A Role for Adenosine Deaminase in Drosophila Larval Development

Tomas Dolezal¹, Eva Dolezelova², Michal Zurovec², Peter J. Bryant¹*

1 Developmental Biology Center, University of California, Irvine, California, United States of America, 2 Institute of Entomology and University of South Bohemia, Ceske Budejovice, Czech Republic

Adenosine deaminase (ADA) is an enzyme present in all organisms that catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine. Both adenosine and deoxyadenosine are biologically active purines that can have a deep impact on cellular physiology; notably, ADA deficiency in humans causes severe combined immunodeficiency. We have established a Drosophila model to study the effects of altered adenosine levels in vivo by genetic elimination of adenosine deaminase-related growth factor-A (ADGF-A), which has ADA activity and is expressed in the gut and hematopoietic organ. Here we show that the hemocytes (blood cells) are the main regulator of adenosine in the Drosophila larva, as was speculated previously for mammals. The elevated level of adenosine in the hemolymph due to lack of ADGF-A leads to apparently inconsistent phenotypic effects: precocious metamorphic changes including differentiation of macrophage-like cells and fat body disintegration on one hand, and delay of development with block of pupariation on the other. The block of pupariation appears to involve signaling through the adenosine receptor (AdoR), but fat body disintegration, which is promoted by action of the hemocytes, seems to be independent of the AdoR. The existence of such an independent mechanism has also been suggested in mammals.

Introduction

Adenosine deaminase (ADA) is an enzyme present in all organisms that catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine. It is a critically important enzyme for human survival because its congenital absence causes severe combined immunodeficiency disease (SCID). ADA deficiency accounts for about 20% of all types of SCID [1]. It is one of the most severe human immunodeficiencies and is associated with depletion of all three major categories of lymphocytes: T cells, B cells, and natural killer cells, resulting in impaired cellular immunity and decreased production of immunoglobulins [2]. Without intervention, the affected individuals die from opportunistic infections within the first few months of life.

ADA occurs as a soluble monomer in all human cells, but also exists as “ecto-ADA,” bound to the membrane glycoprotein CD26/dipeptidyl peptidase IV, and it has been suggested that this form of ADA regulates extracellular adenosine levels [3]. ADA deficiency is accompanied by greatly elevated levels of the ADA substrates adenosine and deoxyadenosine, both of which are biologically active purines that can have a deep impact on cellular physiology. Adenosine is not just a metabolite; it is also a signaling molecule that regulates numerous cellular functions by binding to G protein-coupled adenosine receptors (A1, A2a, A2b, and A3 in mammals) that can regulate intracellular cyclic adenosine monophosphate [4]. Deoxyadenosine is a cytotoxic metabolite released by various cell populations that undergo programmed cell death; it can kill cells through a mechanism that includes disturbances in deoxynucleotide metabolism [5].

Extracellular adenosine is now considered an important stress hormone that is released in excessive amounts in the vicinity of immune cells during both systemic and cellular stress [6]. The predominant source of extracellular adenosine during systemic activation of the stress system is the sympathetic nervous system [7]. Specific inflammatory stimuli such as bacterial products are also capable of triggering adenosine release from immune cells [8]. These data are in line with evidence demonstrating a dramatic increase in extracellular adenosine levels under conditions associated with multiple organ failure, which is the cause of 50%–80% of all deaths in surgical intensive care units [6].

ADA is not the only adenosine deaminase in mammalian cells. Recently, the cat eye syndrome critical region protein 1 (CECR1) gene was identified and shown to encode a protein representing a subfamily of proteins related to but distinct from classical ADAs [9]. The duplication of a small region of chromosome 22 containing this gene is associated with “cat eye syndrome,” a disorder characterized by hypoplastic kidneys, congenital heart malformation, and anomalous pulmonary venous connections. The founding member of this subfamily is encoded by insect-derived growth factor (IDGF) [10], and homologs have been described in various organisms [11–14].

We have previously found six Drosophila genes with sequence similarity to the CECR1 subfamily [15]. Their
products are mitogenic on *Drosophila* cells, and at least two of them (ADGF-A and ADGF-D) exhibit strong ADA activity, which is necessary for their mitogenic function. We therefore named them adenosine deaminase-related growth factors (ADGFs). We also demonstrated that adenosine functions as a negative signal for cell proliferation and concluded that ADGFs stimulate cell growth in vitro by depletion of extracellular adenosine [16]. *Drosophila* also contains a gene, termed *Ada*, with sequence similarity to human ADA, but as we have previously shown the product of this gene is most likely not an active ADA [16].

In this report we show that a null mutation in *Drosophila* ADGF-A gene leads to dramatically increased levels of adenosine and deoxyadenosine in the larval hemolymph. This increase leads to larval death associated with the disintegration of fat body and the development of melanotic tumors. We present a detailed analysis of the hematopoietic defects associated with the *adgf-a* mutation, show a genetic interaction of this mutation with signaling through the *Drosophila* adenosine receptor (AdoR, encoded by the gene *CG9753*) and with regulation of premetamorphic changes by ecdysone, as well as a genetic interaction of ADGF-A with a major innate immunity regulator—the Toll signaling pathway.

**Results**

**Mutation in the ADGF-A Gene Causes Larval Death and Melanotic Tumors**

We produced mutations in five of the six ADGF genes by homologous recombination mutagenesis [17] and showed that loss of the most abundantly expressed gene, ADGF-A, leads to death in the larval or pupal stage. Under optimal conditions (20–30 isolated homozygous larvae per vial), about 60% of larvae homozygous for the *adgf-a* mutation reach the third instar. Development during the third larval instar is significantly delayed, and wandering homozygous larvae usually appear 2 d after their heterozygous siblings, which start wandering at about 5 d of development. Some homozygous third-instar larvae can be found alive in the vial even after 10 d of development. Mutant third-instar larvae show fat body disintegration (Figure 1A and 1B) and multiple melanotic tumors (Figure 1C), predominantly in the caudal part of the body and accompanied by disintegration of the fat body. Melanization of the lymph glands was never observed in these larvae, and the imaginal discs and brain appear normal. Less than 30% of homozygous larvae eventually pupate. Homozygous pupae usually die soon after pupariation; in some cases they develop normal head and thorax imaginal structures; however, abdominal parts usually do not develop. There is also an abnormal curvature (to the right) of the pupal abdomen (Figure 1D). Less than 2% of mutant pupae develop normally and eventually emerge as adults without any obvious abnormalities besides the abdominal curvature; some of them are sterile.

To confirm that the mutant phenotype is caused solely by a mutation in the ADGF-A gene, we created transgenic flies carrying the ADGF-A gene under a heat-shock promoter (HS-ADGF-A). The *adgf-a* homozygous flies carrying the HS-ADGF-A construct showed survival rates significantly higher than *adgf-a* even without heat shock, probably due to leaky expression of the HS-ADGF-A construct (Figure 2A). However, while non-heat shocked animals still produced many melanotic tumors, only 22% of animals that were heat shocked as late embryos/early first instar developed these tumors (Figure 2B). This result confirms that the mutant phenotype is caused by the mutation in the ADGF-A gene. This conclusion is further supported by the even more efficient rescue achieved by expression of transgenically provided ADGF-A in the lymph glands using the Gal4/UAS system (see below).

**The adgf-a Mutant Phenotype Is Associated with Elevated Levels of Adenosine and/or Deoxyadenosine**

Using liquid chromatography and mass spectrometry of deproteinated hemolymph samples, we measured adenosine concentrations in hemolymph of mutant and wild-type third-instar larvae. The adenosine concentration in the *adgf-a* mutant was 1.14 ± 0.26 μM compared to less than 0.08 μM in the wild type, and the deoxyadenosine concentration in mutants was 1.66 ± 0.99 μM compared to an undetectable level in the wild type.

**The Catalytic Activity of ADGF-A Is Required for Its Function**

To test whether the function of ADGF-A in vivo is also dependent on its catalytic activity, we produced two versions
of the UAS-ADGF-A construct [18]: one carrying wild-type cDNA of ADGF-A and one carrying an ADGF-A cDNA with a mutation causing a substitution of two amino acids (H386G and A387E) in the catalytic domain [16]. Two different lines carrying the wild-type UAS-ADGF-A expression construct together with an Actin-Gal4 driver (providing ubiquitous expression) both completely rescued the mutant phenotype, whereas larvae with UAS-ADGF-A but without the driver showed the typical mutant phenotype. However, neither of the two lines carrying the mutated version of the UAS-ADGF-A (producing full-length protein detected by anti-myc antibody; see Materials and Methods) showed any rescue of the mutant phenotype. This result therefore demonstrates that the catalytic activity of ADGF-A is required for its function in vivo.

Hemocyte Development Is Affected in the adgf-a Mutant

We investigated the number and morphology of hemocytes (blood cells) in the hemolymph of the adgf-a late third-instar larvae (Figures 3 and 4). These larvae contain an average of seven-fold more hemocytes in circulation than wild-type larvae (Figure 3). In contrast to normal larval plasmocytes, which remain rounded after settling down on the substrate (Figure 4A), most of the cells in the adgf-a mutant (more than 75%) are strongly adhesive and, after they are deposited in a drop of hemolymph on a microscope slide, develop filamentous and membranous extensions (Figure 4B–4D). An average of 7% of hemocytes in the adgf-a mutant are lamellocytes (Figures 3 and 4E), large flat cells that are not present in circulation of wild-type larvae under normal conditions [19]. Crystal cells were also detected, which, with mutant larvae carrying several hundred while there are fewer than a hundred of these cells in the wild type (Figure 5). The lymph glands normally do not release hemocytes into the hemolymph before metamorphosis [20]; instead, they are released during metamorphosis when the lymph glands disperse [19]. However, the lymph glands of adgf-a mutant larvae are already dispersed in the late third instar. This process is similar to normal metamorphic changes, in which the hemocytes are first released from the front lobes, and the posterior lobes disperse later.

To analyze hemocytes in living larvae, we used the Hemolectin marker (Hml) [21]. We compared the number and distribution of hemocytes stained by GFP in flies carrying hml-Gal4 UAS-GFP in wild-type and mutant backgrounds. While there are relatively few hemocytes, mostly free-floating in the hemolymph, in early third-instar wild-type larvae (see Figure 4I), a much higher number of hemocytes, which are mostly attached to the tissues under the integument...
(described as sessile hemocytes in [19]), was observed in mutant larvae (see Figure 4J). A similar behavior was detected later in wild-type larvae, toward the end of the third instar (see Figure 4H). At this stage, the Hml marker disappeared from the most of the hemocytes in mutants (see Figure 4F and 4G).

The adgf-a Mutant Phenotype Is Rescued by Expression of ADGF-A in the Lymph Glands

To distinguish which tissues require ADGF-A expression for proper development, we tested for rescue of adgf-a lethality by expressing ADGF-A in specific subsets of larval tissues. A transgenic line carrying the UAS-ADGF-A construct on Chromosome II was crossed to lines expressing the Gal4 driver [18] in different tissues (Table 1). Since ADGF-A is normally expressed in the larval lymph glands [16], and the mutant phenotype is characterized by abnormal hemocyte development, special consideration was given to lines expressing the Gal4 driver in the lymph glands and/or circulating hemocytes. No line expressing the Gal4 driver exclusively in the lymph glands has been reported, so we used a combination of lines sharing in common the feature of Gal4 driver expression in the lymph glands. The results (see Figure 2 and Table 1) clearly demonstrate that expression of ADGF-A in all lobes of developing lymph glands (but not in circulating hemocytes) reduces the number of hemocytes in the hemolymph to almost normal levels (see Figure 3). The number of hemocytes is also reduced, but to a lesser extent in larvae rescued by Cg-Gal4/UAS-ADGF-A. However, when assayed by survival rate and melanotic tumor formation, the rescue by Cg-Gal4 is full and similar to that of e33C-Gal4 (see Figure 2). The difference in effectiveness may be explained by the different expression patterns of the drivers. Cg-Gal4 is expressed only in certain compartments of lymph gland lobes containing relatively mature hemocytes, and strongly in most circulating hemocytes [22, 23]. The C564-Gal4 driver is not expressed as strongly as e33C-Gal4, but is still uniformly expressed in the lymph glands; it also fully rescued the mutant phenotype. We have tried two different insertions of the Dot-Gal4 construct. The Dot-Gal411C insertion on Chromosome II, which shows weak expression [24], did not rescue the phenotype, but a Dot-Gal443A insertion on Chromosome X, which shows stronger expression, rescued approximately half of the mutant animals (Figure 2). Nearly all rescued individuals were males, suggesting that expression of the Gal4 driver was influenced by X-chromosome dosage compensation, and expression in females heterozygous for Dot-Gal4 was not strong enough for rescue.

Expression of ADGF-A in salivary glands and fat body (as well as in other tissues) is not required for full rescue, as
demonstrated by use of the Cg-Gal4, Dot-Gal4, but especially by e33C-Gal4 driver, and is also not sufficient to rescue the phenotype at all, as demonstrated by T110-Gal4 and Lsp2-Gal4 (Table 1).

Since ADGF-A is strongly expressed in embryonic mesoderm [16], we have tried to rescue the phenotype by the expression of ADGF-A in embryonic and larval muscle cells using the Dmef2-Gal4 driver [25]. No rescue of the phenotype, including body shape of escaping pupae, was observed.

The only line showing significant (but not complete) rescue of adgf-a survival without expression in the lymph glands was GawB5015 (see Figure 2), which expresses the Gal4 driver very strongly and specifically in the ring gland and salivary glands (as well as very weak and spotty expression in imaginal discs [unpublished data]). However, expression of ADGF-A driven by GawB5015 does not prevent the formation of melanotic tumors (see Figure 2B).

Ablation of Hemocytes in Mutant Larvae Reduces Fat Body Disintegration and Melanotic Tumor Formation

The l(3)hematopoiesis missing (l[3]hem) mutation reduces cell division in larval proliferating tissues and thus dramatically reduces the number of hemocytes in larvae. It also suppresses the hemocyte overproliferation and associated defects observed in the hopscotchTumorous-lethal mutant [26]. We therefore used the l(3)hem1 mutation to test whether the reduction of hemocyte number in the adgf-a mutant affects the phenotype. We recombined this mutation onto the chromosome containing the adgf-a mutation and found that in homozygous l(3)hem1, adgf-a double mutants the number of hemocytes is significantly reduced compared to the adgf-a single mutants (see Figure 3). Furthermore, while 90% of adgf-a mutant larvae showed disintegration of fat body, only 40% of l(3)hem1, adgf-a double mutants (total number of counted animals was 82) show the disintegration (Figure 6A). Similarly, melanotic tumor formation is significantly suppressed by l(3)hem1, with only 55% of double mutants showing melanotic tumors compared to more than 83% in adgf-a (Figure 6A). However, the delay in development and block of pupariation (Figure 6B), as well as the pupal body shape, were not influenced by this mutation. This shows that the effect on hemocyte development is related to only one other aspect of the adgf-a phenotype—namely, fat body disintegration—and the developmental arrest of adgf-a mutants is probably independent of this process.

Block in Activation of Macrophages Suppresses Disintegration of Fat Body

Previous results suggest that fat body disintegration might be caused by the action of hemocytes. Embryonic macrophages express the scavenger receptor encoded by croquemort (crq), which allows them to bind and remove apoptotic corpses [27]. We therefore tested whether a mutation in the crq gene would block the suggested interaction between hemocytes and fat body in adgf-a mutant larvae. We used the mutation

| Table 1. Gal4 Drivers—Expression Pattern and Rescue of the adgf-a Phenotype |
| --- |
| Gal4 Line | Average Survival Rate (%) | Expression in: | Lymph Glands | Embryonic Hemocytes | Salivary Glands | Fat Body | Lymph Gland Expression Details | Expression in Other Tissues |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Cg | 94 | + | + | – | + | Only mature cells | No |
| e33C | 89 | + | +/- | – | – | Strong, uniform | Malpighian tubules, trachea, optic lobes, gut, brain |
| c564 | 88 | + | – | + | + | Medium, uniform | Imaginal discs, gut, brain |
| GawB5015 | 44 | – | – | + | + | Ring gland, imaginal discs |
| Dot43A | 38 | + | – | + | – | Variable expression | Proventriculus, pericardial cells |
| T110 | 16 | – | – | + | – | Malpighian tubules, gut, brain, imaginal discs; weakly ring gland |
| Dmef2 | 0 | – | – | + | – | Embryonic and larval muscles |
| Lsp2 | 0 | – | – | – | + | No |

DOI: 10.1371/journal.pbio.0030201.t001
were not detected in the number of crystal cells was not increased and lamellocytes untranslated exon of body or melanotic tumor formation (Figure 6A). Even the double-mutant larvae showed either disintegration of fat body and macrophage-like cells (which are still present in double mutants) suppresses the fat body disintegration, further strengthening the hypothesis that the disintegration is caused by hemocytes. In addition, the absence of lamellocytes and the normal number of crystal cells in the double mutant strongly suggest that the differentiation of these cells and thus melanotic tumor formation is a secondary reaction to fat body disintegration, rather than a primary effect of the adgf-a mutation.

**Mutation in a Putative Adenosine Receptor Suppresses the Block of Pupariation in adgf-a**

We have identified a putative homolog of the mammalian adenosine receptor family in the *Drosophila* genome, AdoR, and produced a null mutation in this gene using homologous recombination (adoR; ED, unpublished data). The adoR mutants are fully viable. We used this mutant to test the hypothesis that the increased level of adenosine in the adgf-a mutant contributes to the mutant phenotype by its effect on signaling through the adenosine receptor. The results show that introducing the adoR mutation into the adgf-a background significantly increases pupariation, as well as adult emerging rate, compared to the adgf-a single mutant (Figure 6B). When the earlier lethality was avoided by picking up larvae after molt to the third instar, the pupariation rate of adoR, adgf-a double mutant was comparable to wild type as well as to the single adgf-a mutant treated with ecdysone (Figure 7A). Development during the third instar is much less delayed in the double mutant, with most of the larvae pupariating within 1 d after their heterozygous siblings (Figure 7A).

The adoR mutation also significantly reduced melanotic tumor formation in the adgf-a mutant (see Figure 6A), but disintegration of the fat body appeared at the same rate as in the single mutant (see Figure 6A). While the number of macrophage-like cells in circulation is not significantly changed in the double mutant, the number of lamellocytes is decreased (see Figure 3), but the number of crystal cells is normal (see Figure 5A and 5C).

These results demonstrate that adenosine signaling through the adenosine receptor is involved in the developmental arrest of adgf-a mutant, but that it does not play a role in fat body disintegration and macrophage differentiation.

**Hormonal Regulation in the adgf-a Mutant**

The delayed development and low pupariation rate in the adgf-a mutant larvae (see Figures 2A and 7A) could be caused by an effect on hormonal regulation of development. The main source of developmental hormones in the *Drosophila* larva is the ring gland, composed of the prothoracic gland, corpus allatum, and corpus cardiacum [28]. The prothoracic gland releases the steroid molting hormone ecdysone, which is converted to an active form, 20-hydroxyecdysone (20E), by the fat body as well as some of the target organs [29]. The block of pupariation in the adgf-a mutant suggested that the

---

**Figure 6.** Suppression of the adgf-a Mutant Phenotype by Mutations in Other Genes

(A) Percentage of late third-instar larvae with melanotic tumors (black bars) and fat body disintegration (green bars). The x-axis (which is shared with [B]), shows the genotype. The y-axis shows the percentage of larvae with tumors and fat body disintegration.

(B) Survival rate of double mutants compared to single adgf-a mutant. The y-axis shows the percentage of the pupae (blue bars) and adult flies (purple bars) demonstrating the larval and pupal survival, respectively. Each experiment was repeated at least four times (with 20–30 animals in each vial) and the standard error is shown.

DOI: 10.1371/journal.pbio.0030201.g006

---

The double mutants showed a lower number of circulating hemocytes than the single mutant, but there was still a significant increase in this number compared to wild type (see Figure 3), and the cells showed increased clumping. None of the double-mutant larvae showed either disintegration of fat body or melanotic tumor formation (Figure 6A). Even the adgf-a mutant larvae heterozygous for the crq mutation (crq/CyO GFP; adgf-a/adgf-a) showed significant suppression of the fat body disintegration, with most of the tissue staying compact in bigger pieces and never disintegrating to single adipose cells; melanotic tumors were rarely observed. This shows that the block of the putative interaction between fat body and macrophage-like cells (which are still present in double mutants) suppresses the fat body disintegration, further strengthening the hypothesis that the disintegration is caused by hemocytes. In addition, the absence of lamellocytes and the normal number of crystal cells in the double mutant strongly suggest that the differentiation of these cells and thus melanotic tumor formation is a secondary reaction to fat body disintegration, rather than a primary effect of the adgf-a mutation.
level of ecdysone in these larvae might not be sufficient to initiate pupariation. To test this possibility, we tried to rescue the phenotype by feeding mutant larvae 20E, which can initiate pupariation in the ed1 mutant, which has an extremely low level of ecdysone [30,31]. The results (Figure 7A) clearly demonstrate that the adgf-a mutant larvae are responsive to ecdysone and that this treatment restores the pupariation frequency to almost wild-type level. The delay in development is also significantly reduced (Figure 7A).

Since the adgf-a mutant shows certain precocious metamorphic changes (macrophage differentiation and fat body disintegration), we speculated that a reduced ecdysonoid level could be caused by precocious degeneration of the prothoracic part of the ring gland. However, the overall structure of the ring gland is not visibly affected even in the oldest larvae (10 d, i.e., 5 d after the heterozygous siblings pupariated) with a fully disintegrated fat body (Figure 7B and 7C).

We also used a transgenic line carrying the Sgs3-GFP construct, which was previously used to monitor the effects of ecdysteroid levels on glue protein expression in salivary glands [32]. All analyzed adgf-a mutant larvae carrying the Sgs3-GFP construct showed normal expression of Sgs-GFP in salivary glands (Figure 7D). Mutants that pupariated usually showed typical GFP expectoration, indicating the presence of a high premetamorphic peak of ecdysteroids (Figure 7E). In some cases, GFP was secreted into the lumen of salivary glands, but was not expectorated (Figure 7F), which is similar to the defect seen in animals expressing the dominant-negative form of ecdysone receptor driven by the Sgs3-Gal4 driver [33]. These results demonstrate that the target tissues of adgf-a mutants are normally responsive to ecdysteroids and that they are probably capable of releasing ecdysteroids, although the level of ecdysteroids might vary.

ADGF-A Genetically Interacts with Toll Signaling Pathway

The antimicrobial response of Drosophila includes at least two distinct signaling pathways [34]—the Toll signaling pathway, which leads to the activation of two nuclear factor kappa B (NF-kB) factors, Dorsal-related immunity factor (DIF) and dorsal (DL); and the immune deficiency protein pathway activating the third NF-kB factor, Relish (REL). A zygotic null mutation in cactus (cact; a Drosophila inhibitor of NF-kB) leads to hyperproliferation of hemocytes, melanotic tumor formation, disintegration of fat body, and slower larval development, with 60% larval lethality, as well as a thin body-shape phenotype [35]. All of these phenotypes are strikingly similar to the abnormalities seen in adgf-a mutants, which was our first clue as to a possible interaction of ADGF-A with the Toll signaling pathway. We hypothesized that the activity of ADGF-A is suppressed by Toll signaling, resulting in similar phenotypes of the adgf-a mutation and constitutive activation of Toll pathway.

To test this hypothesis, we crossed transgenic flies carrying ADGF-A gene under the control of a heat-shock promoter on Chromosome II (HS-ADGF-A) with cactE8 (a lethal allele of cact on Chromosome II, which, in combination with cactP13, results in a zygotic null combination, or, with cactP13, results in zygotic hypomorphic combination).

Overexpression of ADGF-A in animals with a hypomorphic cact combination (cactE8/cactP13) increased the adult survival rate almost 4-fold (Figure 8A). The rescue could be increased by multiple heat shocks before pupariation to 7-fold (unpublished data). The suppression of melanotic tumor formation is also significant (from more than 80% down to 26%, Figure 8B). The most severe cact null mutation (cactE8/cactP13), leading to developmental arrest in larvae (less than 8% pupate), is partially rescued in animals with over-expression of ADGF-A when the pupariation rate is increased 3-fold (Figure 8A).

These results demonstrate that ADGF-A overexpression can partially rescue the effects of constitutively active Toll signaling in larvae, mainly the developmental arrest, but also...
Discussion

ADA Deficiency in Drosophila Causes Abnormal Hemocyte Development, Melanotic Tumor Formation, Fat Body Degeneration, and Delayed Development

We have established an ADA deficiency model in Drosophila in order to study the effects of altered adenosine levels in vivo. We produced a loss-of-function mutation in the ADGF-A gene, which produces a product (ADGF-A) with ADA activity. When homozygous, the mutation causes abnormal hemocyte development, leading to melanotic tumor formation [36], as well as fat-body disintegration associated with death during the larval stage or delayed transition to the pupal stage of development. In agreement with our previous study using cells cultured in vitro [16], here we have shown that ADA enzymatic activity is essential for ADGF-A function in vivo, when this function is assayed by testing for rescue of the mutant phenotype. Just as increased levels of both ADA substrates, adenosine and deoxyadenosine, are found in blood of SCID patients [5], adgf-a mutant larvae also have elevated levels of adenosine and deoxyadenosine, indicating that the mutant phenotype is caused by disturbance in the turnover of these nucleosides.

Expression of ADGF-A only in the lymph glands is sufficient to fully rescue the mutant phenotype, indicating that the hemocytes within the lymph glands play a major role in regulation of adenosine levels in the hemolymph. A similar regulatory role has also been attributed to blood cells in humans [5]. This suggests a function for ADGF-A within the lymph gland. However, ADGF-A behaves as a soluble growth factor and could be released from the lymph gland to activate targets elsewhere in the larval body. Our results show that ADGF-A functions by limiting the level of extracellular adenosine, and in this way the protein could have a systemic function even if it were restricted to its tissue of origin. Although our tests did not exclude a role for ADGF-A in circulating hemocytes (which constitute a separate lineage from the lymph gland hemocytes [20]), we showed that expression of ADGF-A in circulating hemocytes is not required for rescue of the adgf-a mutant phenotype, since e33C-Gal4/UAS-ADGF-A—which expresses ADGF-A in the lymph gland but not in circulating hemocytes—fully rescued the phenotype.

ADGF-A Is Involved in Hemocyte Differentiation in the Lymph Glands

Late third-instar larvae homozygous for the adgf-a mutation contain, on average, seven times more hemocytes in circulation than wild-type larvae, and most of these cells show strong adhesive properties compared to normal larval plasmatocytes, which remain rounded after settling down on the substrate. Although these cells share other characteristics with plasmatocytes, they are normally not seen in circulation until they are released from the lymph glands at the onset of metamorphosis under the regulation of ecdysone to serve as phagocytes for histolysing tissues during metamorphosis—thus, they are referred to as pupal macrophages [19]. In agreement with the presence of these cells in circulation, at least the first lobes of the lymph glands are usually completely dispersed in late third-instar mutant larvae. This indication of precocious metamorphic changes [36] in the mutant is further supported by the finding that hemocytes aggregate in a segmental pattern in early rather than late third instar (see Figure 4H–4J), and that the hemocytes lose expression of Hemolectin in late third-instar larvae rather than at the onset of metamorphosis (see Figure 4G) [21].

Recent studies show that the Toll signaling pathway, which is already known to be involved in the control of innate immunity of both Drosophila and mammals [34], may also be involved in the control of hemocyte differentiation in the Drosophila larva. Constitutive activation of Toll signaling leads to developmental arrest and hematopoietic defects associated with the melanotic tumor formation, in the case of hypomorphic cact mutants.
Adenosine Deaminase-related Growth Factor-A (ADGF-A)

Figure 9. Schematic Map of the ADGF-A Gene with Promoter Analysis

The ADGF-A gene contains four exons and two transcriptional starts [17,47]. We analyzed sequences preceding both transcriptional starts for the presence of known transcriptional factor binding sites using the software program Gene2Promoter (Genomatix Software GmbH). Selected sites are represented by color bars in approximate positions of promoter regions. The legend under the sequence shows the names of transcription factors binding to matching colored binding sites.

DOI: 10.1371/journal.pbio.0030201.g009

with melanotic tumor formation [35], similar to the phenotype of the adgf-a mutant. Our work also shows that forced expression of the ADGF-A gene can rescue the effects of overactive Toll signaling, suggesting that ADGF-A might function downstream of Toll signaling to control its effects. This conclusion is consistent with the existence of a putative binding site for Dorsal (one of two known effectors of Toll signaling) in the ADGF-A promoter (Figure 9). It will be important to explore this connection further, since recent studies suggest an interaction between adenosine signaling and the NF-kB signaling pathway, which is the mammalian counterpart of the Toll pathway [37].

Precocious Fat-Body Disintegration Caused by Mutant Hemocytes

One of the most remarkable features of the adgf-a mutant phenotype is the disintegration of the fat body in third-instar larvae, another indication of precocious metamorphic changes since the disintegration normally occurs much later, during pupal life. Furthermore, our study of this mutant provides strong evidence that the fat body disintegration is promoted by the action of hemocytes. Fat body disintegration was significantly suppressed when the hemocyte number was reduced using the l(3)hem1 mutation [26], and fully blocked by the croquemort (crq) mutation [27] which affects a CD36-related receptor (Croquemort) expressed on macrophages and required in phagocytosis of apoptotic cells. Human CD36 is a scavenger receptor which, in combination with the macrophage vitronectin receptor and thrombospondin, binds apoptotic cells. A similar role of Croquemort for removing histolyzing tissues during Drosophila metamorphosis has not yet been tested, but seems likely since the crq mutant used in this study [crqKG01679] is lethal in pupae.

The idea that hemocytes are involved in fat body disassociation in Drosophila is further supported by work on the flesh fly Sarcophaga. Natori’s group showed that proteinase cathepsin B was released from pupal hemocytes when they interacted with the fat body, and that this enzyme digested the basement membrane of the fat body, causing the tissue to dissociate [38,39]. They also showed that the interaction of hemocytes with the fat body is mediated by a 120-kDa membrane protein localized specifically on pupal hemocytes [40]. This protein was suggested to be a scavenger receptor, but it does not seem to be homologous to Drosophila Croquemort (unpublished data). Work by Franc et al. [27] is consistent with the idea that more than one scavenger receptor is involved in this process.

Possible Signaling Role for Adenosine

The precocious metamorphic changes that appear to occur in response to elevated adenosine in the adgf-a mutant larvae lead to the suggestion that adenosine may act as a regulatory signal for these processes during normal development. One possibility is that adenosine acts as a downstream effector of ecdysone-regulated prepupal changes, and that the increase in adenosine concentration is mediated by ecdysone-induced down-regulation of ADGF-A expression. This is supported by the presence of multiple sites for ecdysone-inducible transcription regulators in the ADGF-A promoter (Figure 9). Adenosine could serve as a signal for macrophage differentiation, and the lack of adenosine deaminase activity due to the adgf-a mutation could cause precocious differentiation of these cells in mutant larvae. We are now carrying out direct tests of the idea that the differentiation of hemocytes in mutant larvae is caused by elevated adenosine. If confirmed, this effect would have general significance, since in ADA-deficient mice, inflammatory changes in the lungs include an accumulation of activated alveolar macrophages [41], and this could also be mediated by elevated adenosine.

Elevated Adenosine Delays Development and Inhibits Pupariation

The elevated adenosine in the adgf-a mutant larvae leads to precocious changes (hemocyte differentiation and fat body disintegration) resembling those normally occurring at the time of metamorphosis, but it also is associated with an apparently opposite effect, in that it causes a significant delay in progress through the third larval instar and a decrease in the frequency of successful pupariation (formation of the puparium from the larval cuticle), which is one of the earliest steps in metamorphosis. We conclude that the mutation has additional effects on the hormonal regulation of development.

One possible explanation for the developmental delay and failure to pupariate is that the adgf-a mutation affects the production or release of ecdysteroid hormones from the major endocrine organ of the Drosophila larva—the ring gland. This is supported by the fact that pupariation rate and survival of the adgf-a mutant can be significantly improved by expression of transgenic ADGF-A in the ring gland and
salivary glands. We suggest that this somehow interferes with the regulation of hormone release. Other mutants with hormonal dysregulation show delayed larval development and failure to pupariate [42,43]. Presumably the elevated adenosine in the adgf-a mutant blocks the production or release of ecdysone from the ring gland by an unknown mechanism. This idea is supported by our finding that both pupariation rate and survival of the adgf-a mutant can also be improved by feeding the mutant larvae with 20E in the diet (see Figure 7A). Thus it is clear that the adgf-a mutant is arrested in development due to an effect of the mutation on hormone production from the ring gland.

The arrest of development in the adgf-a mutants was significantly suppressed by loss of the adenosine receptor caused by the ador mutation: larvae simply homozygous for adgf-a pupated after two or more days, whereas larvae also homozygous for ador pupated within 1 d after their heterozygous siblings (see Figure 7A). Therefore, adenosine signaling through the AdoR must play a role in the developmental arrest of the adgf-a mutant, and this is most likely mediated by signaling to the ring gland, where AdoR is expressed (ED, unpublished data). The mutation in AdoR does not block macrophage differentiation and fat-body disintegration, so this effect must involve another, as yet uncharacterized mechanism independent of AdoR signaling.

Work using adenosine-receptor deficient mammalian cells also suggested the existence of a novel, undefined adenosine signaling mechanism [44]. However, we cannot exclude the role of elevated deoxyadenosine in these effects. Drosophila, now with the advantage of the well-characterized adgf-a mutant, could serve as an ideal model system in which to investigate this mechanism.

Concluding Remarks

In our previous work using cells cultured in vitro, we showed that, as in mammals, adenosine can block proliferation and/or survival of some Drosophila cell types [16]. In the present work, we have established a Drosophila model to study altered levels of adenosine and deoxyadenosine in vivo, and we have shown that loss of ADGF-A function causes an increase of these nucleosides in larval hemolymph. Although the adgf-a mutation leads to larval or pupal death, we have shown that this is not due to the adenosine or deoxyadenosine simply blocking cellular proliferation or survival, as the experiments in vitro would suggest. Rather, this mutation leads to an increase in number of hemocytes at the end of larval development due to the differentiation and release of hemocytes from the lymph glands. Hemocytes also differentiate and are released from the lymph glands during systemic infection [19]. Together with our result suggesting an interaction between Toll signaling and ADGF-A, this leads to the hypothesis that adenosine controls hemocyte differentiation in response to infection, and that it signals through the adenosine receptor to postpone the next developmental step, metamorphosis. This would be consistent with the role of adenosine as a "stress hormone" in mammals [6]. A similar process of hemocyte differentiation and release from the lymph glands normally takes place at the onset of metamorphosis, when pupal macrophages remove histolyzing tissues. The ADGF-A promoter contains consensus binding sites for effectors of both Toll and ecysdine signaling. This raises the possibility that adenosine plays a role in the control of metamorphosis as well as in the response to stress.

Materials and Methods

Fly strains and genetics. For standard procedures, flies were raised at 25 °C on a standard cornmeal-agar-yeast-molasses diet supplemented with 0.5% Nipagin to retard mold growth. Oregon flies were used as the wild-type Drosophila strain, but in most cases the y w strain was used as a control since most mutations were carried in the y w background. A mutation in the ADGF-A gene on Chromosome III was obtained as described earlier [17]. In this study, the mutation described as adgf-a\textsuperscript{null} was used in all experiments and is referred to here as adgf-a. A mutation in the adenosine receptor gene on Chromosome III was produced by the ends-out targeting method [45]. HS-ADGF-A, UAS-ADGF-Amyc, and UAS-mutADGF-A\textsuperscript{myc} construct (see description below) were produced by a modified P-element transformation method [46, HS-ADGF-A, UAS-ADGF-A\textsuperscript{myc}[2A], UAS-ADGF-A\textsuperscript{myc}[7A], UAS-mutADGF-A\textsuperscript{myc}[1A], and UAS-mutADGF-A\textsuperscript{myc}[3B], all insertions on Chromosome II, were isolated and used in this work. The following markers and mutations were obtained from the Bloomington stock center, accessible at http://fly.bio.indiana.edu (stock numbers provided in parentheses): Hml-GFP marker (Hml-Gal4/UAS-GFP) expressing GFP in embryonic and larval hemocytes on Chromosome II (BL-6357), the cact\textsuperscript{mel} mutation on Chromosome III (BL-6184), and the cact\textsuperscript{G00570} mutation in the cact gene on Chromosome II (BL-14990). Mutants in Toll signaling pathway were obtained from Dr. S. Govind: cat\textsuperscript{ss} cat\textsuperscript{1} cat\textsuperscript{2} mutations in the cact gene on Chromosome II. The Gal4/USP [18] system was used for precision expression. The following were obtained from the Bloomington stock center (stock numbers in parentheses): cGab-Gal4 on Chromosome II (BL-7011), P\textsuperscript{[w\textsuperscript{+}\textsuperscript{m} = GamB]} 5015 on II (BL-2721), P\textsuperscript{[w\textsuperscript{+}\textsuperscript{m} = GamB]} 564 on II (BL-6982), P\textsuperscript{[w\textsuperscript{+}\textsuperscript{m} = GamB]} T110 on II (BL-6098), Hml-Gal4 on II (BL-6396), Dot-Gal4\textsuperscript{u} on X (BL-6098), Dot-Gal4\textsuperscript{u} on II (BL-6092), and Lsp2-Gal4 (BL-6537) on III. The P\textsuperscript{[m24-GAL4] cact\textsuperscript{3C}}Gal4 E77C lethal insertion on Chromosome III was obtained from Dr. N. Perrimon’s lab, and the Dmef2-Gal4 driver on II from Dr. A. Michelson. Expression information of these Gal4 drivers is provided in Table 1. A stock carrying the ubiquitous actin-Gal4 driver (P\textsuperscript{[actin-Gal4]} UAS-GFP) was obtained from Dr. R. Sousa. To recognize homozygous larvae, balancer chromosomes with the GFP marker were used: CyO P\textsuperscript{[w\textsuperscript{+}\textsuperscript{m} = Ubi-GFP.S65T]} PAD1 (BL-4559) and TM3 P\textsuperscript{[w\textsuperscript{+}\textsuperscript{m} = ActGFP]} MAR2 Ser (BL-4888). Transgenic flies SgGFP-1 (insertion on Chromosome X) and SgGFP-2 (insertion on Chromosome II) were obtained from Dr. A. Andres. For expression of ADGF-A using the HS-ADGF-A construct, flies were heat shocked as late embryos/early first instars at 37 °C for 30 min. In all rescue experiments, 30 freshly hatched homozygous first-instar larvae were selected using a GFP dissection microscope and transferred into fresh vials (at least four vials for each variant). They were left to develop at 25 °C and examined as wandering third-instar larvae, pupae, and adults.

Ecdysone treatment. Mutant larvae were raised on plates with yeast paste at 25 °C and transferred to vials with glucose-yeast medium (control) or with glucose-yeast medium containing 20-hydroxyecdysone (H-5142, Sigma-Aldrich, St. Louis, Missouri, United States) at a concentration of 0.5 mg/ml shortly after the L2/L3 molt. Numbers of puparia were counted at 12-h intervals after the 120-h time point (when the first control larvae start to pupariate). The ecd\textsuperscript{0} flies (Bloomington stock BL-2198) served as a control for the functional 20E diet [31]: flies were raised at 22 °C (permissive temperature for the temperature-sensitive ecd\textsuperscript{0} mutation) and transferred to vials with control or 20E-containing diet and raised at 29 °C (restrictive temperature).

Fat body observation. Living late third-instar larvae were washed and examined in PBS using a standard dissecting microscope with transmitted light. For finer analysis, the fat body was dissected from larvae in PBS and observed using a dissecting microscope. GFP-stained fat body was observed in living, etherized larvae in PBS solution on a standard microscopic slide with a coverslip under a fluorescence microscope.

Hemocyte counts and observations. Circulating hemocytes were obtained by opening two late third-instar larvae in 30 μl of PBS. This allowed us to collect all hemolymph from the larvae in a defined volume. The solution with circulating hemocytes was mixed by gently pipetting, and part was transferred into the chamber of an improved Neubauer hemocytometer. Cell number was recounted to one animal equivalent. Hemocyte morphology was observed by differential
interference contrast microscopy of living cells in Shields and Sang Insect Medium (Sigma-Aldrich) obtained by the same procedure as for counting. To observe hemocyte morphology, samples were analyzed at 10 min after the deposition of solution with hemocytes, in order to allow the cells to adhere to the surface of the slide. Crystal cells were visualized by heating larvae at 60 °C for 10 min in a beaker bath [46]. GFP-stained hemocytes were observed in living, etherized larvae in PBS solution on a standard microscope slide with a coverslip under the fluorescence microscope or by deposition of hemocytes in PBS as for counting and observing under the fluorescence microscope.

**Transgenic stocks.** Wild-type cDNA for ADGF-A was amplified by PCR using proofreading DNA polymerase (ProofStart; Invitrogen, Carlsbad, California, United States) from a pOT2 vector containing the ADGF-A EST-clone (GH08276) using the following primers: 5′-CTGCTGAGAATGGTCGGCAAATCAGCGC-3′ (5′ end primer with XbaI tail) and 5′-GCTGATCATTACAGTGCGCTGTTGAGCGGAGG-3′ (5′ end primer with BclI tail). The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin, United States), and the resulting plasmid (ADGF-A-pGEM) was cut by NotI/SpeI restriction enzymes. The ADGF-A fragment was then cloned into the pCaspSspl HS vector cut by NotI/XbaI to obtain the HS-ADGF-A construct. The myc tag was added to the C terminus of the ADGF-A protein for detection by anti-c-Myc antibody (Sigma-Aldrich). To produce a UAS-ADGF-Amyc construct, the ADGF-A fragment was amplified (by ProofStart from pOT2 vector) using the following primers: 5′-AATCTCGAGACCATCATGTGCGGAGGATCCTGC-3′ (5′ end primer with XbaI tail) and 5′-GCTGATCATTACAGTGCGCTGTTGAGCGGAGG-3′ (5′ end primer with BclI tail). The fragment was cut by XhoI/XbaI and cloned into the pUAST vector modified by MZ. The sequence encoding the catalytic domain, shown to abolish adenosine deaminase [16], was inserted in a sense orientation at the 5′ end of hemolymph was diluted in 99% PBS as for counting and observing under the fluorescence microscope.

Adenosine and deoxyadenosine concentrations measurement. The detection method used liquid chromatography and mass spectrometry (LC/MS method) of deproteinized hemolymph samples. Larval hemolymph was collected from several larvae and centrifuged to pellet the hemocytes. 1 μl of hemolymph was diluted in 99 μl of buffer. The sample was introduced in CH3CN-0.05% TFA (50:50) for 3 min by a microsyringe (Hamilton) at 3 μl/min via an RP-C18 (150 mm × 5 mm Symmetry C8 column at 50 °C) using the electrospray MSN mass spectra obtained by the collision-induced dissociation of the MH+ ion and its product ions in a series of MS/MS experiments that were performed with the ion trap mass spectrometer. The sugar moiety was cleaved off the adenosine molecule and produced ion with a molecular weight of 136 (adenine), which was then detected by MS.

**Supporting Information**

**Accession Numbers**

The FlyBase (http://flybase.bio.indiana.edu) accession numbers for the genes and proteins discussed in this paper are: Ada (FBgn0013766), ADGF-A (FBgn0036752), ADGF-A cDNA (FBpt0018801), adgf-a mutation (FBal0157461), adgf-a mutell (FBal0098650), ADGF-D (FBgn0038172), AdoR (the CG9753 gene; FBgn0039747), c54-4Gal4 (FBti0002929), c54-11Gal4 (FBti0001509), c54-12Gal4 (FBal0097060), c54-14 (FBti0001513), cacten (FBgn0002950), CcGal4 (FBal0002912), c635C-Gal4 (FBti0001529), c54-13 (FBti0002928), c54-10Gal4 (FBti0001527), c54-12Gal4 (FBti0002924), Dot-Gal4 transgenic (FBpt0018800), C0555-Gal4 (FBal0010873), Ssp2-Gal4 (FBti0018351), Relish (FBgn0014108), Sg3-4-3GST (FBpt0013370), Sg3-4GAL4 (FBpt0016397), and T110-Gal4 (FBti0002605).

**Acknowledgments**

We are especially grateful to Petr Simik and his lab for performing the measurement of adenosine concentration. We thank Rui Sousa, Subha Govind, Andrew J. Andres, and the Bloomington Stock Center for fly stocks; and Michal Gazi and other colleagues for critical reading of the manuscript and discussions. We are also grateful to our research technician Ruzenka Kuklova for her help. This work was supported by grants from the United States National Science Foundation (grant number 440860-21065), the Grant Agency of the Czech Republic (grant number 204/04/1205), the Grant Agency of the Czech Academy of Sciences (grant number A5007107), the Ministry of Education, Youth, and Sports of the Czech Republic (grant number MSM6007665801), and the National Institute of Health (grant number R01NS051219). We are especially grateful to Petr Simek and his lab for performing the measurement of adenosine concentration. We thank Rui Sousa, Subha Govind, Andrew J. Andres, and the Bloomington Stock Center for fly stocks; and Michal Gazi and other colleagues for critical reading of the manuscript and discussions. We are also grateful to our research technician Ruzenka Kuklova for her help. This work was supported by grants from the United States National Science Foundation (grant number 440860-21065), the Grant Agency of the Czech Republic (grant number 204/04/1205), the Grant Agency of the Czech Academy of Sciences (grant number A5007107), the Ministry of Education, Youth, and Sports of the Czech Republic (grant number MSM6007665801), and the National Institute of Health (grant number R01NS051219).

**Competing interests.** The authors have declared that no competing interests exist.

**Author contributions.** TD, ED, and MZ conceived and designed the experiments. TD, ED, and MZ performed the experiments. TD analyzed the data. TD, MZ, and PJB wrote the paper.

**References**

1. Aldrich MB, Blackburn MR, Kellemes RE (2000) The importance of adenosine deaminase for lymphocyte development and function. Biochem Biophys Res Commun 272: 311–315.
2. Buckley RH, Schiff RI, Schiff SE, Markert ML, Williams LW, et al. (1997) Human severe combined immuno deficiency: Genetic, phenotypic, and functional diversity in one hundred eight infants. J Pediatr 130: 378–387.
3. Richard E, Alam SM, Arredondo-Vega FX, Patel DD, Hershfield MS (2002) A novel gene encoding a membrane-bound extracellular signaling molecule expressed exclusively in testis of Drosophila melanogaster. J Biol Chem 277: 19720–19726.
4. Bai Y, Shin J, Hershfield MS, Mitchell BS (2001) Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. In: Vogelstein B. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill. pp. 2385–2311
5. Hershfield MS, Mitchell BS (2002) Adenosine deaminase: a potential mediator of immunosuppression in multiple organ failure. Curr Opin Pharmacol 2: 440–444.
6. Scheper B, Doda M, Barany M, Hasko G (2000) Ischemic-like condition releases norepinephrine and purines from different sources in superfused rat spleen strips. J Neuroimmunol 111: 45–54.
7. Bodin P, Burnstock G (1998) Increased release of ATP from endothelial cells during acute inflammation. Inflamm Res 47: 351–354.
8. Riazi MA, Brinkmann-Mills P, Nguyen T, Pan H, Phan S, et al. (2000) The human homolog of insect-derived growth factor, CECR1, is a candidate gene for features of cat eye syndrome. Genomics 64: 277–285.
9. Hershfield MS, Mitchell BS (2001) Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. In: Vogelstein B. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill. pp. 2385–2311
10. Bai Y, Shin J, Hershfield MS, Mitchell BS (2001) Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. In: Vogelstein B. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill. pp. 2385–2311
11. Hershfield MS, Mitchell BS (2001) Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. In: Vogelstein B. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill. pp. 2385–2311
12. Li S, Aksoy S (2000) A family of genes with growth factor and adenosine deaminase activity. J Biol Chem 275: 36934–36941.
13. Akalal DB, Nagle GT (2001) Mollusk-derived growth factor: Cloning and
developmental expression in the central nervous system and reproductive tract of Aplysia. Brain Res Mol Brain Res 91: 163–168.

15. Zurovec M, Dolezal T, Gazi M, Bryant PJ (2001) ADGFs—Growth factors with enzymatic activity. A Dros Res Conf 42: 69.

16. Zurovec M, Dolezal T, Gazi M, Pavlova E, Bryant PJ (2002) Adenosine deaminase-related growth factors stimulate cell proliferation in Drosophila by depleting extracellular adenosine. Proc Natl Acad Sci U S A 99: 4403–4408.

17. Dolezal T, Gazi M, Zurovec M, Bryant PJ (2003) Genetic analysis of the ADGF multigene family by homologous recombination and gene conversion in Drosophila. Genetics 165: 653–666.

18. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.

19. Lanot R, Zachary D, Holder F, Meister M (2001) Postembryonic hematopoesis in Drosophila. Dev Biol 230: 243–257.

20. Holt A, Bossinger B, Strasser T, Janning W, Klapper R (2003) The two origins of hemocytes in Drosophila. Development 130: 4955–4962.

21. Goto A, Kadowaki T, Kitagawa Y (2003) Drosophila hemolymph gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev Biol 264: 592–591.

22. Asha H, Nagy I, Kovacs G, Stetson D, Ando I, et al. (2003) Analysis of ras-hem1 mutation blocks proliferation and hematopoiesis in Drosophila. A Dros Res Conf 42: 69.

23. Evans CJ, Banerjee U (2004) Molecular genetic analysis of the dorothy enhancer has Tinman binding sites and drives hopscotch-induced tumor formation. Genesis 34: 23–28.

24. Kimbrell DA, Hice C, Bolduc C, Kleinhesslkin K, Beckham K (2002) The Dorothy enhancer has Tinman binding sites and drives hopscotch-induced tumor formation. Genesis 34: 23–28.

25. Ranganayakulu G, Schulz RA, Olson EN (1996) Wingless signaling induces degeneration of the prothoracic glands during the larval-pupal-adult transformation in Drosophila melanogaster. J Morphol 207: 1151–1161.

26. Leung SS, Govind S (2003) The l(3)hem1 mutation blocks proliferation and development of hematopoietic precursors in the Drosophila larva. A Dros Res Conf 44: A321.

27. Franc NC, Heitzler P, Ezekowitz RA, White K (1999) Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila. Science 284: 1991–1994.

28. Dai JY, Gilbert LI (1991) Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of Drosophila melanogaster: A cytophysiological analysis of the ring gland. Dev Biol 144: 309–326.

29. Riddiford LM (1995) Hormones and Drosophila development. In: Bate M, Arias AMeditors. The development of Drosophila melanogaster. Plainview: Cold Spring Harbor Laboratory Press. pp 899–939

30. Garen A, Kavart L, Lepesant J-A (1977) Roles of ecdysone in Drosophila development. Proc Nail Acad Sci U S A 74: 5089–5105.

31. Gaziova I, Bonnete PC, Henrich VC, Jindra M (2004) Cell-autonomous roles of the ecdysoneless gene in Drosophila development and oogenesis. Develop 131: 2715–2725.

32. Biyasheva A, Do TV, Lu Y, Vaskova M, Andres AJ (2001) Glue secretion in the Drosophila salivary gland: A model for steroid-regulated exocytosis. Dev Biol 231: 254–254.

33. Cherbas L, Xu X, Zhuimulev E, Belyaeva E, Cherbas P (2003) ECR isoforms in Drosophila: Testing tissue-specific requirements by targeted blockade and rescue. Development 130: 271–284.

34. Hoffmann JA, Reichhart JM (2002) Drosophila innate immunity: An evolutionary perspective. Nat Immunol 3: 121–126.

35. Qiu P, Pan PC, Govind S (1998) A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis. Development 125: 1909–1920.

36. Rizki MTM (1957) Tumor formation in relation to metamorphosis in Drosophila melanogaster. J Morphol 100: 459–472.

37. Hershfield MS (2005) New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. Eur J Immunol 35: 25–30.

38. Kurata S, Saito H, Natori S (1992) The 29-kDa hemocyte proteinase dissociates fat body in Sarcophaga. Dev Biol 153: 113–121.

39. Yano T, Takahashi N, Kurata S, Natori S (1995) Regulation of the expression of cathepsin B in Sarcophaga peregrina (flesh fly) at the translational level during metamorphosis. Eur J Biochem 234: 39–43.

40. Hori S, Kobayashi A, Natori S (2000) A novel hemocyte-specific membrane protein of Sarcophaga (flesh fly). Eur J Biochem 267: 5397–5403.

41. Blackburn RM, Volmer JB, Thresher JL, Zhong H, Crosby JR, et al. (2000) Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. J Exp Med 192: 159–170.

42. Zhou X, Riddiford LM (2002) Broad specifies pupal development and mediates the “status quo” action of juvenile hormone on the pupal-adult transformation in Drosophila and Manduca. Development 129: 2259–2269.

43. Zhou X, Zhou B, Truman JW, Riddiford LM (2004) Overexpression of broad: A new insight into its role in the Drosophila prothoracic gland cells. J Exp Biol 207: 1151–1161.

44. Apasov S, Chen JF, Smith P, Sitkovsky M (2000) A(2A) receptor dependent and A(2A) receptor independent effects of extracellular adenosine on murine thymocytes in conditions of adenosine deaminase deficiency. Blood 95: 3859–3867.

45. Park S, Lim JK (1995) A microinjection technique for ethanol-treated eggs and a mating scheme for detection of germ line transformants. Drosoph Inf Serv 76: 187–189.

46. Rizki TM, Rizki RM, Grell EH (1980) A mutant affecting the crystal cells in Drosophila melanogaster. Wilhelm Roux’s Arch 188: 91–99.

47. Maier SA, Podemski L, Graham SW, McDermid HE, Locke J (2001) Characterization of the adenosine deaminase-related growth factor (ADGF) gene family in Drosophila. Gene 280: 27–36.