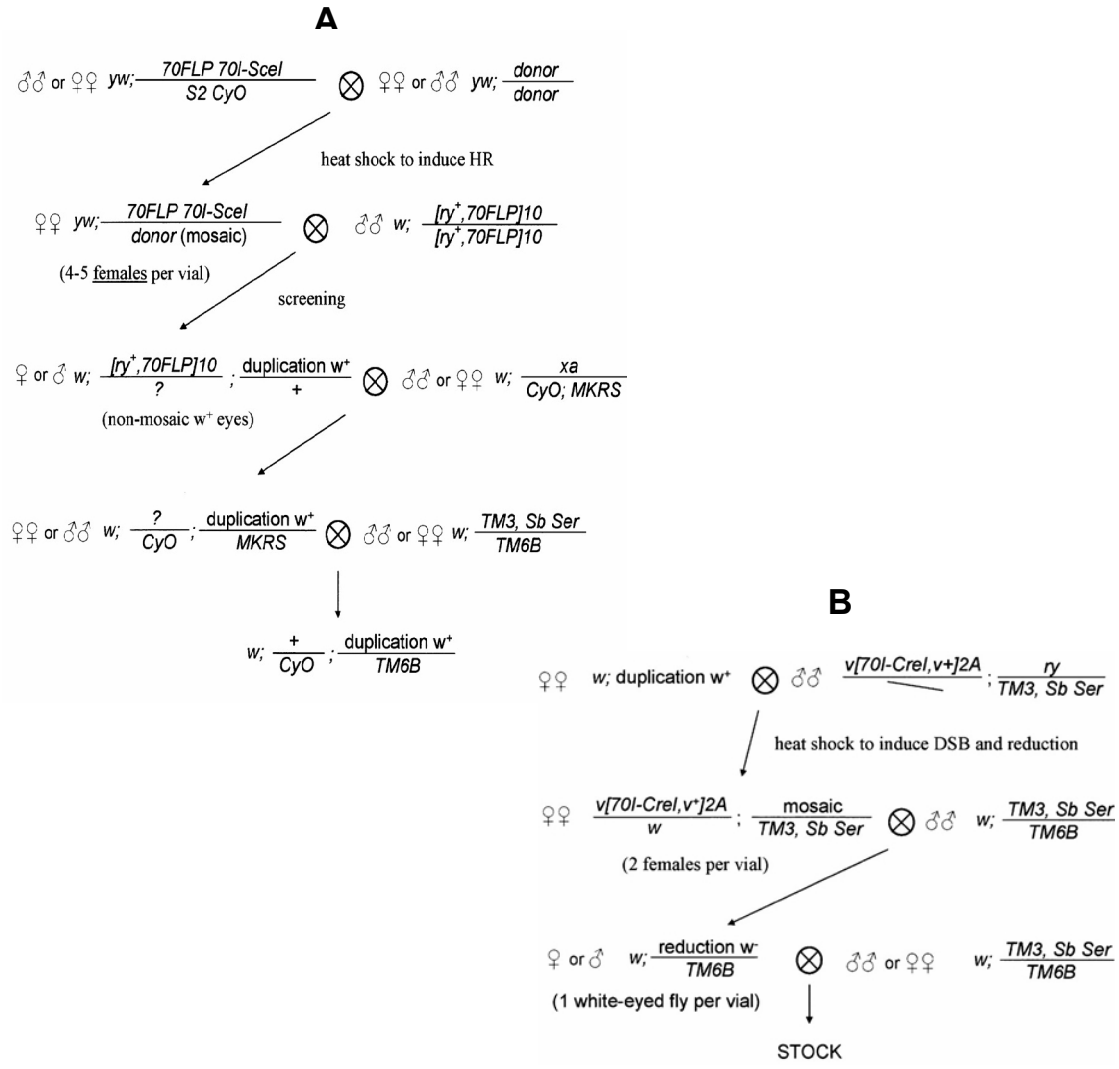

FIGURE 2.—Rapid targeting scheme used (A) for the first step of homologous recombination and (B) for the second step of reduction of the duplication to a single copy. Adapted from DOLEZAL *et al*. 2003.

Ends-out strategy is more straightforward variant of HR to generate a mutant allele, invented three years later (GONG and GOLIC 2003). In a single step of crossing the donor DNA is inserted into the target gene. I-*Sce*I site is designed out of the homologous sequence, so when DSB occurs, donor change to omega form and perform two crossovers for plasmid integration. In donor construct, marker gene is located inside the homologous sequence therefore the insertion is genetically marked and results in disruption of target gene. This positive selection can be useful for tracking the mutant allele in crosses but on

the other hand, it may cause undesirable side effects on the regulation of the target locus or its neighbors. This type of replacement is faster but less precise than the ends-in variant and it is usually used for gene disruption, gene rescue or making deletions.

As we previously mentioned, HR offers many possibilities for exploitation in gene targeting. Therefore we decided to use ends-in homologous recombination method (RONG and GOLIC 2000) to precisely replace the *ADGF-A* coding sequence with the coding sequence of a reporter gene (destabilized Green Fluorescent Protein, *dGFP*) leaving all the surrounding endogenous regulatory sequences intact (Figure 3).

In the following chapter, we describe molecular details of the replacement procedure.
This part of my thesis includes the extension of results published as supplemental information in "Expression of Drosophila adenosine deaminase in immune cells during inflammatory response" paper presented in Part II.

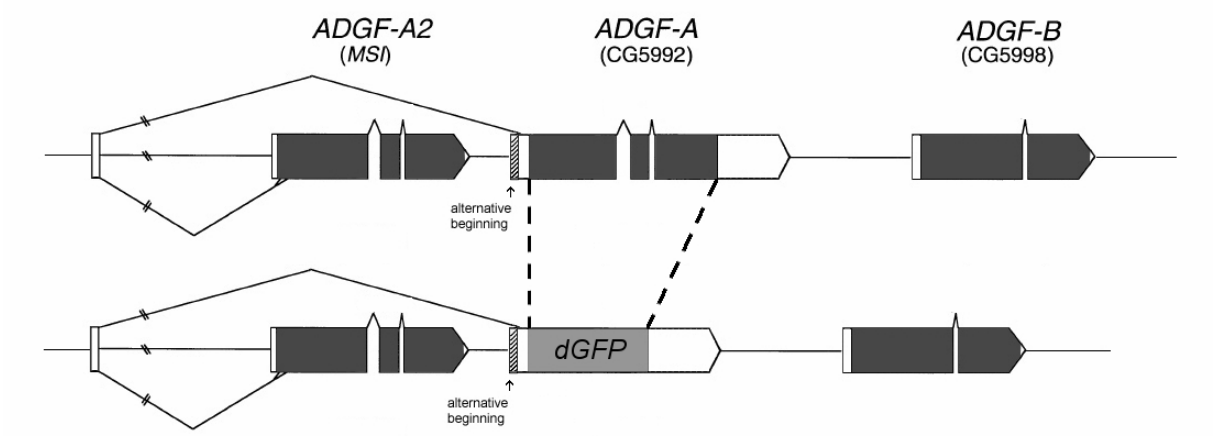

FIGURE 3.—Schematic diagram of the *ADGF-A* coding sequence replacement by the coding sequence of a reporter gene (encoding the destabilized GFP). The arrangement of three *ADGF* genes on the wild-type chromosome III is shown on top. The bottom part shows gene organization in the reporter system. Coding sequences are depicted by the dark boxes, white boxes indicate 5` and 3` untranslated regions of the genes. Introns are represented by chevron-shaped lines, intergenic spaces by horizontal lines. Dashed lines depict the replacement. 5'-3' orientation of all three genes is from left to right on the diagram. The *ADGF-A* gene shares the first exon with the *ADGF-A2* gene but it also uses an alternative transcription start, specific only for the *ADGF-A* gene marked by an arrow.

Results

Tato část práce (strana 23-30) obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě.

Discussion

Tato část práce (strana 31-34) obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě.

Materials and Methods

Tato část práce (strana 35-36) obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě

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Part II

Expression of Drosophila adenosine deaminase in immune cells during inflammatory response

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PLoS one

Expression of Drosophila Adenosine Deaminase in Immune Cells during Inflammatory Response

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Abstract

Extra-cellular adenosine is an important regulator of inflammatory responses. It is generated from released ATP by a cascade of ectoenzymes and degraded by adenosine deaminase (ADA). There are two types of enzymes with ADA activity: ADA1 and ADGF/ADA2. ADA2 activity originates from macrophages and dendritic cells and is associated with inflammatory responses in humans and rats. Drosophila possesses a family of six ADGF proteins with ADGF-A being the main regulator of extra-cellular adenosine during larval stages. Herein we present the generation of a GFP reporter for ADGF-A expression by a precise replacement of the *ADGF-A* coding sequence with *GFP* using homologous recombination. We show that the reporter is specifically expressed in aggregating hemocytes (Drosophila immune cells) forming melanotic capsules; a characteristic of inflammatory response. Our vital reporter thus confirms ADA expression in sites of inflammation in vivo and demonstrates that the requirement for ADA activity during inflammatory response is evolutionary conserved from insects to vertebrates. Our results also suggest that ADA activity is achieved specifically within sites of inflammation by an uncharacterized post-transcriptional regulation based mechanism. Utilizing various mutants that induce melanotic capsule formation and also a real immune challenge provided by parasitic wasps, we show that the acute expression of the ADGF-A protein is not driven by one specific signaling cascade but is rather associated with the behavior of immune cells during the general inflammatory response. Connecting the exclusive expression of ADGF-A within sites of inflammation, as presented here, with the release of energy stores when the ADGF-A activity is absent, suggests that extra-cellular adenosine may function as a signal for energy allocation during immune response and that ADGF-A/ADA2 expression in such sites of inflammation may regulate this role.

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Introduction

Extra-cellular adenosine is an important regulatory molecule with a low physiological concentration that can rapidly increase during tissue damage, inflammation, ischemia or hypoxia. In damaged tissues and inflammatory responses, extra-cellular adenosine is a product of ATP degradation, mediated by a cascade of ectoenzymes. Both ATP and adenosine stimulate purinergic receptors, and their release, signaling and progressive decrease regulate the onset of the acute inflammatory response, the fine-tuning of ongoing inflammation and its eventual down-regulation [1]. However the overall regulation of inflammatory responses by ATP and adenosine is quite complex, especially in mammalian adaptive immunity, and thus many questions regarding the roles of ATP and adenosine persist.

An important step in this regulation is the degradation of adenosine by adenosine deaminase (ADA), an enzyme that converts adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. Generally, two types of enzymes with the ADA activity are known; the ADA1 family proteins and adenosine deaminase-related growth factors (ADGF) or ADA2-like proteins. ADA1 is present both in prokaryotes and eukaryotes and has been studied for long time, mainly because its deficiency in humans causes severe combined immunodeficiency (SCID) syndrome. Although the most studied function of ADA1 is the reduction of toxic intracellular levels of adenosine, especially for lymphocytes [2], ADA1 has also been detected as an ectoenzyme associated with cell surface receptors [3].

Although ADA2 activity in human plasma was discovered long time ago [4], the protein responsible for this activity was only recently isolated [5]. ADA2 (ENSG00000093072) is a secreted protein and adenosine seems to be its only substrate [6]. ADA2 activity is significantly increased in the pleural effusions of tuberculosis patients [7] and the serum of patients infected with HIV [8]. It was also shown that macrophages are the source of ADA2 activity during inflammatory responses in rats [9]. Zavialov *et al.* [10] showed that ADA2 protein secreted by monocytes undergoing differentiation is the only source of ADA activity from these cells. They further revealed that human ADA2 promotes $CD4^+$ T cell-dependent differentiation of monocytes to macrophages and their subsequent proliferation and that this role of ADA2 is independent of its ADA catalytic function.

ADA2 belongs to the family of ADGF proteins, first characterized in insect [11]. The ADGF-A protein (FBgn0036752)

from *Drosophila melanogaster* is similar to secreted human ADA2 [12] and both proteins share all structure domains, including those considered unique to ADA2 [13]. However, ADGF-A, as with other ADGFs from lower species, has a higher affinity for adenosine (similar to ADA1) than human ADA2. According to Zavialov *et al.* [13], human ADA2 may have become specialized during evolution to be an adenosine deaminase specifically active in sites of high adenosine concentration and lower pH, typified by sites of inflammation.

We have showed that *ADGF-A* mRNA is expressed in the Drosophila hematopoietic organ, called the lymph gland [12] and that this expression is required for larval survival [14]. Drosophila hematopoiesis and cellular immunity are much simpler than in vertebrates, nevertheless both systems share many features [15]. The main component of cellular immunity in flies is represented by plasmatocytes that are macrophage-like cells with phagocytic activity. These cells are responsible for the inflammatory response to tissue damage (clearance of tissue debris and healing) and infection. In similarity to vertebrate systems, these macrophage-like cells are attracted to sites of injury where they adhere and become phagocytic [16]. This ability to recognize and adhere to damaged or "nonself" tissue is an ancestral feature of blood cells. In the case of larger objects, such as parasitic wasp eggs, specialized cells called lamellocytes (large flat cells) differentiate from prohemocytes and encapsulate the foreign object, thus isolating it from the rest of the body cavity. The intruding object is then destroyed by melanization; an important immune mechanism in arthropods utilizing toxic quinone substances and other short-lived reaction intermediates [17]. These substances are also involved in the formation of more long-lasting products such as the melanin that physically encapsulates pathogens. Furthermore, reaction intermediates in the melanin pathway participate in the wound healing process by the formation of covalent links in damaged tissues and results in sclerotization.

Since hemocyte *ADGF-A* mRNA expression is required for larval survival, we were interested in the regulation of its expression. However, because there is no available antibody against ADGF-A, we decided to produce a vital GFP reporter for its expression using homologous recombination. Here we show that this reporter is expressed in vivo, in aggregating larval hemocytes at sites of inflammation and that the acute expression of the ADGF-A protein is most probably regulated at post-transcriptional level.

Materials and Methods

Drosophila strains and culture

The AGFP reporter was produced as described in File S1. The line depicted as *AGFP[23]*, was used for the experiments presented herein; this line was kept as *w; AGFP[23]/TM6B* and *w; AGFP[23]/TM3 Act>GFP Ser* and crossed to the wild-type *Oregon R* strain to obtain *AGFP[23]/+*. Additional lines used in this work were as following: *Oregon-R* as wild-type strain, *w; adgf-a^{karel}/TM6B*, *w; cactus[E8]/CyOGFP*; *AGFP[23]/TM6B*, *w; cactus[IIIG]/CyOGFP*, *w; cactus[D13]/CyOGFP* and *y[1] v[1] hop[Tum]/FM7c*. All flies were raised on corn meal/sucrose/agar medium and kept at 25°C.

Wasp parazitation

w; AGFP[23]/TM6B flies crossed to *Oregon-R* flies and only *Oregon-R* flies were left to lay eggs for 12 hours in regular food-containing vials. The second-instar larvae were then immunized by a parasitic wasp *Leptopilina boulardi* for two hours. The wasps were then discarded and the infected larvae permitted to continue their development for another 24–48 hours at 25°C. GFP fluorescence was then analyzed in encapsulated wasp eggs dissected from the third-instar larvae, as described below.

GFP fluorescence analysis

GFP fluorescence was analyzed in dechorionated embryos, whole and dissected larvae and pupae and in adult flies using fluorescent either 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.

Expression analysis by RT-PCR and Real-time PCR

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, Ohio) according to manufacturer's instructions. RNA was isolated from *w; AGFP[23]/TM3 Act>GFP Ser* stock in the following hours after egg laying: 14–18 h (embryos), 34–38 h (first-instar larvae), 58–62 h (second-instar larvae), 82–86 h (third-instar larvae) and from the third-instar wandering larvae, white prepupae, pupae 26–34 h after pupation and three days-old adult males and females. cDNA was generated from 2.4 µg of RNA treated with TURBO DNase I (Ambion, Austin, TX) using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) with oligo(dT) primering. *Actin* amplification was used to determine the final dilution of cDNA from each sample for the PCR reactions which were performed using the following primers: 5′-TACCCCATTGAGCACGGTAT-3′ and 5′-GGTCATCTTCTCACGGTTGG-3′ for *Actin*, 5′-AATCGGAGCTCCTCAATCGC-3′ and 5′-GCTACACATTGATCCTAGC-3′ for *AGFP*, 5′-AGGTTCTCATCCACAGTGG-3′ and 5′-CGGACTACTACTACAAAGC-3′ for *ADGF-A*.

Third-instar wandering larvae of the following genotypes were used for Real-time PCR analysis: *AGFP[23]/+*, *AGFP[23]/AGFP[23]*, *cactus[IIIG]/cactus[E8]*; *AGFP[23]/+*, *cactus[D13]/cactus[E8]*; *AGFP[23]/+*. Total RNA was isolated (1) from 60 whole larvae using TRI Reagent and (2) from hemocytes as follows: 15–30 larvae were dissected one at a time in Ringer solution in 1.5-ml tubes and the hemolymph collected (the remaining body was discarded). The hemolymph were then centrifuged for 5 min at 1500×g, the supernatant discarded and total RNA isolated from the collected hemocytes using the RNAqueous-Micro kit (Ambion, Austin, TX). The RNA was then treated with TURBO DNase I. The concentration of RNA was determined by NanoDrop ND1000 (Thermo Fisher Scientific Inc., Waltham, MA) analysis and cDNA synthesized using the SuperScript III Reverse Transcriptase kit with oligo(dT) primering. The cDNA was then treated with RNaseH (Takara, Japan). Samples were analyzed by Real-time PCR using 1× Syber green Supermix (Biorad, Hercules, CA) and 5 pmol of each primer in CFX96 Real Time System C1000 (Biorad) as triplicate measurements. The following primers were used: 5′-CTTCATCCGCCACCAGTC-3′ and 5′-CACGTTGTGCACCAGGAA-3′ for *Rp49*, 5′-GGATCCCCCAGTCAACGG-3′ specific for *ADGF-A*, 5′-TGCTTCTGCTAGGATCAATGTGTA-3′ specific for *AGFP* and 5′-CTGAGTGGATGCGAATGAGAGTG-3′ common for *ADGF* and *AGFP*. Biorad CFX Manager software was used to quantify transcript levels by comparison to relative standard curves generated for each gene by serial (5×) dilutions of Drosophila genomic DNA. Levels of *ADGF-A* and *AGFP* were normalized with *Rp49* levels from the same cDNA samples and plotted as *ADGF-A*

or *AGFP* expression level relative to *Rp49*. Results are shown as mean ± SEM of three independent experiments. The experimental significance was determined using a Student's *t* test utilizing the STATISTICA 6 software (StatSoft) package.

Results

Generation of the *ADGF-A* reporter system

We used homologous recombination to produce a GFP reporter for the analysis of endogenous, in vivo, levels of *ADGF-A* expression by the precise replacement of the *ADGF-A* coding sequence with that of *GFP* (Figure 1). This therefore ensured that all the surrounding regulatory sequences, including 5′ and 3′ untranslated regions, of *ADGF-A* locus remained intact. We utilized a destabilized version of the Green Fluorescent Protein (*dGFP*) [18], that makes it possible to analyze dynamic changes in *ADGF-A* expression since the dGFP does not accumulate in cells as it is turned-over in around 2–4 hours.

We used the *'ends-in'* version of homologous recombination [19] that permitted precise exchange of the coding sequences in two steps and without leaving any traces of the recombination process. The first recombination step produced a duplication in the *ADGF-A* containing region, with the *ADGF-A* coding sequence at one side and the *dGFP* coding sequence on the other side (File S1). The second recombination step resulted in a reduction (File S1) of this configuration, thus producing the sequence assembly as shown in Figure 1. Details of the whole procedure are described in File S1.

We obtained a total 12 lines exhibiting the correct replacement as judged by the successful hybridization of the *dGFP* probe to DNA fragments of correct size in Southern blotting analysis (Figure 2). We named this replacement as *AGFP* (for *ADGF-A GFP* reporter). The Southern blot results were further confirmed by PCR and sequencing (data not shown). In two lines (8-4 and 74-3; Figure 2), the second recombination event occurred downstream of the *ADGF-A/dGFP* sequences restoring a wild-type gene arrangement (File S1). This was confirmed by PCR analysis resulting in an amplification of the *ADGF-A* fragment size instead of the shorter *dGFP* sequence (data not shown).

Since the replacement of the *ADGF-A* coding sequence results in a null mutation of this gene we examined the homozygous *AGFP* or heterozygous *adgf-a/AGFP* larvae and found that they exhibited the same phenotype as the originally described *adgf-a* mutant [14] (see further); thus providing confirmation at the phenotypic level that the replacement successfully occurred within the *ADGF-A* locus.

Expression of *AGFP* mRNA

Our molecular characterization (Figure 2 and File S1) confirmed that the coding sequence of the *ADGF-A* gene had been accurately replaced by the coding sequence of *dGFP*. Therefore, we further analyzed if the expression of *AGFP* reporter mRNA corresponded to the endogenous *ADGF-A* expression.

ADGF-A mRNA is normally expressed throughout all stages of development [12] (Figure 3) and similarly, the *AGFP* mRNA was also expressed at all stages (Figure 3). This demonstrated that our *AGFP* reporter expression system was able to faithfully report the temporal expression of the endogenous *ADGF-A* gene.

Moreover Figure 4A demonstrates that the overall levels of mRNA are similar ($p = 0{,}286$) for both *ADGF-A* and *AGFP* transcripts when measured in samples obtained from the *AGFP[23]/+* heterozygous wandering larvae; where *ADGF-A* is expressed from wild-type chromosome and *AGFP* from the modified one.

We had previously shown that the expression of *ADGF-A* in the larval hematopoietic system is crucial for larval survival [14]. In agreement with this observation, Figure 4C demonstrates that the expression of *ADGF-A* in larval hemocytes is ~10 times stronger compared to overall larval expression. This cell type-specific expression pattern is also represented in the expression of the *AGFP* reporter (Figure 4B, $p = 0{,}344$), further confirming the fidelity of the created reporter system in relaying the normal pattern of endogenous *ADGF-A* expression.

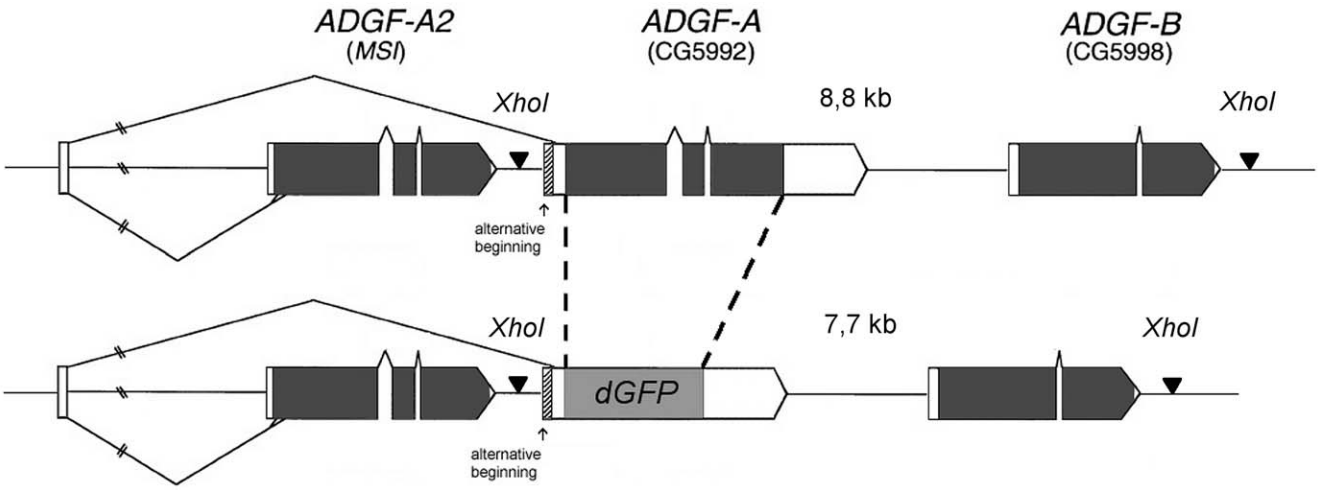

Figure 1. Schematic diagram of the *ADGF-A* coding sequence replacement by the coding sequence of a reporter gene encoding the destabilized GFP. The arrangement of three *ADGF* genes on the wild-type chromosome III is shown on the top. The bottom panel shows gene organization in the reporter system. Coding sequences are depicted by dark boxes and white boxes indicate 5′ and 3′ untranslated regions of the genes. Introns are represented by chevron-shaped lines, intergenic spaces by horizontal lines. Dashed lines depict the replacement. 5′-3′ orientation of all three genes is shown from left to right. The *ADGF-A* gene shares the first exon with the *ADGF-A2* gene but it also uses an alternative transcription start site, specific only for the *ADGF-A* gene marked by an arrow. *Xho*I restriction sites in the sequence are depicted by triangles and the fragment lengths after the digestion are shown above each sequence in kilobases.

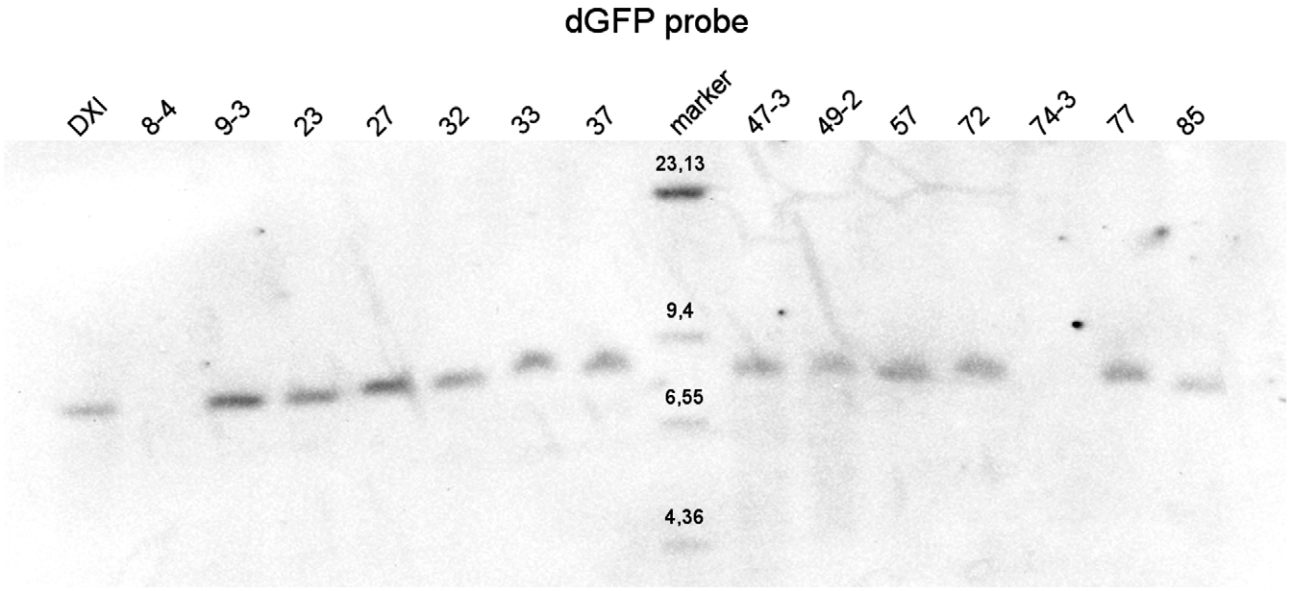

Figure 2. Southern blot analysis of the second recombination step. Genomic DNA was digested with *Xho*I restriction enzyme and the membrane was hybridized with the *dGFP* probe. The size of the *Xho*I fragment hybridised with the *dGFP* probe, after the successful replacement is 7.7 kb (Figure 1 and File S1) - the *DXI* line with duplication was used as a control for the fragment size (File S1). Sizes in kilobases are depicted above each band of the DIG-labeled marker.
doi:10.1371/journal.pone.0017741.g002

ADGF-A expression analysis by the AGFP reporter

Utilizing our AGFP reporter we analyzed the in vivo *ADGF-A* expression by observing the fluorescence of dGFP in the *AGFP*/+ animals at all developmental stages. Surprisingly, we did not detect any fluorescence above background levels in any tissue/cells (data not shown), including larval hemocytes where we could detect mRNA in relatively high quantities. We examined several independent versions of our reporter line, including *AGFP[23]* and *AGFP[72]*, that were analyzed thoroughly (data not shown).

We also tried to detect the dGFP protein in fixed *AGFP*/+ embryos and larvae by immuno-fluorescence using an anti-GFP antibody. We hoped this would overcome both the background autofluorescence that is close to the green spectrum (by using secondary antibody conjugated to the red flourophore, Cy3) and potentially low levels of dGFP protein present by signal amplification achievable using a polyclonal antibody. We also tried to detect the dGFP protein in whole larval and hemocyte lysates by western blotting. However, we were unable to detect the dGFP protein by any of these methods (data not shown), suggesting that there is either no expression of the AGFP reporter or that expression levels fall below our detection thresholds.

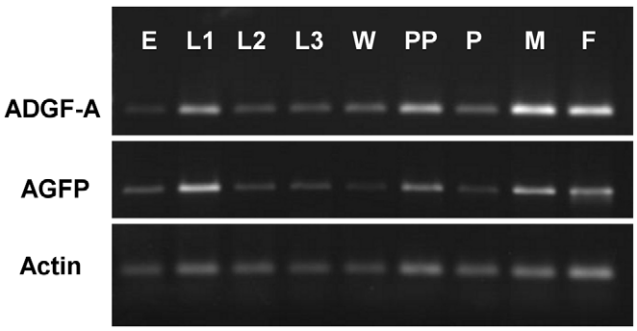

Figure 3. Developmental profile of *ADGF-A* and *AGFP* mRNA expression by RT-PCR. The total RNA was isolated from *w; AGFP[23]/TM3 Act>GFP Ser* individuals at the following stages: embryos (E), first-instar larval stage (L1), second-instar larval stage (L2), third-instar larval stage (L3), wandering larvae (W), prepupae (PP), pupae (P), male adults (M) and female adults (F). RNA was reverse transcribed and amplified by PCR using *ADGF-A* or *AGFP* specific primers. *Actin* cDNA was amplified as template control.
doi:10.1371/journal.pone.0017741.g003

Interestingly, we did detect GFP fluorescence in aggregating hemocytes of *AGFP* homozygous animals (Figure 5B,D,F). *AGFP* homozygotes are effectively *adgf-a* gene mutants and thus present the same mutant phenotype that includes melanotic capsule formation associated with differentiating hemocytes [14]. The strongest GFP expression was detected in the melanotic capsules (Figure 5F). These capsules are formed by aggregating plasmatocytes and lamellocytes, with inside melanization. While most of the circulating, *i.e.* non-adhered plasmatocytes and lamellocytes did not express GFP (Figure 5C), the aggregating cells did (Figure 5B). This was especially apparent in large aggregates (Figure 5D) that eventually formed melanized capsules (Figure 5F).

It was possible that this observed fluorescence was due to an increased expression of *AGFP* mRNA in *adgf-a* deficient, *AGFP* homozygous larvae. Therefore we measured *AGFP* mRNA levels in *AGFP* homozygous and heterozygous larvae and hemocytes. We did not detect any significant differences in mRNA levels ($P = 0,286$ for larvae and $p = 0,45$ for hemocytes, respectively) between homozygotes and heterozygotes (heterozygotes levels were doubled due to a presence of only one *AGFP* allele) (Figure 6A,B). It is important to note that there was quite high variability in the mRNA levels of homozygotes most likely attributable to variability in the mutant phenotype. This is because development was quite delayed making it difficult to collect larvae at precisely the same stage. More valuable results for comparison were obtained using *cactus* mutants (see below).

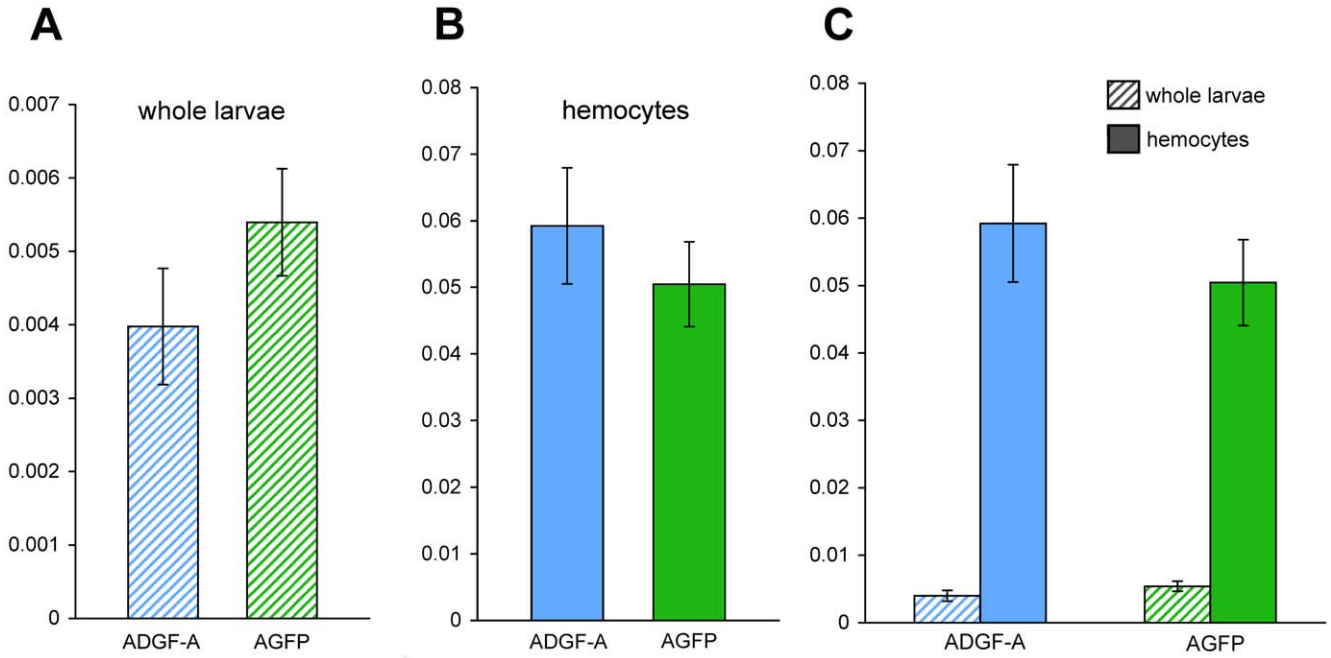

Figure 4. Real-time PCR analysis of the *ADGF-A* and *AGFP* expression in the *AGFP[23]/+* larvae. (A) Expression level in whole larvae and (B) circulating hemocytes. (C) Combined expression in whole larvae and in hemocytes plotted on one graph to demonstrate the difference in levels. Data represent mean values ± SEM, normalized relative to the endogenous control.
doi:10.1371/journal.pone.0017741.g004

AGFP expression in *cactus* mutants

The melanotic capsule phenotype of the *adgf-a* mutant is similar to another phenotype caused by the constitutive activation of Toll signaling [20]. This can be achieved by a zygotic mutation in the *Cactus* gene (FBgn0000250), an inhibitor of the Rel transcription factors *Dorsal* and *Dif*, that activate Toll target genes. Therefore we tested if *cactus* mutations would lead to the expression of our *AGFP* reporter.

Similarly to *AGFP* homozygous larvae, both hypomorphic (*cactus[E8]/cactus[IIIG]*) and null (*cactus[E8]/cactus[D13]*) mutant combinations [20] together with *AGFP[23]/+* showed GFP fluorescence in aggregating hemocytes and especially in the outer border of melanotic capsules (data not shown and Figure 5G). Since this result was obtained in *AGFP[23]/+* heterozygotes, it demonstrated that it was not the *adgf-a* mutant phenotype, nor the presence of two *AGFP* copies that are required for AGFP reporter expression. Rather it was the behavior of hemocytes and particularly their aggregation that causes the expression of dGFP protein.

We also assayed the *AGFP* mRNA levels in the *cactus* mutant background. We did not detect significant changes in *AGFP* mRNA expression when comparing hemocytes with and without *cactus* mutations (Figure 6A). When the *AGFP* levels were compared in whole larvae, the amount was however increased in the *cactus* mutants (Figure 6B). Since we did not observe GFP expression in any other cells/tissues of the *cactus* mutants, besides the hemocytes, this difference was most likely caused by the increased number of hemocytes per larva in the *cactus* mutants [20].

AGFP expression in *hop^{Tum}* mutant

The *hop^{Tum}* mutation (FBal0005547) results in the hyperactivity of the Jak kinase Hopscotch [21], leading to the aggregation of hemocytes and the formation of melanotic capsules. In similarity to *AGFP* homozygotes and *cactus* mutants, *hop^{Tum}* mutants also promote the expression of the AGFP reporter in hemocyte aggregates that were forming melanotic capsules (Figure 5H).

AGFP expression in larvae infected by parasitic wasps

As we know that the AGFP reporter was expressed in larvae harboring different mutations that induced melanotic capsule formation, we used a parasitic wasp *Leptopilina boulardi* to test its expression during a real immune challenge that would result in melanotic capsule formation. When the parasitic wasps lay their eggs in Drosophila larvae, the plasmatocytes attach themselves to the surface of the egg and thereafter the egg is encapsulated by lamellocytes and destroyed by melanization [22]. We found that the AGFP reporter started to produce a dGFP signal during the encapsulation process (Figure 7B) and it was strongly expressed in fully encapsulated and melanized eggs (Figure 7C). This result revealed that our AGFP reporter system was able to inform us as to the in vivo expression of ADGF-A status in response to a *bona fide* immune challenge within a living animal.

Discussion

Generation and verification of reporter

The adenosine deaminase activity of ADGF-A is an important regulator of extra-cellular adenosine in Drosophila larvae [14]. Therefore we decided to make a vital GFP reporter system which would allow us to observe dynamic changes in the ADGF-A expression in vivo.

This work demonstrated that it was possible to use the 'ends-in' based method of homologous recombination [23] to specifically and precisely replace a target gene sequence with that of a reporter (or other heterologous sequence). We used this approach to precisely exchange the entire coding-sequence of the *ADGF-A* gene with that of the *dGFP* reporter, leaving intact all the regulatory

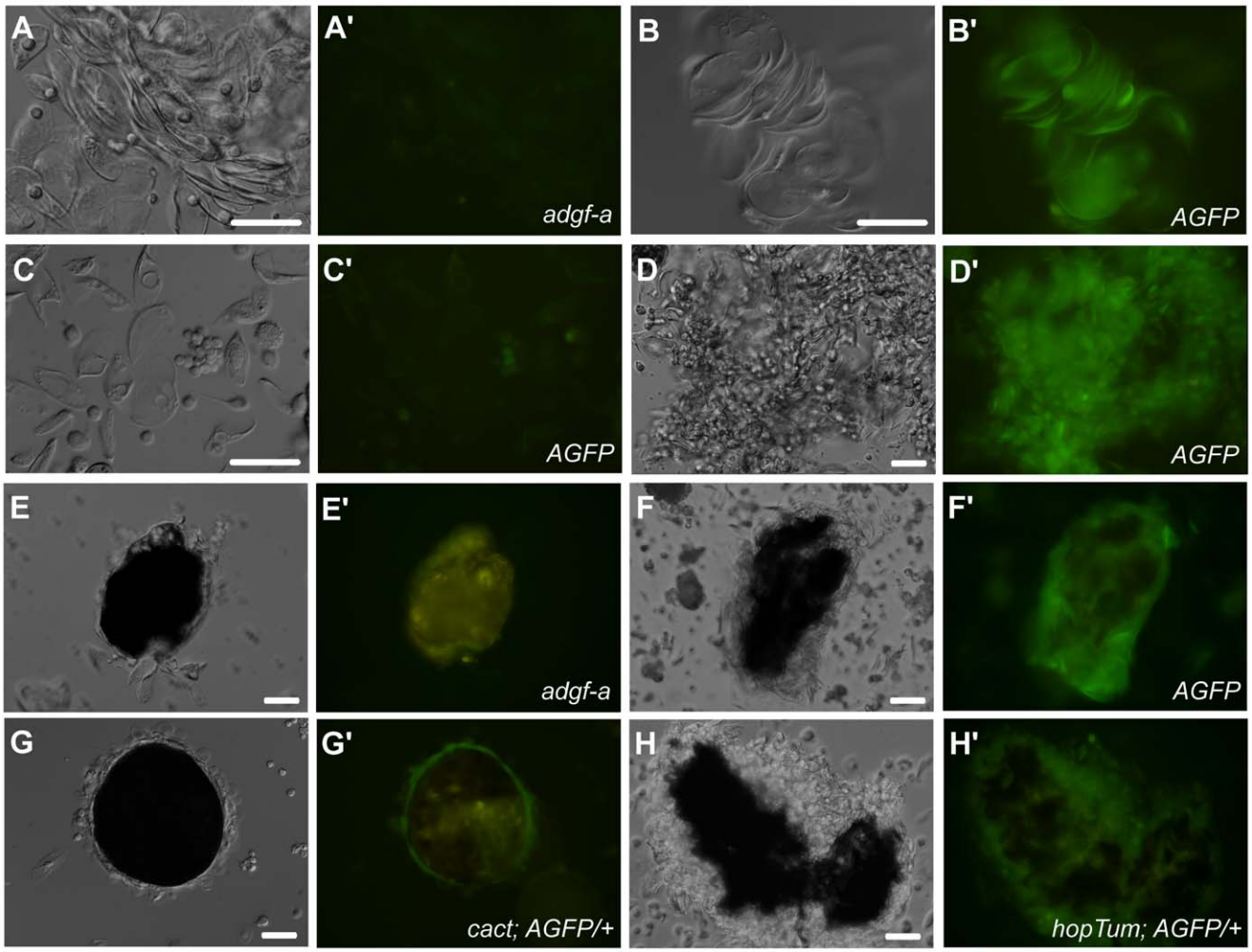

Figure 5. AGFP expression in hemocytes and melanotic capsules obtained from the late third-instar larvae. (A) Aggregating lamellocytes from the *adgf-a* mutant larvae without AGFP as a control to account for autofluorescence. (B) Aggregating lamellocytes from *AGFP[23]* homozygotes showing GFP fluorescence. (C) Non-aggregating plasmatocytes and lamellocytes from *AGFP[23]* homozygotes that rarely displayed any GFP fluorescence. (D) A clump of plasmatocytes and lamellocytes from *AGFP[23]* homozygotes exhibiting strong GFP expression. (E) A melanotic capsule from the *adgf-a* mutant showing only yellowish autofluorescence of the melanized region. (F) GFP fluorescence in hemocytes surrounding a melanotic capsule from *AGFP[23]* homozygotes. (G) A melanotic capsule from the null *cactus* mutant with AGFP reporter (*cactus[E8]/cactus[D13]*; *AGFP[23]*/+) showing GFP expression in surface lamellocytes. (H) A melanizing clump with lamellocytes and plasmatocytes expressing GFP in hop^{Tum}; *AGFP[23]*/+ mutant. Scale bar (50 μm) is shown in white on DIC images. Genotypes are shown on corresponding fluorescent micrographs distinguished with an apostrophe. All fluorescent images were captured using the same exposure settings.
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sequences, including the whole 5′ and 3′ UTRs, of the locus. This allowed us to faithfully reproduce the natural expression pattern of ADGF-A in a manner offering maximal fidelity, especially when compared with more conventional methods of transgenic reporter fly generation that rely on random sites of genomic integration.

Our molecular and phenotypic characterizations of recombination events, coupled with the developmental and the hemocyte-specific expression profiling clearly demonstrated the successful creation of the reporter. Furthermore that it faithfully recapitulates the expression pattern of the endogenous *ADGF-A*. Although newer, and arguably less laborious, strategies to target genes [24] or to tag proteins utilizing novel recombineering strategies [25] are now becoming available, it is important to note that our reporter does represents the first faithful reporter of ADGF-A expression to be published. We anticipate that this will be of use to the wider Drosophila community, especially in light of the absence of anti-ADGF-A antiserum.

Although reporter-derived *AGFP* mRNA was present in all developmental stages (and in similar levels to the endogenous ADGF-A transcripts), we did not detect any GFP protein expression at any developmental stage under normal growth conditions. This may be because the expression levels were too low to be detectable using the available methodology. Additionally in the case of heterozygous animals, the reporter was only expressed from one of the two alleles, thus lowering the potentially detectable expression to ~50% compared to normal endogenous ADGF-A expression. However, the detectable GFP fluorescence only at the sites of hemocyte aggregation in both AGFP homozygous and heterozygous animals suggests that heterozygosity of the reporter was not the reason. We also used a destabilized version of GFP that is reported to undergo degradation within a couple hours [18]. Whilst this allowed us to observe the important dynamic changes in *ADGF-A* expression, it probably also lowered the overall reporter signal and as a consequence its sensitivity,

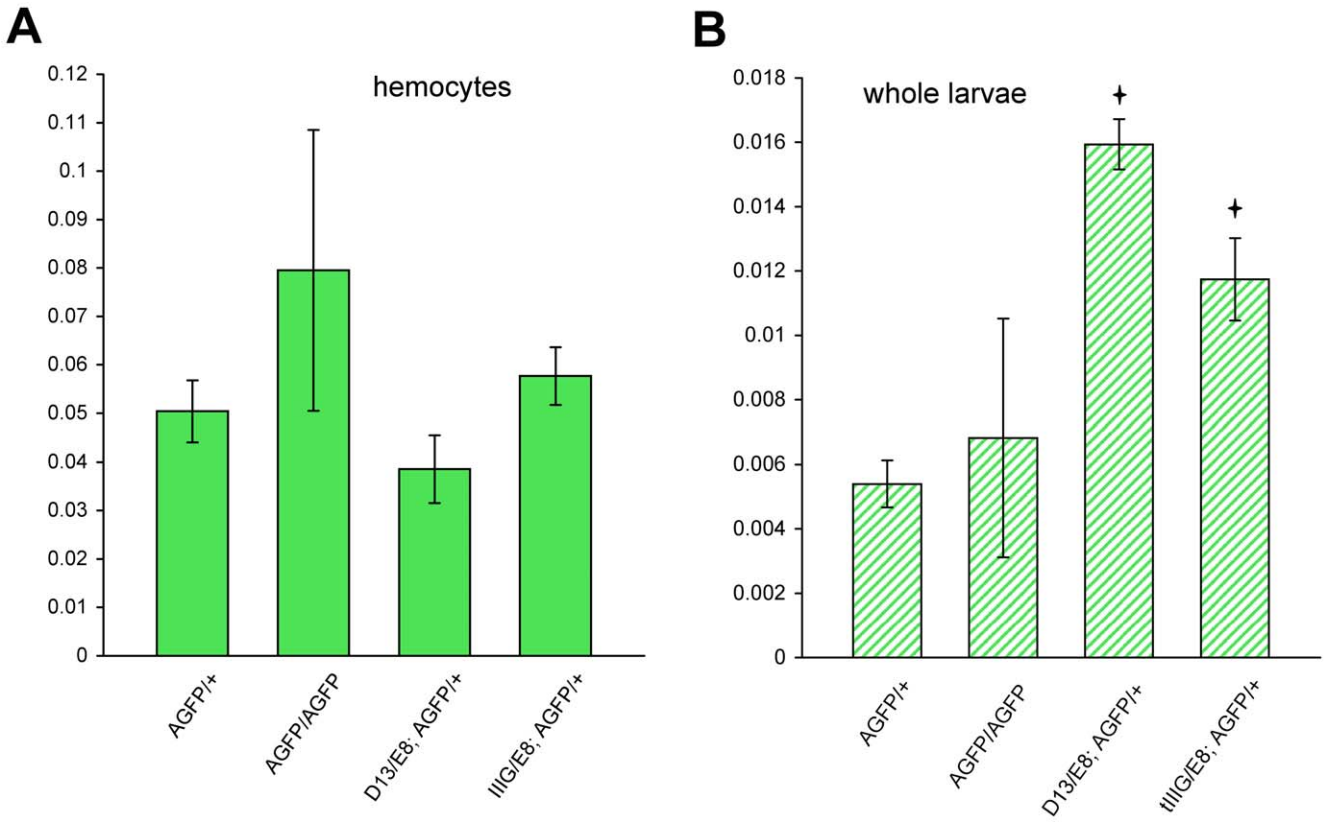

Figure 6. Real-time PCR analysis of *AGFP* mRNA expression. (A) Expression in hemocytes and (B) in wandering larvae. Data represent the mean values ± SEM, normalized relative to the endogenous control. The following genotypes are shown: *AGFP[23]/+*, *AGFP[23]/AGFP[23]*, *cactus[D13]/cactus[E8]*; *AGFP[23]/+* and *cactus[IIIG]/cactus[E8]*; *AGFP[23]/+*. Columns indicated by asterisks are significantly different from *AGFP[23]/+* control (hemocytes: p(*AGFP/AGFP*) = 0,45; p(*D13/E8*) = 0,27; p(*IIIG/E8*) = 0,45; larvae: p(*AGFP/AGFP*) = 0,2862; p(*D13/E8*) = 0,00028; p(*IIIG/E8*) = 0,0063).
doi:10.1371/journal.pone.0017741.g006

especially when compared with more stable GFP variants. Therefore we can not conclude that there is no expression of ADGF-A/AGFP at the protein level under normal growth conditions but rather if there is any expression, it is certainly very low.

Expression of reporter in site of inflammation

In agreement with the previously reported expression of *ADGF-A* mRNA in the hematopoietic organ [12], we found that the *ADGF-A/AGFP* mRNA was quite abundant in hemocytes under normal physiological conditions; typified by circulating and sessile non-activated macrophage-like cells called plasmatocytes. Nevertheless, we rarely observed GFP fluorescence in these cells. However we found that GFP expression was induced in hemocytes during the formation of melanotic capsules, a type of immune response typical of insects [17]. The purpose of melanization is to either isolate and destroy larger foreign bodies that are too big to be phagocytosed or its involvement in the healing of larger wounds. Melanotic capsule formation can be considered a form of inflammatory response since it involves the recruitment and adherence of immune cells (including the macrophage-like plasmatocytes and specialized insect cells called lamellocytes) to large invading objects, as required. Furthermore, both plasmatocytes and lamellocytes expressed the AGFP reporter as they became adhesive and especially in the site of melanotic capsule formation, *i.e.* the site of inflammation.

We used four different ways to induce melanotic capsule formation in larvae. Firstly, in the *adgf-a* mutant, it was induced by a disintegration of endogenous larval tissue, the fat body [14,26]. Secondly, in the *cactus* mutant, it was induced by constitutive activation of Toll signaling leading to lamellocytes differentiation and the spontaneous aggregation of both plasmatocytes and lamellocytes with consequent melanotic capsule formation [20]. Thirdly, in the hop^{Tum} mutant, hyperactivation of the JAK/STAT signalling cascade led to melanotic capsule formation [21]. Lastly, melanotic capsule formation was induced by a genuine immune reaction to an egg deposited by a parasitic species of wasp. This last approach demonstrated that the observed AGFP reporter expression did not occur as a consequence of the genetic manipulations used in the other three cases, but rather was indicative of a *bona fide*, in vivo immune response. In all four cases the AGFP reporter was only expressed in the adherent hemocytes of the melanotic capsule, thus indicating that the ADGF-A protein expression is tightly associated with hemocyte function during the immune reaction regardless of the inducing factor and signaling cascades involved.

Is ADGF-A expression post-transcriptionally regulated?

We did not detect any significant increases in *AGFP* mRNA levels in hemocytes isolated from larvae forming melanotic capsules (and expressing abundantly detectable GFP fluorescence), when compared to hemocytes from *AGFP/+* larvae with no melanotic capsules (and exhibiting a lack of GFP fluorescence). In addition, the *ADGF-A/AGFP* mRNA was quite abundant in these unchallenged wild-type hemocytes, corresponding to ~10% of the mRNA levels of the ribosomal house-keeping protein gene, *Rp49*.

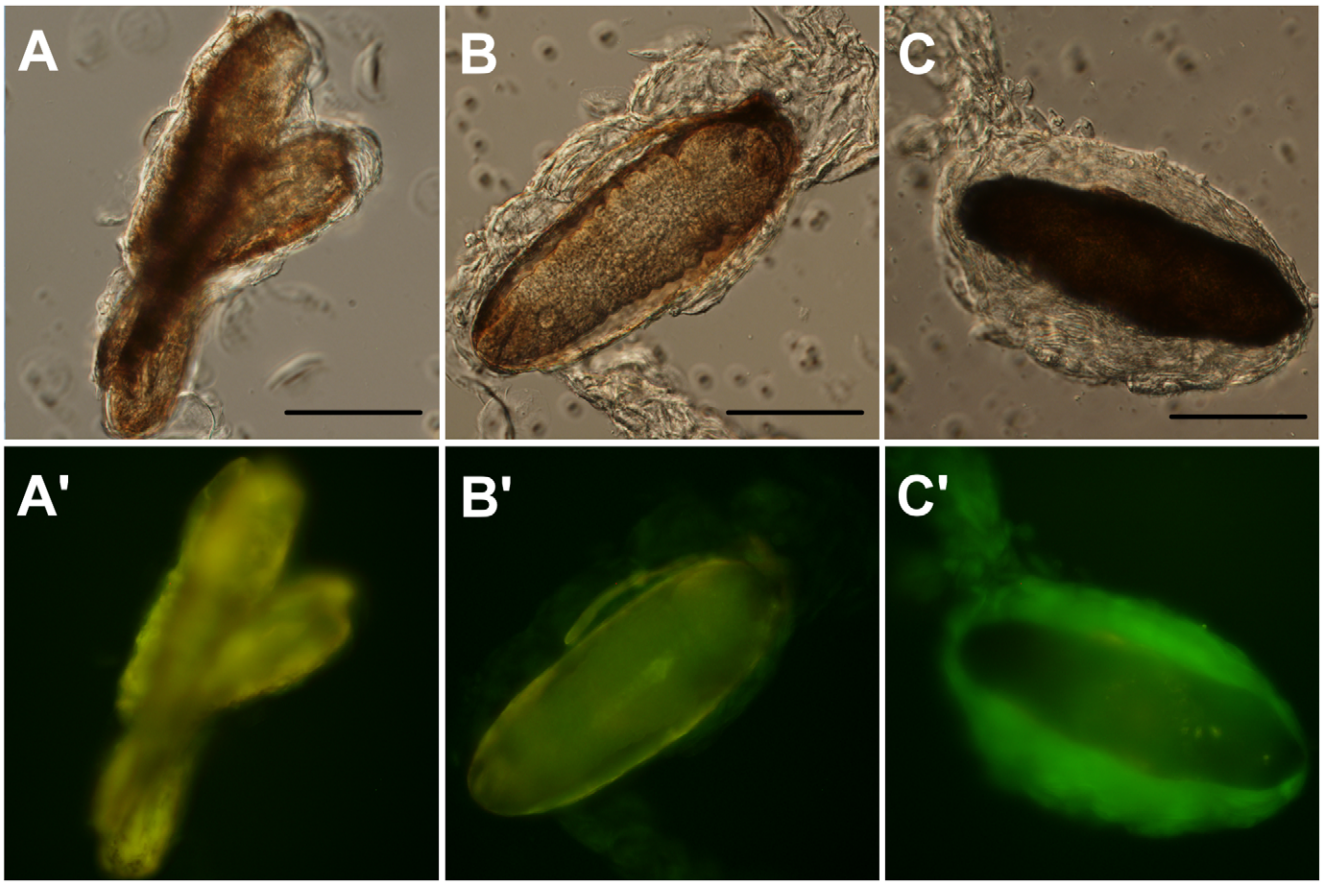

Figure 7. Encapsulation of parasitic wasp egg. (A) Partially encapsulated wasp eggs from *Oregon-R* larva as a control displaying only yellow auto-fluorescence of the wasp eggs. (B) Partially encapsulated wasp egg from *AGFP[23]/+* larva showing a faint but clearly visible green fluorescence of AGFP reporter in the encapsulating lamellocytes. (C) A fully encapsulated and melanized wasp egg from *AGFP[23]/+* larva exhibitting strong AGFP expression in the encapsulating material. DIC (top with 100-μm black scale bar) and corresponding fluorescence (bottom labeled by letter plus an apostrophe) images are shown in each panel.
doi:10.1371/journal.pone.0017741.g007

These results suggest the possibility of a post-transcriptional regulative mechanism of ADGF-A/AGFP protein expression. It should be stressed that both 5′ and 3′ UTRs of the *ADGF-A* mRNA were preserved in the *AGFP* reporter mRNA; only the coding sequence was replaced. Therefore, the potential for *adgf-a* gene UTR-mediated post-transcriptional regulation of the GFP reporter sequence exists. Since adenosine is readily transported across the plasma membrane by nucleoside transporters [27], an intriguing possibility may be provided by the potential for a riboswitch mechanism, similar to that present in prokaryotic adenosine deaminase based regulation [28]. Riboswitches have mostly been described as mechanisms of bacterial regulation that enable rapid responses to environmental stimuli. They typically act via the binding of specific ligands (*e.g.* purine molecules) to riboswitch regulatory elements in 5′ UTR of mRNA's, thus prompting or inhibiting their translation. It is therefore possible that when hemocytes find themselves in environments of high concentrations of extra-cellular adenosine, they take the adenosine up and it then binds to a riboswitch within the 5′ UTR of the *ADGF-A* mRNA, that subsequently activates the translation of the ADGF-A protein. Accordingly, we did observe increases in the expression of our AGFP reporter system, when isolated but non-adhered hemocytes were challenged by a dose of exogenous adenosine (data not shown). However we were unable to obtain quantifiable data due to the inherently weak AGFP signal in our system. Furthermore, it is also possible that the activation or adherence of hemocytes also play a role in this regulation that we are unable to yet model. Nevertheless, it will be very important to further explore the mechanism of this post-transcriptional regulation evidently at work in our system and investigate whether a similar mechanism is also operating in mammalian systems.

What is the role of ADGF/ADA2 in the site of inflammation?

There are at least two ways ADA2 can influence the inflammatory response. Firstly, by its catalytic-independent signaling function and secondly, via regulating adenosine levels through its adenosine deaminase enzymatic activity. It is not known if Drosophila ADGF-A exerts a signaling function similar to human ADA2. This function might be an evolutionary adaptation in vertebrates since the signaling function is associated with adaptive immunity [10] and includes cells that are not present in the innate immunity of Drosophila. Interestingly, insect wounds do not undergo the same burst of cellular proliferation and differentiation that characterizes mammalian wounds healing [29]. However, ADGF-A does shares all the protein domains, including the putative receptor binding domain, with human ADA2 [13] and thus the potential signaling role of ADGF proteins in insects should to be addressed.

The role of ADGF/ADA2 in the site of inflammation is certainly linked to its catalytic activity (the conversion of extra-cellular

adenosine to inosine). There are at least two important roles of extra-cellular adenosine during inflammatory response; to mitigate the severity of the potentially harmful response by its anti-inflammatory role and to readjust the energy 'supply-to-demand' ratio by stimulating additional blood flow and glucose release from stores.

Changes in the relative amounts of ATP and adenosine form the core of inflammatory response regulation, and act through the purinergic receptors [1]. The release of ATP into the extra-cellular space acts a potently pro-inflammatory, 'danger-associated molecular pattern' (DAMP) signal. Such inflammatory processes are associated with a significant increase in the expression of ecto-5′nucleotidase that acts to rapidly convert ATP into adenosine [30]. Furthermore, in vertebrates, adenosine itself is a strongly anti-inflammatory molecule [1] and acts later to down-regulate inflammation. Therefore during acute inflammation, increased extra-cellular adenosine is rapidly metabolized to inosine by adenosine deaminase. Indeed, a close correlation can be observed between inflammation and local increases in adenosine deaminase activity [7,9]. Our results in Drosophila confirm this correlation in vivo and suggest that adenosine plays a similar role during the inflammatory response of insects as in vertebrates.

However, lowering extra-cellular adenosine levels may have an important function beyond the site of inflammation. Extra-cellular adenosine is traditionally regarded as a local signal due to its usually rapid metabolism; therefore only exerting localized effects on cells/tissues surrounding its site of production. However, studies in the rat model suggest it can act over longer distances in a hormone-like manner [31]. In this rat model, lower-limb ischemia causes the muscular accumulation of both extra-cellular adenosine and inosine that upon reperfusion are rapidly released into the circulating blood. It is these plasma nucleosides that then promote hepatic glucose release and eventual hyperglycemia, via the activation of A3 adenosine receptor on hepatocytes. We recently demonstrated that extra-cellular adenosine can also act as an anti-insulin hormone stimulating a release of glucose from stores in the Drosophila model [26]. Therefore a connection between the work presented here (demonstrating the quite exclusive expression of ADGF-A in sites of inflammation) and our previous study (showing the hyperglycemic effect caused by the deficiency of ADGF-A associated with increased extra-cellular adenosine [26]) could lead to a reappraisal of the roles of extra-cellular adenosine and its regulation by ADGF-A/ADA2. Accordingly, damaged tissues and sites of inflammation generating significant amounts of extra-cellular adenosine, may serve as sites of hormone production that alert an organism towards the appropriate allocation of energy reserves towards mounting a necessary immune reaction. Such a mechanism would need to be under tight control as not to precipitate harmful hyperglycemia and eventually uncontrolled loss of energy reserves. Therefore, once the stimulus for the inflammation is under control, marked by the presence of sufficient activated immune cells at the inflammation site, the signal for energy release should be suppressed. This could explain why ADGF-A protein is only expressed in fully adhered immune cells at site of inflammation *i.e.*, to dampen this important but potentially dangerous signal. The deficiency of ADGF-A protein that causes hyperglycemia and progressive loss of energy reserves in flies [26] illustrates the potential importance of this regulatory circuit. The ability of extra-cellular adenosine to stimulate glucose release over longer distances [31] and the expression of ADA2 in sites of inflammation [7,9] suggest that similar roles of adenosine and ADA2 in energy allocation could be applicable to mammalian systems.

To better understand the immunomodulatory roles of ATP and adenosine it is important to monitor dynamic changes in the expression of their receptors and the enzymes regulating their in vivo concentrations. This is especially applicable in a time when increasing attention is being paid to the role of adenosine system in inflammation and its involvement in, for example, the pathophysiology of inflammatory bowel diseases [30]. Our work demonstrates that Drosophila could serve as a valuable and convenient model for visualization of such dynamic changes an in vivo context.

Conclusions

Our work reports the creation of a functional in vivo expression reporter for ADGF-A using precise gene replacement homologous recombination procedures. Results of this work confirm the expression of ADGF/ADA2 enzymes in the site of inflammation by showing that ADGF-A expression occurs specifically in the adhered immune cells of such sites in Drosophila. This supports the view that the inflammatory response and its regulation are evolutionary ancient and Drosophila and mammalian systems share common mechanistic features. Our model also suggests that the expression of adenosine deaminase during inflammation response might be regulated at post-transcriptional level, therefore potentially providing a means of rapid responding regulation. Our previously uncharacterised observations of ADGF-A expression highlight its potential regulatory role in energy allocation stimulated by extra-cellular adenosine.

Supporting Information

File S1 Details of design and production of AGFP reporter by homologous recombination.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MN TD. Performed the experiments: MN. Analyzed the data: MN TD. Contributed reagents/materials/analysis tools: TD. Wrote the paper: MN TD.

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Expression of Drosophila adenosine deaminase in immune cells during inflammatory response

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SUPPORTING INFORMATION S1

Details of design and production of AGFP reporter by homologous recombination

Materials and methods

Design of homologous recombination construct and cloning: We used Drosophila *yw* strain genomic DNA to amplify three fragments. Using primer F1 5´-gcg gcc gcc atg aaa tcc aca tca agg gg-3´ (introducing *Not*I site to 5´ terminus) and primer R1 5´-tgc tca cca tga tga gcg gta gat ttc gtt ag-3´ (introducing 9 bases from the *dGFP* coding sequence to 5´ terminus) we amplified a 4.3-kb long fragment 1 containing the *ADGF-A2* gene and promotor sequence of the *ADGF-A* gene. With primer F3new 5´-gct ag c acg gtt ggt ggc ag-3´ (introducing *Nhe*I site to 5´ terminus) and primer R3new2 5´- ggg ccc ata tct ctt cct tct tga cgg-3´ (introducing *Apa*I site to 5´ terminus) we amplified a 2.2-kb fragment 3 including a short part of the *ADGF-B* gene and the upstream sequence. A 6-kb fragment 4 was amplified with primer F4 5´- ggg ccc cgt tgg gcg cca ttt tgt gc-3´ (introducing *Apa*I site to 5´ terminus) and primer R4new 5´-ggt acc gtc gtc ctg ttc ctg ttt cc-3´ (introducing *Kpn*I site to 5´ terminus) covering the *ADGF-B* gene and the downstream and upstream sequences.
From plasmid Casper4 containing the *dGFP* sequence (provided by Tamas Lukacsovich) we amplified with primer F2 5´-acc gct cat cat ggt gag caa ggg cga gg-3´ (introducing 10 bases from the *ADGF-A* promoter to 5´ terminus) and primer R2new 5´-gct ag c tac aca ttg atc cta gc-3´ (introducing 5 bases from the *ADGF-A* terminator and *Nhe*I site to 5´terminus) a 861-bp fragment 2 containing *dGFP* coding sequence.
Fragments 1 and 2 were amplified using standard PCR and then combined in 2 steps by recombinant PCR. In the first step 5 µl of each fragment from unpurified PCR reactions were added to 30-µl PCR mixture without primers and annealed for five PCR cycles. In the second step 40 µl of unpurified PCR mixture from the first step was added to 60 µl of PCR mixture with the outer primers F1 and R2new and combined fragments were amplified for additional five cycles. A 5.2-kb recombinant fragment was then purified from agarose gel and cloned into the pGEM-T Easy vector (Promega).
Fragments 3 and 4 were also cloned into pGEM-T Easy. Fragment 3 was combined using inserted restriction sites to recombinant fragment and together re-cloned from pGEM-T Easy to pBLUESCRIPT II SK+ vector. An I-*Sce*I site was inserted in to the fragment 4 by annealing oligonucleotides Sce-ApaI 5´-cta ggg ata aca ggg taa t-3´ and Sce-REM 5´- cta gat tac cct gtt atc cct agg gcc-3´ (introducing *Apa*I and *Nhe*I site compatible overhangs) and ligating into *Apa*I and *Nhe*I sites of fragment 4, destroying the *Nhe*I site. Introduced I-*Sce*I sequence replaced ~1.9 kb of the 5'-end sequence originally amplified in fragment 4 (combination with the 3' end of fragment 3 deleted ~1.4 kb region in the *ADGF-B* gene in the final). Fragment 4 with I-*Sce*I site was then transferred from pGEM-T Easy to the construct in pBLUESCRIPT II SK+ using *Apa*I and *Kpn*I sites. The assembled construct was finally transferred from

pBLUESCRIPT II SK+ to pTV2 plasmid (provided by Yikang Rong and Kent Golic) using *Not*I and *Kpn*I sites (Figure S1A). The *dGFP* sequence and I-*Sce*I site was verified by sequencing and restriction analysis.

Fly stocks: In the first step of homologous recombination (HR) we used *yw; P[ry+, 70FLP]4 P[v+, 70I-SceI]2B Sco/S(2)Cyo* and *w[1118]; P[ry+, 70FLP]10* (constitutively active FLP recombinase, homozygous on the second chromosome) fly stocks. In the second step of HR we used *v P[70I-CreI, v+]2A; ry/TM3 Ser* stock. All stocks were provided by Yikang Rong and Kent Golic. The stocks *yw; Xa/Cyo; MKRS* and *w; TM3 Sb Ser/TM6B* were used for the mapping of insertions and to establish the recombinant lines.
DNA construct for HR was injected in to the *yw* embryos using the modified P-element-mediated transformation procedure (PARK and LIM 1995). To induce HR we used the rapid targeting scheme (RONG and GOLIC 2001), described in details in DOLEZAL *et al*. (2003).

Molecular analysis of the targeted events: The first step of HR was analyzed using standard methods of DNA isolation, PCR analysis, restriction and southern blot analysis. DNA from all tested lines (homozygous or heterozygous adults) were digested with restriction enzyme *Xho*I and hybridized with a 1.6-kb ADGF-A RNA-probe or with a 864-bp dGFP RNA-probe labeled by DIG (Maxiscript *in vitro* transcription kit, Ambion Austin, TX) (Figure S1B). After the second step of HR, selected lines were tested for the presence of *dGFP*. Genomic DNA was isolated from heterozygous adults and digested by *Xho*I and *Kpn*I restriction enzymes. Southern blot was hybridized with the dGFP RNA-probe. Recombination events were further verified by PCR analysis and sequencing.

Results and commentaries

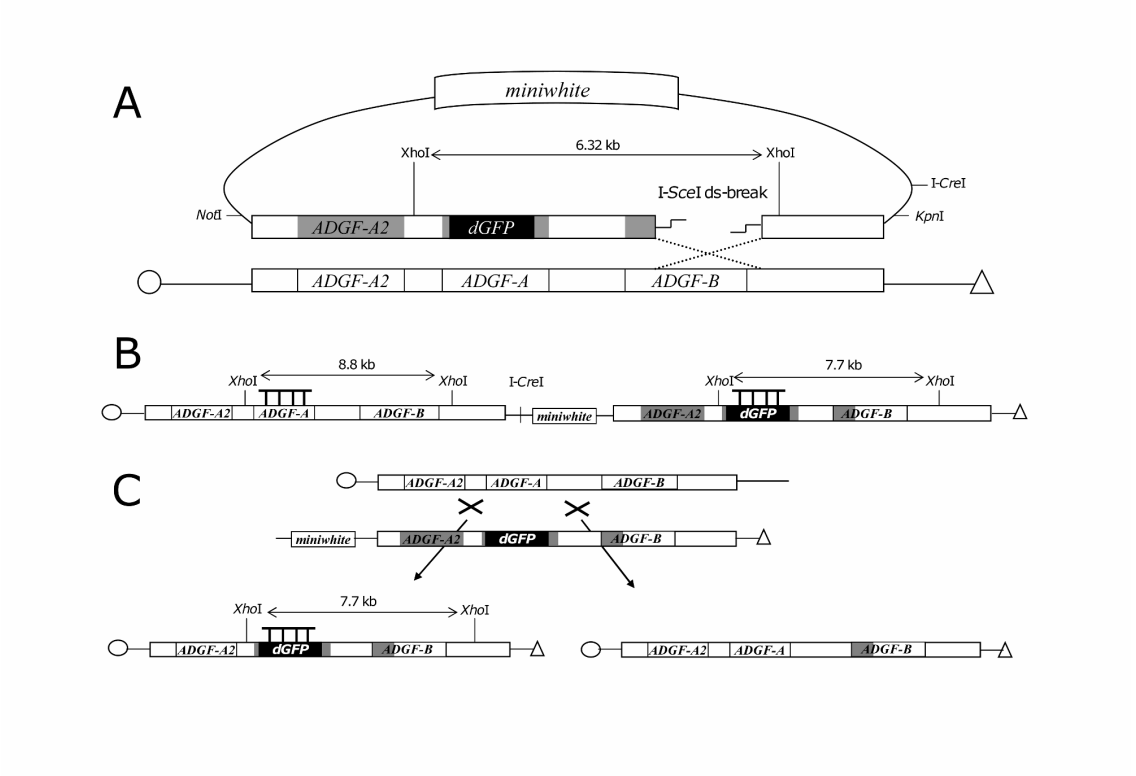

Figure S1. Design of targeting construct and two steps of homologous recombination. (A) The first step of homologous recombination and construct design. The *ADGF-A* coding sequence (from start to stop codon containing two short introns) was exchanged by coding sequence of *dGFP*. I-*Sce*I site replaced ~1.4-kb distal region of the *ADGF-B* gene starting 2.2 kb downstream of the *dGFP* stop codon. 4-kb long homologous sequence distal from *ADGF-B* was placed on the other side of I-*Sce*I site. The whole homologous region with *dGFP* and I-*Sce*I site was cloned into the pTV2 vector containg *miniwhite* marker. I-*Sce*I-induced double-strand break in donor DNA (top) stimulates recombination with the homologous sequence on chromosome III (drawn as rectangle). (B) The structure of an expected duplication after the first step of HR produced by recombination between the donor DNA and the target locus. The centromeric side (represented by circle) of the duplication contains wild-type gene arrangement, on the telomeric side (represented by triangle) the *ADGF-A* gene is replaced by the *dGFP* sequence. (C) The second step of HR. I-*Cre*I is used to produce DSB between the duplicated regions resulting in a reduction event. Recombination occurring upstream of the *ADGF-A/dGFP* sequences results in the chromosome carrying *dGFP* sequence (left). Recombination downstream of the *ADGF-A/dGFP* sequences leads to wild-type gene arrangement (right). Positions of the *Xho*I restriction sites and the corresponding sizes of the fragments on southern blot are indicated above the sequences; binding of the ADGF-A and dGFP probes are marked by comb-like structures. Grey boxes represent gene sequences including untranslated regions originated from the donor construct; white boxes indicate chromosomal gene sequences; the black box represents *dGFP* coding sequence and box with *miniwhite* represents an eye-color marker used for screening.

For the first step of homologous recombination (HR) we used a donor line (M2) with the P element targeting construct inserted on the second chromosome to avoid the complication with the remaining FRT sequence and P-element repeats (targeted sequence is on chromosome III). We recovered 15 potential recombinant lines from total 241 screened vials.

In 7 of those lines (Table S1) the events were mapped to the third chromosome where the *ADGF-A* gene is located, while remaining 8 events were mapped to the second chromosome and thus labeled as non-targeted events and discarded. All targeted lines were tested by southern blot and PCR analysis for the changes in the targeted region (Figure S2). We digested genomic DNA by an *Xho*I restriction enzyme and used probes for the *ADGF-A* and the *dGFP* sequences. This allowed us to distinguish between the centromeric side of the duplication with 8.8 kb if the *ADGF-B* sequence was properly repaired (see further), the telomeric side of the duplication with 7.7 kb (with the *dGFP* replacement and again the proper *ADGF-B* repair) and the original or non-targeted construct insertion with 6.3 kb (i.e. with the *dGFP* sequence and the deletion in the *ADGF-B* region; see Figure S1 for details).

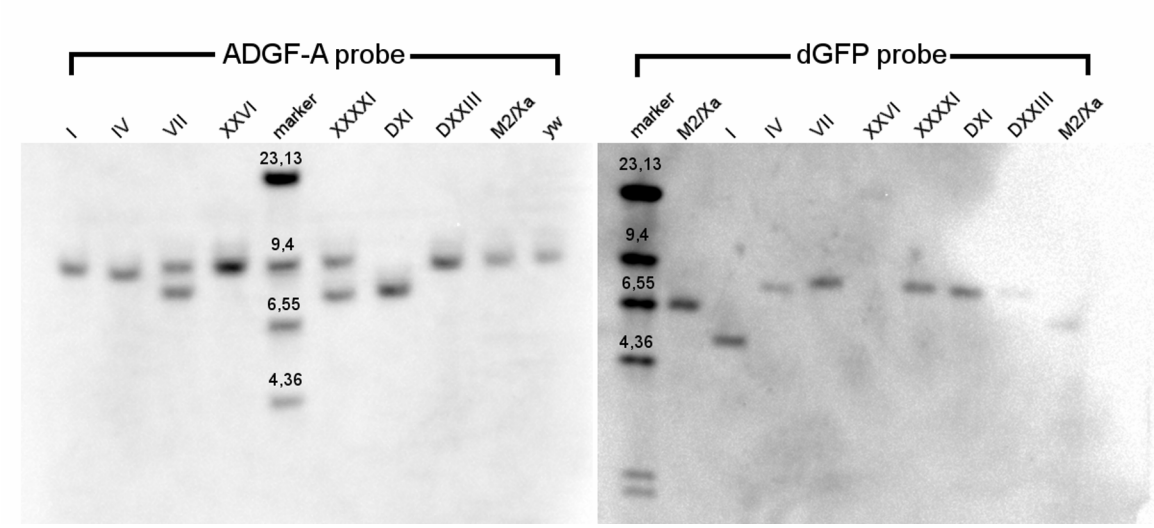

Figure S2. Southern blot analysis of lines with targeted events after the first step of homologous recombination. Genomic DNA was isolated from *yw* control flies, M2/Xa line with the original insertion of the donor construct and from heterozygous (lines I, IV, VII, XXVI and XXXXI) and homozygous (lines DXI and DXXIII) adult flies with targeted events. DNA was digested with *Xho*I restriction enzyme and hybridized with probes specific for the *ADGF-A* or the *dGFP* sequence (see also scheme on Figure S1 for the fragment sizes). Sizes in kb are depicted above each band of the DIG-labeled marker (Roche, Mannheim, Germany).

Four of the tested lines detected by the *ADGF-A* probe (labeled as I, IV, XXVI and DXXIII) produced the expected fragments for centromeric side of the duplication. Three duplications (VII, XXXXI and DXI) produced approx. 1.5-kb shorter fragments on this side of the duplication indicating deletions in the vicinity of the original I-*Sce*I site (in the cases of the VII and XXXXI lines the DNA originated from heterozygous flies explaining the presence of the additional wild type fragments). The I-*Sce*I site in the donor construct was accompanied by a deletion in the *ADGF-B* region compared to the homologous wild-type sequence (see Material and methods and Figure S1A for details). Therefore we expected that in some cases the repair of this gap during HR process might be imperfect. An additional PCR and sequencing analysis of the XXXXI and DXI lines confirmed deletions in the *ADGF-B* region. In the case of DXI line we detected a deletion of a 1.2-kb region in the *ADGF-B* gene and downstream sequence which was shorter and shifted compared to the original deletion in the donor construct. A 1.4-kb deletion found in XXXXI line was precisely the same size and location as in the original donor construct including the I-*Sce*I site in this duplication. The

deletion occurrence in the vicinity of double strand break had been previously discussed by RONG and GOLIC (2000) and EGLI *et al*. (2003). In the case of the DXI duplication, the deleted region formed probably as a result of non homologous joining of sequence placed on the right side of I-*Sce*I in donor construct (Figure S1A) to the *ADGF-B* targeted region during the crossing-over mechanism. The other side of the duplication with *dGFP* was repaired by re-synthesis using chromosomal sequence as a template to the wild-type state. Possible explanation for a character of the XXXXI duplication with preserved I-*Sce*I site is an existence of the second donor construct during G2 phase of the cell cycle. Events explainable by the presence of the second donor construct during recombination process were previously documented (RONG and GOLIC 2000; DOLEZAL *et al.* 2003). LANKENAU *et al*. (2003) also found such events and offered two possible mechanism for I-*Sce*I site preservation: dimerization of donor construct with only one double strand break and subsequent resection of the ends and classical double strand break repair or using BIR (break-induced recombination) model where the intact I-*Sce*I site is explained by an endogenous double strand break in the target gene but not in a donor construct. In the case of the XXXXI duplication the double strand break would occur downstream of the *ADGF-B* gene that match with donor construct sequence (Figure S1A). Detailed characterization of the duplication event VII was not performed.

For the second step of HR we chose five lines - IV, VII, XXXXI, DXI, DXXIII - carrying duplications in the *ADGF-A* region and crossed them with the line carrying the CreI endonuclease. The progeny was heat-shocked early in their development to induce CreI expression resulting in a loss of the *miniwhite* marker (Figure S1C). Selected white-eyed flies were crossed individually to establish balanced stocks. After I-*Cre*I mediated reduction event, we recovered totally 53 lines (Table S1); 14 of them were subjected for further southern blot and PCR analysis. Genomic DNA was isolated from heterozygous lines and digested by two different restriction enzymes *Xho*I and *Kpn*I; only *Xho*I is shown, *Kpn*I confirmed results with *Xho*I in all cases. We used *Kpn*I because the site for this enzyme was localized outside the boundaries of homology with the donor construct and thus confirmed that all tested events were targeted.

Mutations causing an early lethality most likely occurred in lines 9-3, 47-3 and 32 during the recombination processes. The homozygous viability of reductions 23 and 27 originating from the same duplication as line 32 as well as the viability of the original duplication stock DXI indicate that some changes appeared during the second recombination step leading to reduction 32. On the other hand, mutations leading to early lethality in lines 9-3 and 47-3 most likely occurred already during the first step of HR. This is supported by two observations. First, it is the absence of homozygotes in the original duplication stock IV. Second, it is homozygous lethality in line 8-4 which originated from the same duplication as lines 9-3 and 47-3 but the *dGFP* sequence was not detected in this line and thus we expected that the wild-type gene arrangement would be associated with normal viability. In comparison, reduction 74-3 from a different duplication where the wild-type gene arrangement was also restored expressed the wild-type phenotype.

TABLE S1

SUMMARY OF TARGETED EVENTS AND THEIR MOLECULAR AND PHENOTYPIC CHARACTERIZATION

Dupli-cation	Southern blot analysis of duplications	Total number of isolated reductions	Reductions analyzed by Southern blot	Southern blot results	Phenotype of homozygotes
I	GFP side ~2.5 kb shorter	0	0	-	-
			8-4	no GFP signal	early lethality
			9-3	expected size	early lethality
IV	duplication as expected	15	47-3	expected size	early lethality
			49-2	expected size	*adgf-a* mutant
			57	expected size	*adgf-a* mutant
VII	ADGF-A side ~1.5 kb shorter	17	74-3	no GFP signal	wild type
XXVI	GFP side - no signal	0	0	-	
			72	expected size	*adgf-a* mutant
			77	expected size	*adgf-a* mutant
XXXXI	ADGF-A side ~1.5 kb shorter	6	85	expected size	*adgf-a* mutant
			23	expected size	*adgf-a* mutant
			27	expected size	*adgf-a* mutant
DXI	ADGF-A side ~1.5 kb shorter	12	32	expected size	early lethality
			33	expected size	*adgf-a* mutant
DXXIII	duplication as expected	3	37	expected size	*adgf-a* mutant
total		**total**	**total**		
7 / 241		**53**	**14**		

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Conclusions

This thesis has contributed to clarification of conditions and regulation level of ADGF-A expression in *Drosophila melanogaster*; it presents new application of ends-in homologous recombination method for monitoring of protein expression. The results are summarized in published paper and in unpublished data.

The main conclusions are as follows:

Ends-in homologous recombination method was for the first time used to create a functional reporter system for monitoring in vivo protein expression.

ADGF-A protein is expressed at very low levels under physiological conditions, but its expression is strongly increased during immune response.

ADGF-A enzyme expression occurs in the site of inflammation, especially in the adhered immune cells.

Expression of ADGF-A during inflammation is most likely subject to post-transcriptional regulation enabling a rapid response regulation.

Exclusive expression of ADGF-A protein in site of inflammation as presented in this work together with effects caused by the lack of ADGF-A suggest that this expression is important for regulation of adenosine role in stimulation of glucose-release during immune response.

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The goal: Methods of Drosophila immunization using parasitic wasp *Leptopilina boulardi*

2007 Laboratory of Prof. Bruno Lemaitre, EPFL, Lausanne, Switzerland
The goal: Methods of Drosophila immune response activation (bacterial and fungi cultures)

Presentations:

Novakova, M., Dolezal, T. Visualizing ADGF-A expression using GFP reporter system. 50th annual Drosophila Research Conference. Chicago, Illinois, USA, March 4-8, 2009.

Fenckova, M., **Novakova, M**., Dolezal, T. Extracellular adenosine functions as a stress signal in Drosophila. 20th European Drosophila Research Conference. Wien, Austria, September 12-14, 2007.

Novakova, M., Jungova, R., Zurovcova, M. Nucleotide variation within the *Idgf* multigene family of *Drosophila melanogaster*. Population Genetics Group Meeting. University of Edinburgh, UK. December 13-16, 2005.

Novakova, M., Zurovcova, M. Nucleotide variation of two physically linked *Idgf* genes of *Drosophila melanogaster*. Population Genetics Group Meeting. University of Reading, UK. December 14-17, 2004.

Publications:

Novakova, M., and Dolezal, T., 2011 Expression of Drosophila adenosine deaminase in immune cells during inflammatory response. PLoS ONE **6:** e17741.