Mutation in the *Drosophila melanogaster* adenosine receptor gene selectively decreases the mosaic hyperplastic epithelial outgrowth rates in *wts* or *dco* heterozygous flies

Roman Sidorov · Lucie Kucerova · Istvan Kiss ·
Michal Zurovec

Received: 5 October 2014 / Accepted: 25 November 2014 / Published online: 21 December 2014
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Abstract Adenosine (Ado) is a ubiquitous metabolite that plays a prominent role as a paracrine homeostatic signal of metabolic imbalance within tissues. It quickly responds to various stress stimuli by adjusting energy metabolism and influencing cell growth and survival. Ado is also released by dead or dying cells and is present at significant concentrations in solid tumors. Ado signaling is mediated by Ado receptors (AdoR) and proteins modulating its concentration, including nucleoside transporters and Ado deaminases. We examined the impact of genetic manipulations of three *Drosophila* genes involved in Ado signaling on the incidence of somatic mosaic clones formed by the loss of heterozygosity (LOH) of tumor suppressor and marker genes. We show here that genetic manipulations with the *AdoR*, equilibrative nucleoside transporter 2 (*Ent2*), and Ado deaminase growth factor-A (*Adgf-A*) cause dramatic changes in the frequency of hyperplastic outgrowth clones formed by LOH of the *warts* (*wts*) tumor suppressor, while they have almost no effect on control *yellow* (*y*) clones. In addition, the effect of AdoR is dose-sensitive and its overexpression leads to the increase in *wts* hyperplastic epithelial outgrowth rates. Consistently, the frequency of

mosaic hyperplastic outgrowth clones generated by the LOH of another tumor suppressor, *discs overgrown* (*dco*), belonging to the *wts* signaling pathway is also dependent on AdoR. Our results provide interesting insight into the maintenance of tissue homeostasis at a cellular level.

Keywords Cell competition · Adgf-A · Ent2 · LATS1 · Warts · Discs overgrown

Introduction

Adenosine (Ado) is a ubiquitous metabolite that plays a prominent role as a paracrine homeostatic signal of metabolic imbalance within tissues [1]. Ado can be formed extracellularly by the dephosphorylation of adenine nucleotides that are released from stressed or damaged cells [2]. Ado is also formed intracellularly from ATP and transported to the extracellular space via bidirectional nucleoside transporters [2]. The adenosine gradient then acts in neighboring cells, allowing them to deal more effectively with potentially stressful events [1].

While there is a lot of information on the effects of Ado and various metabolically stable adenosine receptor (AdoR) analogs at the organismal and organ levels, our knowledge of adenosine signaling in maintaining tissue homeostasis at a cellular level remains poor. Recent studies have shown that Ado transport, metabolism, and signaling are all parts of the Ado homeostatic signaling, which plays a more important biological role in diverse organisms than previously thought. For example, the local concentration of extracellular Ado was shown to provide a signal for the localization of hematopoietic progenitor cells in the cortical zone of the *Drosophila* lymph gland [3] or to regulate the regeneration of pancreatic beta cells in zebrafish and mice in vivo [4].

Electronic supplementary material The online version of this article (doi:10.1007/s11302-014-9435-2) contains supplementary material, which is available to authorized users.

R. Sidorov · L. Kucerova · M. Zurovec
Biology centre AS CR,
Branisovska 31, 370 05 Ceske Budejovice, Czech Republic

I. Kiss
Biological Research Centre HAS,
Temesvari krt. 62, Szeged 6726, Hungary

M. Zurovec (✉)
Faculty of Natural Sciences, University of South Bohemia,
Branisovska 31, Ceske Budejovice, Czech Republic
e-mail: zurovec@entu.cas.cz

AdoRs, which belong to the G-protein-coupled receptor family [5] mediate most of the physiological effects of extracellular adenosine in vertebrates and invertebrates. Four distinct AdoR isoforms were identified in mammals. Two of them are positively coupled to adenylate cyclase (A2A and A2B) and the other two (A1 and A3) negatively regulate the enzyme. *Drosophila* has a single AdoR, which is positively coupled to adenylate cyclase [6]. Its sequence shows about 38 % identity with the N-terminal region of 350 amino acids of the A2A human homologue [7]. *Drosophila* AdoR mutants are viable and show no obvious phenotypic changes [7, 8].

Several other conserved gene products are involved in adenosine signaling and modulate the extracellular concentration of adenosine, including nucleoside transporters and Ado deaminases [9, 10]. Our previous research showed genetic interactions between them including compensatory changes in the expression of the *equilibrative nucleoside transporter 2* (*Ent2*) in the *AdoR* mutants and *adenosine deaminase growth factor A* (*Adgf-A*) in the *Ent2* mutants [11]. The *Adgf-A* mutant is homozygously lethal, causing elevated levels of extracellular Ado [12, 13]. Null mutants in *Ent2* are also homozygously lethal, while *Ent2* hypomorphs show similar defects in synaptic transmission and associative learning as the *AdoR¹* mutant [11].

Since Ado exerts a broad range of cytoprotective, growth-promoting, and immunosuppressive effects and was observed at a high concentration in a number of human tumors, it was suggested to be an important factor regulating tumor growth [14–17]. In this report, we examined the effect of three *Drosophila* genes involved in Ado signaling on the incidence of somatic mosaic clones, including *AdoR*, *Ent2*, and *Adgf-A*. We show that genetic manipulations with these genes cause dramatic changes in the frequency of hyperplastic outgrowth clones by the loss of heterozygosity (LOH) of the *warts* (*wts*) tumor suppressor gene, while they have no or very little effect on control clones. The *Drosophila* *wts* clones are formed in the epithelium of imaginal discs, neural epithelium, and histoblast nests [18, 19]. The *wts* homologue in humans is called *LATS1* and its downregulation has been reported in a number of tumors [20]. This report is the first to demonstrate how changes in Ado signaling influence the frequency of the *wts* mosaic hyperplastic epithelial outgrowth clones in *Drosophila*.

Materials and methods

Fly stocks

The *AdoR¹* mutant is viable and fertile and was generated earlier by homologous recombination [12]. We prepared an *AdoR¹* stock isogenized to *white¹¹¹⁸* flies by serial backcrosses.

Two additional *AdoR* alleles were received from public stock collections. The *AdoR* deletion mutant designated *AdoR^{KG0396ex}* was generated by Wu et al. [8]. The mutation-designated *AdoR^{Df(3R)Exel6214}*, which contains a larger deletion located in 3R (99D5-99E2), encompasses approximately 17 genes including the *AdoR* locus and was from the Exelixis collection (Harvard University, USA). The *Drosophila* RNA interference strains carrying upstream activation sequence (*UAS-AdoR^{RNAi-VDRC}*, line number 1385 and *UAS-Ent2^{RNAi-VDRC}*, line number 7618) hairpins were received from the Vienna *Drosophila* RNA interference (RNAi) Center (VDRC). The *UAS-AdoR^{RNAi-TRIP}* (TRIP, Bloomington stock BL-27536) flies were received from the Transgenic RNAi Project of Harvard Medical School. The *wts^{x1}* mutant was received from Dr. Tian Xu (Yale University). The *UAS-AdoR*, *UAS-Ent2*, and *UAS-ADGF-A* strains were constructed earlier [7, 11]. All other *Drosophila* strains, including *Act-Gal4*, *Tub-Gal80*, *en-Gal4*, *y*, discs overgrown (*dco³*) were obtained from the Bloomington *Drosophila* stock center.

Drug treatment

For testing cisplatin and 2,6-diaminopurine (DAP), 10 female and 4 male flies were placed into standard rearing vials for mating and egg laying. After 36–40 h, the parents were transferred to fresh medium. The first instar larvae were treated by adding 0.3 ml of the solutions or suspensions of tested substances onto the surface of the food. Cisplatin, cis-diaminedichloroplatinum (Sigma-Aldrich) was used in a 0.2 mg/ml aqueous (0.67 mM) solution unless otherwise specified. The DAP assays (Sigma-Aldrich) were performed using 12–80 mM (toxicity assay) and 40 mM (Somatic Mutation and Recombination test, SMART) concentrations in 5 % dimethyl sulfoxide, (DMSO, Sigma-Aldrich). Because of its low solubility, DAP was added as a suspension to larval food. The toxicity of paraquat (Sigma-Aldrich, 20 mM solution in 5 % DMSO) was tested on adults for 3 days (males, 24 h after eclosion). Control flies were treated with the equivalent concentration of DMSO. Lethality was expressed in the percentage of surviving flies (with respect to the original number of embryos taken to the experiment).

SMART and clonal analysis

SMART, as described by Stern [21], is based on LOH and mitotic crossing-over. We used a variation of SMART based on flies heterozygous for marker genes (*y/+* or *wts^{x1}/+*), in which the mosaic clones can be detected over the entire body surface of the adults and more than 70 % of the *wts* hyperplastic outgrowths are found in larval wing imaginal discs or in adult wings, notum, and sternopleura [22]. We examined either spontaneous or mutagen (cisplatin, diaminopurine, paraquat)-induced somatic recombination and compared the

frequencies of clones that appeared in the examined genetic backgrounds ($AdoR^+$, $AdoR^I$ or $AdoR^+/AdoR^I$). Cisplatin showed a strong statistically significant recombinogenic effect ($P < 0.01$) at the doses that had low toxicity (Fig. S1A). We therefore chose cisplatin to induce mitotic recombination in most of our experiments.

The wts^{x1} clones were classified into four size categories as follows: (1) “Smallest detectable”—The clones of the minimal detectable size (~20 cells, based on the wing hair counting), (2) “Small”—Readily detectable, 4–5 times bigger than the smallest (~100 cells), (3) “Average”—3–10 times bigger than the small (up to one third of the total organ size), and (4) “Large”—2–3 times bigger than the average (up to the total size of the organ).

In order to label the clones with green fluorescent protein (GFP) and/or to express various transgenes, we used the mosaic analysis with a repressible cell marker (MARCM) system [23], based on the loss of Gal80 expression in clones and subsequent derepression of Gal4-dependent transcription (Figs. 1b–f and S4). For the MARCM expression in wts^{x1} or $AdoR^I$ clones, we used the *Gal80* transgene localized in the cytological position 84B, in the chromosomal arm 3R. In $wts^+ Tub-Gal80/wts^{x1}$ heterozygous flies, the mosaic wts^{x1} clones lose the repressor and express *UAS* constructs driven by the

Act-Gal4 driver. The following *UAS* constructs were used: *UAS-GFP*, *UAS-Adgf-A*, *UAS-Ent2*, *UAS-Ent2^{RNAi}*, *UAS-AdoR*, and *UAS-AdoR^{RNAi}*.

Adult flies were screened for hyperplastic outgrowths or color markers under a binocular microscope. The GFP clones were detected with a fluorescent microscope in dissected imaginal discs from wandering stage third instar larvae.

Quantitative PCR

Total RNA was extracted from 60 *Drosophila* adults using the RiboZol RNA extraction reagent (AMRESCO) followed by NucleoSpin RNA II kit (Macherey-Nagel) including an on-column rDNase I digestion step. Total RNA (1000 ng) was reverse transcribed using PrimeScript Reverse Transcriptase (Takara) with oligo(dT)₁₇ primer. Quantitative PCR was performed using the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). Primer sequences are listed in Table S1. RT-qPCR was performed in triplicates and all results are presented with means and SEM from four independent biological samples. We used *rp49* and *Rack1* messenger RNA (mRNA) levels for normalization.

Fig. 1 Examples of somatic mosaic clones in adult flies and larval imaginal discs; **a–c** adult flies, **d–f** imaginal discs. The clones are indicated by arrows. **a** yellow (black arrows point to yellow bristles) and wts^{x1} clones (red arrow) on the thorax of a $y/+$; $wts^{x1} AdoR^I/+$ + imago. **b** A large wts^{x1} GFP clone (white arrow) in the wing (*Act-Gal4* *UAS-GFP/+* +; + $wts^{x1}/Tub-Gal80$ +); **c** non-hyperplastic GFP clone (white arrow) in proboscis labeled using the MARCM system (*Act-Gal4* *UAS-GFP/+* +; +/*Tub-Gal80*). **d** *UAS-AdoR^{RNAi-VDRC}* *UAS-GFP*

clone (white arrow) in the imaginal disc of third instar larva *Act-Gal4* *UAS-GFP/+*; *AdoR^{RNAi-VDRC}/Tub-Gal80* genotype; **e** wts^{x1} clone (white arrow) labeled with GFP using the MARCM system in the imaginal disc of an *Act-Gal4* *UAS-GFP/+* +; + $wts^{x1}/Tub-Gal80$ + third instar larva. **f** GFP-labeled clone (white arrow) using the MARCM system in the imaginal disc of third instar larva (*Act-Gal4* *UAS-GFP/+* +; +/*Tub-Gal80*); **d–f** Nuclei are marked by DAPI stain (blue)

Statistical data evaluation

Clone frequency p (%) was calculated as (number of clones)/(total number of flies) $\times 100$. Clone frequencies were compared using Student's t test and Fisher's correction $\phi = 2 \times \arcsin(\sqrt{p})$ for frequency. The t test was calculated for confidence intervals (CI) for $P < 0.05$ and $P < 0.01$, if appropriate. These intervals are shown as error bars in the figures unless stated otherwise. Low-sample t -criterion correction was applied to samples of fewer than 500 flies, according to Urbakh [24]. We applied the χ^2 criterion to assess the lethality of various heteroallelic *AdoR* mutant combinations.

Results

AdoR mutant flies are homozygous viable

We reported earlier that the homozygous *AdoR*^{*l*} mutation generated by homologous recombination was viable and able to partially rescue the lethality of adenosine deaminase growth factor-A (*Adgf-A*) mutants [12]. The mutation *AdoR*^{*KG0396ex*} lacking the entire *AdoR* coding region was also reported to be homozygous viable [8]. In contrast, the survival of *AdoR*^{*RNAi-VDRC*} flies with long hairpin constructs was dramatically reduced when driven by the *Act-Gal4* driver. We conclude that the lethality of the *AdoR*^{*RNAi-VDRC*} construct was presumably caused by off-target effects. The larger deletion *AdoR*^{*Df(3R)Exel6214*} was homozygous lethal in our experiments.

To verify that there is no negative effect of *AdoR* mutations on fly viability, we prepared heteroallelic combinations of various *AdoR* mutations. As shown in Table S2, the frequencies among the offspring of all examined combinations, including *AdoR*^{*l*}/*AdoR*^{*l*}, *AdoR*^{*l*}/*AdoR*^{*KG03964ex*}, *AdoR*^{*KG03964ex*}/*AdoR*^{*Df(3R)Exel6214*}, and *AdoR*^{*l*}/*AdoR*^{*Df(3R)Exel6214*}, were in accordance with the expected Mendelian ratios, showing no significant deviation from the expected frequencies. Our results confirmed that the *AdoR*^{*l*} and *AdoR*^{*KG03964ex*} mutations have no negative effect on fly viability under standard rearing conditions.

AdoR^{*l*} strongly reduces *wts*^{*x1*} clone frequency in SMART

In order to examine the effect of the loss of *AdoR* function on the incidence of somatic mosaic clones in flies *in vivo*, we adapted a robust test, originally designed for assaying the mutagenicity of chemicals, or the effect of genetic background on the frequency of mutations [25, 26]. The mutations are visualized as somatic mosaic clones generated by LOH of the recessive allele of the marker gene. In our experiments, it was advantageous to induce clone formation with mutagens since the spontaneous LOH occurred at a relatively low frequency.

In this way, we were able to reduce the number of flies in the experiments and still reach statistical significance.

First, we compared the effect of *AdoR*^{*l*} and *AdoR*⁺ backgrounds on the rate of somatic mosaic clones labeled with two different types of markers, *wts*^{*x1*} and *y*. The *wts*^{*x1*} is a mutant allele of the *wts* tumor suppressor causing epidermal tissue overgrowth that is visible as small hyperplastic outgrowths, whereas *y* causes yellow (*y*) cuticles and bristles in the adult (Fig. 1a, b). The mosaic clones were induced with cisplatin as described in “Experimental Procedures”. The results are shown in Fig. 2a. The frequency of *wts*^{*x1*} clones in *AdoR*^{*l*} flies was much lower than in *AdoR*⁺ flies; only 4.5 % compared to 65.9 %, respectively. In contrast, *y* mosaic clones occurred at a frequency of 11.1 % in *AdoR*⁺ flies, whereas in *AdoR*^{*l*} flies (*y*/+; *AdoR*^{*l*} *wts*^{*x1*}/*AdoR*^{*l*} +) it reached 18.5 % (similarly for *y*/+; *AdoR*^{*l*}/*AdoR*^{*l*} flies). The higher frequency of *y* mosaic clones might be connected to the involvement of *AdoR* in the control of DNA repair or detoxification. The dramatically decreased *wts*^{*x1*} hyperplastic outgrowth clone frequency in *AdoR*^{*l*} flies was unexpected.

To show that the observed difference in the incidence of mosaic clones carrying *wts*^{*x1*} and *y* markers was not dependent on cisplatin induction, we examined the frequency of spontaneous mosaic clones for both markers. The spontaneous level of *wts*^{*x1*} clones also dropped by sevenfold in *AdoR*^{*l*} homozygous flies, compared to the *AdoR*⁺ controls (from 6.7 to 0.9 %, respectively) showing that the reduction of *wts*^{*x1*} clone frequency was not caused by cisplatin. The spontaneous frequencies of *y*/*y* clones were slightly below the test sensitivity in both fly strains (Fig. 2a).

To further prove that the drop of *wts*^{*x1*} clone frequency in *AdoR*^{*l*} flies was not caused by cisplatin, we used two other genotoxic compounds for SMART, diaminopurine (DAP), and paraquat. As expected, both DAP and paraquat produced higher clonal rates in both *AdoR*⁺ and *AdoR*^{*l*} flies than the spontaneous frequency. Again, the frequencies of *wts*^{*x1*} mosaic clones induced by DAP and paraquat in the *AdoR*^{*l*} homozygous flies (1.8 and 3.3 %, respectively) were 7–2.7-fold lower compared to those in the corresponding *AdoR*⁺ flies (12.6 and 8.8 %, respectively). The results are shown in Fig. 2b. The chemicals were also assayed for toxicity. The results showed that DAP and paraquat have a much higher impact on fly viability than cisplatin (Fig. S1).

These results suggested that the *AdoR*^{*l*} mutation had different effects on the frequency of hyperplastic outgrowth and non-tumor mosaic clones represented by the *wts*^{*x1*} and *y* markers, respectively. The effect was independent of the mutagen used and also occurred spontaneously. We did not observe any differences in *wts*^{*x1*} clone morphology when comparing *AdoR*⁺ and *AdoR*^{*l*} flies. The differences in SMART results can be expected because chemicals differ in their genotoxicity, toxicity, and which pathways they activate in different target tissues. The dramatically decreased *wts*^{*x1*}

Fig. 2 The effect of AdoR on the frequency of somatic mosaic clones. **a** The incidence of *wts*^{x1} and *y* somatic mosaic clones in *AdoR*⁺ and *AdoR*¹ flies. The gray and empty bars represent spontaneous and 0.67 mM cisplatin-induced *wts*^{x1} and *y* mosaic clone frequencies, respectively, in homozygous *AdoR*⁺ (left side, *y*/+; *wts*^{x1}/+) and *AdoR*¹ (right side, *y*/+; + *AdoR*¹/+ *AdoR*¹) flies. Error bars show 95 % confidence interval (CI). The numbers above each bar indicate the number of flies tested. Most *wts*^{x1} clones appeared in adult wings, notum, and sternopleura. The yellow clones appear in lower frequency since they must cover a bristle precursor cell for being detected. **b** The *wts*^{x1} clone frequency induced by three chemicals in *AdoR*⁺ and *AdoR*¹ flies. The *wts*^{x1} clones were induced by 0.67 mM cisplatin (CP), 20 mM paraquat (PQ), and 40 mM diaminiopurine (DAP); the solvents (marked in brackets) were water or 5 % aqueous DMSO; CTR control. White and gray bars represent clone frequency in +*AdoR*⁺/*wts*^{x1} *AdoR*⁺ and *wts*^{x1} *AdoR*¹/+ *AdoR*¹ flies, respectively. Error bars show 95 % CI. The numbers above the right of each bar indicate the number of flies tested. **c** *wts*^{x1} clone frequencies in cis- and trans-*AdoR*¹ *wts*^{x1} heterozygous flies. The black bars (left) represent clone frequencies observed in cis- and trans- *AdoR*¹ *wts*^{x1} heterozygous flies. For comparison, *wts*^{x1} clone frequencies observed in homozygous *AdoR*⁺ and *AdoR*¹ flies are shown on the right (white and gray bars, resp.). The *wts*^{x1} clones were induced by 0.67 mM cisplatin. Error bars show 95 % CI. The numbers above each bar indicate the number of flies tested

hyperplastic outgrowth frequency in *AdoR*¹ flies therefore called for further investigation.

Different *wts*^{x1} clone frequencies in cis- and trans-*AdoR* *wts* heterozygotes suggest the need of *AdoR*⁺ after clone formation

Since both *AdoR* and *wts* are localized on the distal tip of the 3R chromosomal arm, mitotic crossing-over break points most frequently occur between the centromere and both loci. To find out whether the *AdoR*¹ mutation affects *wts*^{x1} clone formation by influencing processes such as DNA repair or

affects the survival of already established clones, we compared the frequencies of *wts*^{x1} hyperplastic outgrowths in cis- and trans-heterozygotes for *AdoR*¹ and *wts*^{x1}, which produce qualitatively different *wts*^{x1} clones. The cells in cis- and trans-heterozygotes have almost the same genetic background and conditions; if the *AdoR*¹ affects *wts*^{x1} clone formation we should observe similar hyperplastic outgrowth frequencies in cis- and trans-heterozygotes. Alternatively, if the *AdoR*¹ influences *wts*^{x1} hyperplastic growth or survival, we should observe different *wts*^{x1} clone frequencies because the mitotic recombination produces different clones: in the cis-heterozygous flies for these genes, the *wts*^{x1} clones lack functional *AdoR*, whereas in the trans-heterozygous flies, they contain homozygous *AdoR*⁺ (Fig. S2). The comparison of *wts*^{x1} clone frequency in cis- and trans-heterozygotes for *wts*^{x1} and *AdoR*¹ therefore may provide important information about whether *AdoR*-*wts*^{x1} interactions affect clonal formation or survival. The resulting data are shown in Fig. 2c. The *wts*^{x1} mosaic clones containing *AdoR*⁺ in trans-heterozygotes occurred at high frequency (91.9 %), whereas their rate in cis-heterozygotes lacking functional *AdoR* was very low (4.7 %). Such a difference strongly suggests that functional *AdoR* is needed for *wts*^{x1} hyperplastic clone survival/growth after clone formation.

The low frequency of *wts*^{x1}/*wts*^{x1} clones formed in cis-heterozygotes also means there was no rescue of *wts*^{x1} hyperplastic outgrowth clones by the surrounding *AdoR*¹/*AdoR*⁺ cells. Taking this result into account, the effect of *AdoR*¹ appeared to be cell autonomous. This provided the opportunity for further experiments, which could use the overexpression of the studied genes within somatic clones only.

Fig. 3 The size distribution of *wts^{x1}* hyperplastic outgrowth size classes in two *Drosophila* genotypes. We analyzed 148 spontaneous *wts^{x1}* hyperplastic outgrowths detected among 1574 *wts^{x1}/+* flies and 35 spontaneous *wts^{x1}AdoR¹* hyperplastic outgrowths found among 2267 *wts^{x1}AdoR¹/++* flies. The differences between the average and large clones in *wts^{x1}/+* and *wts^{x1}AdoR¹/++* flies are statistically significant

To better understand the impact of *AdoR¹* on the *wts^{x1}* cells, we also assessed clone sizes. As shown in Fig. 3, we observed significantly higher proportion of larger *wts^{x1}* hyperplastic outgrowths, among the *AdoR¹wts^{x1}/++* flies compared to *wts^{x1}/+* flies.

Confirmation of the specificity of AdoR effect on the frequency of *wts^{x1}* hyperplastic outgrowth clones

To confirm that the observed decrease of the *wts^{x1}* hyperplastic outgrowth clone rate was specific for the loss of the function of *AdoR*, we used *AdoR* silencing through the expression of *UAS-AdoR^{RNAi}* hairpin RNA.

Expression of the *UAS-AdoR^{RNAi-TRIP}* hairpin in the flies using *Act-Gal4* led to the decrease of *AdoR* mRNA level to 33 % of its wild-type level, as shown by qPCR (Fig. S3). These flies had a significantly decreased frequency of *wts^{x1}* clones by 40.0 % (95 % confidence interval, CI ± 0.4 %) compared to control flies without *AdoR* silencing. This result supports the hypothesis stipulating the requirement of functional *AdoR* in the *wts^{x1}* clones for their high frequency. The weaker effect of RNAi-silencing as compared to the *AdoR¹* mutation can be explained by the incomplete downregulation of *AdoR*.

The *AdoR* knockdown experiments were also performed with flies expressing the *UAS-AdoR^{RNAi-VDRC}* construct together with *UAS-GFP*. Since the global expression of *UAS-AdoR^{RNAi-VDRC}* caused lethality, we used the MARCM system (see “Materials and methods” and Fig. S4) and targeted its expression just to *wts^{x1}* clones. The frequency of *GFP AdoR^{RNAi-VDRC} wts^{x1}* clones in adults was about threefold lower than in the control flies without *UAS-AdoR^{RNAi-VDRC}* (Fig. 4a). Consistently, the frequency of *wts^{x1} AdoR^{RNAi-VDRC}* clones assayed in larval imaginal discs also showed similar decrease (but these small samples lack statistical power) (Fig. S5). The rate of control MARCM-labeled *GFP* clones in imaginal discs of *AdoR⁺* larvae with or without *UAS-AdoR^{RNAi-VDRC}* was statistically indistinguishable (27.3, 95 % CI

± 8.5 %, compared to 20.0, 95 % CI ± 9.3 %) (Fig. S5). These results support the notion that the loss of *AdoR* function decreases the frequency of *wts^{x1}* clones and exerts no or very little influence on the frequency of control *GFP* clones.

To further test the specificity of the effect of *AdoR* level on the rate of *wts^{x1}* clones, we used *AdoR* overexpression. Because the ubiquitous overexpression of *UAS-AdoR* in the entire body was lethal [9], we expressed it locally in the *wts^{x1}* mosaic hyperplastic clones using the MARCM system (Fig. S4). We constructed *UAS-AdoR/Act-Gal4 UAS-GFP/+ Tub-Gal80/wts^{x1}+* flies carrying the *UAS-AdoR* and *Act-Gal4* transgenes and compared their *wts^{x1}* clone frequency with that of the control flies lacking *UAS-AdoR* (Fig. 4b). Such *AdoR* overexpression increased the *wts^{x1}* hyperplastic outgrowth clone frequency to 75.4 compared to 66.0 % in *AdoR⁺* flies without *UAS-AdoR* (significant at $P < 0.05$).

Taken together, these data confirms the specific requirement of *AdoR* for *wts^{x1}* hyperplastic outgrowth occurrence and also revealed a dose effect: *AdoR* overexpression further increased the frequency of *wts^{x1}* clones while, conversely, incomplete RNAi-silencing of *AdoR* was not as effective as the null mutation.

AdoR¹ mutation also affects the frequency of *dco³* hyperplastic outgrowth clones

To further confirm the effect of *AdoR¹* on hyperplastic outgrowth clone frequency we tested a mutation in another *Drosophila* tumor suppressor gene *discs overgrown* (*dco³*) under the same conditions. Since the morphological outgrowths of *dco³* clones are more difficult to detect, we also labeled the clones with *GFP* using the MARCM system (*Act-Gal4 UAS-GFP/+ +; dco³/Tub-Gal80 +*).

As shown in Fig. 4c, the frequency of *dco³* mosaic hyperplastic outgrowths in heterozygous *dco³/+* flies with *AdoR⁺* and *UAS-GFP* was about 12.1 % and this rate dropped by approximately a factor of four to 3.5 % in the *AdoR¹ dco³/+* cis-heterozygotes (*Act-Gal4 UAS-GFP/+ +; AdoR¹ dco³/Tub-Gal80 +*). This difference was statistically highly significant ($P < 0.01$). In contrast, in the control experiment, the frequency of *GFP* clones was 1.72 % in the *AdoR¹* mutant flies (*Act-Gal4 UAS-GFP/+ +; AdoR¹/Tub-Gal80 +*), which is comparable to the 1.1 % value observed in flies that were wild type for *AdoR* (*Act-Gal4 UAS-GFP/+ +; +/Tub-Gal80*); the difference was statistically insignificant.

Our results showed that the *AdoR¹* mutation dramatically decreased the frequency of hyperplastic outgrowth clones induced by LOH of another tumor suppressor gene *dco*, suggesting that more types of hyperplastic outgrowth clones may require functional *AdoR* for their growth. The extent of this effect was, however, lower than that observed in the previous experiments with *wts^{x1}* clones, probably reflecting the different growth properties of these hyperplastic

Fig. 4 The frequencies of mosaic clones in various genetic backgrounds. **a** The incidence of *wts^{x1}* GFP clones in adult *AdoR^{RNAi}* and control flies. The UAS transgenes were expressed by MARCM system. The left bar (*AdoR⁺*) *y w*; *act-Gal4 UAS-GFP/+*; *wts^{x1}/tub-Gal80*; the right bar (*AdoR^{RNAi}*) *y w*; *act-Gal4 UAS-GFP/+*; *UAS-AdoR^{RNAi-VDRC} wts^{x1}/tub-Gal80*. Error bars show 95 % CI. The numbers above each bar indicate the number of flies tested. **b** *AdoR* and *Ent2* overexpression and *Ent2* silencing affect the frequency of *wts^{x1}* clones. The *wts^{x1}* clone frequencies, in flies with *UAS-AdoR⁺* (black bar, left), *UAS-Ent2⁺* (left gray bar) and *UAS-Ent2^{RNAi-VDRC}* (right gray bar) constructs expressed using MARCM system (see the text). For comparison, *wts^{x1}* clone frequency observed in homozygous *AdoR⁺* flies is shown on the left (white bar). Error bars show 95 % CI. The numbers above each bar indicate the number of flies tested. **c** The incidence of *dco³* and control

mosaic clones in *AdoR⁺* and *AdoR¹* flies. Black bars (left) the frequency of *dco³* clones in heterozygous *dco³/dco³* flies (*Act-Gal4 UAS-GFP/+*; + *AdoR¹ dco³/Tub-Gal80* ++ and *Act-Gal4 UAS-GFP/+*; + *dco³/Tub-Gal80* +); gray bars (right) control GFP clones labeled by MARCM system in *AdoR¹* and *AdoR⁺* flies (*Act-Gal4 UAS-GFP/+*; + *AdoR¹/Tub-Gal80* + and *Act-Gal4 UAS-GFP/+*; + *Tub-Gal80*). Error bars show 95 % CI. The numbers above each bar indicate the number of flies tested. **d** The ectopic expression of adenosine deaminase ADGF-A rescues *wts^{x1}* *AdoR¹* clones in *UAS-ADGF-A/en-Gal4*; *wts^{x1}AdoR¹/++* flies. The *en-Gal4* was used to drive *UAS-ADGF-A⁺* expression in the posterior wing (gray shading in the schematic wing drawing above the graph indicates the *en* expression pattern). The hyperplastic outgrowth clone rates in the anterior and posterior compartments of 1518 wings examined were 0.2 ± 0.1 (left empty bar) and 2.7 ± 0.1 (right gray bar), respectively

outgrowth types. The frequency of the control GFP-labeled non-tumor clones did not decrease in *AdoR¹* flies in any of the experiments.

The effect of *Ent2* and *Adgf-A* on *wts^{x1}* clone frequency

To find out whether other members of the Ado signaling pathway influence *wts^{x1}* hyperplastic outgrowth frequency, we overexpressed *Ent2* and *Adgf-A*. In addition, we silenced the transporter expression by using *UAS-Ent2^{RNAi}*.

MARCM expression of *UAS-Ent2* in the *wts^{x1}* clones increased the induced mosaic hyperplastic outgrowth rate from 65.8 to 91.6 % ($P < 0.01$) (Fig. 4b). Conversely, the knockdown of *Ent2* expression by RNAi in the *UAS-Ent2^{RNAi}-VDRC* ++/+ *Act-Gal4 UAS-GFP*; *wts^{x1}/++Tub-Gal80*

flies decreased the frequency of *wts^{x1}* hyperplastic outgrowths to 0.9 %. These results showed that both ectopic expression and silencing of the *Ent2* transporter exerted an even stronger effect on *wts^{x1}* hyperplastic outgrowth frequency than the changes in the level of the Ado receptor.

We also expressed *UAS-Adgf-A* in *AdoR¹ wts^{x1}/++* mutants using an *engrailed-Gal4* driver and took advantage of the distinct ectopic expression of the UAS construct in *wts^{x1}* hyperplastic outgrowths in the anterior and posterior compartments of the wing (Fig. 4d). There was a statistically significant increase (13.5 times) of the *wts^{x1}* hyperplastic outgrowth rate in the posterior compartment compared to the intact anterior one—from 0.2 to 2.7 % ($P < 0.01$). The ratio is quite comparable to the 14-fold difference observed in the *wts^{x1}* hyperplastic outgrowth rates between flies with *AdoR⁺* and

*AdoR*¹ homozygous backgrounds (Fig. 2a) suggesting that the overexpression of *Adgf-A* completely rescues the effect of the *AdoR*¹ mutation in *wts*^{x1} clones. Taken together, our results suggest that the *wts*^{x1} hyperplastic outgrowth clone frequency is regulated by adenosine signaling.

Discussion

Fly genetic methods allow the study of interactions between cells in the mosaic clones and their microenvironment. Our results show that AdoR and extracellular adenosine play key roles in the growth control of mosaic hyperplastic outgrowth clones. This conclusion follows from the observations that the frequency of *wts*^{x1} and *dco*³ hyperplastic outgrowth clones in the *AdoR*¹ mutant background is dramatically reduced compared to that in the *AdoR*⁺ flies. In contrast, the rate of non-hyperplastic clones (*yellow* or *GFP*) did not show any reduction, and even showed a significant increase for *y*. To exclude the possible effect of different recombination frequency on different chromosomes we used *yellow* on chromosome X and *Gal 80* on chromosome 3 (segregation of *Gal 80* permits the *UAS-GFP* expression on chromosome 2).

AdoR seems to influence the SMART results by more than one mechanism. The slightly higher proportion of *y* mosaic clones in *AdoR*¹ flies compared to that in the *AdoR*⁺ ones suggests that the loss of functional AdoR may slightly increase the frequency of LOH. It was reported earlier that adenosine receptor agonists reduced the level of mutations in mouse and human cells treated with alkylating agents or H₂O₂ [27]. The elevated level of *y* clones in *AdoR*¹ flies might suggest that the loss of such protective AdoR function slightly increases the rate of somatic mutations.

We found that the lack of functional AdoR causes a dramatic reduction of *wts*^{x1} mosaic hyperplastic outgrowth clone frequency. Our data strongly suggest that AdoR is not needed for *wts*^{x1} clone formation, but rather for their growth/survival. The reduction of *wts*^{x1} clonal rate in *AdoR*¹ or *AdoR*^{RNAi} flies was consistent for clone established by spontaneous mosaicism, as well as for all chemical treatments, but its extent was different for different SMART conditions and also depended on the expression level of AdoR.

Moreover, AdoR interacts with *dco*, another tumor suppressor gene, which is also a member of the *wts* signaling pathway. Both the *wts*^{x1} and *dco*³ mosaic clones form fast-growing hyperplastic outgrowths. The *dco* is *Drosophila casein kinase Iε/δ* and affects the upstream regulator of *wts* called *fat* [28, 29]. *Wts* is a serine/threonine protein kinase belonging to the WTS/LATS tumor suppressor family, which suppresses growth by antagonizing the function of the transcriptional co-activator protein Yorkie (Yki) [30]. Mutations in *wts* causing elevated levels of Yki activity can convert wild type (*WT*) cells into faster growing supercompetitors that are

able to eliminate adjacent *WT* cells [31, 32]. Our results suggest that the *wts*^{x1} and *dco*³ mosaic hyperplastic outgrowths lose their growth advantage in the absence of functional AdoR.

Interestingly, changes in the expression of Ent2 equilibrative nucleoside transporter in *wts*^{x1} clones have a similar cell autonomous effect as changes in AdoR expression. The overexpression of *Ent2* in *wts*^{x1} clones leads to their higher frequency, whereas *Ent2* silencing dramatically decreases *wts*^{x1} clonal rate (Fig. 4a). The normal frequency of *wts*^{x1} clones therefore requires WT function of both AdoR and Ent2, suggesting some form of interaction between them. We previously observed that *AdoR*¹ and *Ent2*³ mutant flies have similar defects in associative learning and synaptic function [11]. We even observed some compensatory changes including a dramatic increase in *Ent2* mRNA in *AdoR*¹ mutants or a reduction of adenosine deaminase transcript levels in *Ent2* mutants [11]. Although we cannot directly measure local extracellular Ado concentrations, we know that Ado is critical for clone survival since the clonal rate can be rescued by overexpressing the extracellular adenosine deaminase *Adgf-A* in *AdoR*¹ *wts*^{x1}/++ mutants (Fig. 4d). The growth restrictions probably appear early in the clonal development. Significant differences in the distribution of *wts*^{x1} hyperplastic outgrowth sizes between *wts*^{x1}*AdoR*¹/++ and *wts*^{x1}/+ flies suggest that larger *wts*^{x1}*AdoR*¹ clones have lower probability for being eliminated than the smaller ones. Alternative explanation that the fewer clones in *wts*^{x1}*AdoR*¹/++ flies exist because the clones have fused is not likely since the overall frequency of *wts*^{x1} clones was too low as well as the proportion of flies having more than one *wts*^{x1} hyperplastic outgrowth.

Earlier, microarray analyses of gene expression in tissue culture cells showed that most of the common *Drosophila* cell lines, including imaginal disc cells Cl.8+, neuroblasts Bg2-c2, or hematopoietic Mbn2 cells seem to have the Hippo/Warts pathway inactivated [33]. Interestingly, the Cl.8+ imaginal disc cell line also has a very low level of functional AdoR [6]. Our previous tissue culture experiments showed that there is an intriguing connection between the AdoR and Ent2 functions with cell survival. The exposure of Cl.8+ cells to extracellular Ado causes a concentration-dependent block of cell division and apoptosis associated with a loss of mitochondrial membrane potential and excessive ATP production [34]. In contrast, other cell types containing functional AdoR, including Bg2-c2 and Mbn2 cells, are able to decrease Ado uptake upon Ado treatment and avoid apoptosis following adenosine stimulation [34, 35]. Cl.8+ cell death could be prevented by blocking adenosine transport or increasing extracellular adenosine deaminase activity but not by blocking AdoR [34]. These results showed that Ado can induce receptor-independent cell death or block of cell growth.

Our earlier experiments showed that global AdoR overexpression in flies causes lethality [9]. Microinjection of *Act*-

Gal4 and *UAS-AdoR* flies with the AdoR antagonist SCH58261 was able to partially rescue the flies [6]. Similarly, the overexpression of AdoR in *Drosophila* C1.8+ and S2 cells in vitro was cytotoxic [6]. These results suggest that the AdoR-mediated signaling can also cause death of *Drosophila* cells. Strong effects of both the Ado transport and AdoR signaling on cell viability were recently also described in the experiments involving the regeneration of pancreatic β -cells in zebrafish in vivo [4].

The simplest model that would explain our data on the occurrence of *wts*^{x1} clones is shown in Fig. 5. We postulate that the extracellular Ado represents two opposing signals,

Fig. 5 Model of adenosine effect on *wts*^{x1} hyperplastic outgrowth survival. Hyperplastic outgrowth cells are shown in orange color, cells with WT phenotype in light gray, green shapes represent AdoR, and blue two-headed arrows denote Ent2. **a** In *AdoR*^{+/wts}/*AdoR*^{+/wts}^{x1} flies, the frequency of *wts*^{x1} mosaic hyperplastic outgrowths is high. The *wts*^{x1} hyperplastic outgrowth cells may behave as supercompetitors and eliminate adjacent *wts*⁺ cells. High level of extracellular Ado released by the outcompeted cells causes elevated AdoR stimulation as well as high Ado uptake in hyperplastic outgrowth cells. **b** Mutation in *AdoR* leads to dramatic decrease of *wts*^{x1} clone frequency, because Ado uptake in those cells is no longer balanced by AdoR signaling. **c** Silencing of *Ent2* in *wts*^{x1} hyperplastic outgrowth clones using RNAi also leads to dramatic decrease of *wts*^{x1} clonal rate (unbalanced AdoR signal). **d** Overexpression of *Adgf-A* in *AdoR*^{1/wts}/*AdoR*^{1/wts}^{x1} flies eliminates the effect of *AdoR* mutation and rescues the *wts*^{x1} clones (example of this effect in the posterior wing compartment is shown in Fig. 4d)

which need to be balanced in fast-growing cells. Such hypothesis is supported by earlier experiments in mice brain showing that Ado uptake induces ATP synthesis and stimulates catabolic reactions, while the signal mediated by the AdoR signaling leads to the downregulation of a number of transcripts encoding metabolic enzymes [1]. It was also shown earlier that *wts*⁻/*wts*⁻ cells behave as supercompetitors and eliminate adjacent *wts*⁺ cells at clonal boundaries [36–38]. In our experiments, *AdoR*^{+/wts}/*AdoR*^{+/wts}^{x1} flies are characterized by a relatively high frequency of *wts*^{x1} hyperplastic outgrowth clones in which the *wts*^{x1}/*wts*^{x1} cells may behave as supercompetitors. The dying *wts*⁺ cells become rich sources of extracellular Ado (Fig. 5a, black arrows). Ado is taken up by the *wts*^{x1} hyperplastic outgrowth cells and this uptake is compensated by high AdoR stimulation (Fig. 5a). This extracellular Ado effect is indirectly supported by the experiments with the ADGF-A overexpression (Figs. 5d and 4d). In flies carrying *AdoR*¹ mutation or *Ent2*^{RNAi}, and therefore lacking such balance, *wts*^{x1} hyperplastic outgrowths cannot grow in most cases (Fig. 5b, c). However, there are some escaper *AdoR*¹ *wts*^{x1} or *Ent2*^{RNAi} *wts*^{x1} clones which probably grow if they reach the critical threshold while providing their own microenvironment. If we overexpress *Adgf-A* in the posterior wing compartment of *AdoR*^{+/wts}/*AdoR*^{1/wts}^{x1} flies using *en-Gal4* and *UAS-Adgf-A*, the *wts*^{x1} hyperplastic outgrowths in this area occur at the high rate suggesting that the depletion of extracellular Ado eliminates the effect of *AdoR* mutation and rescues the *wts*^{x1} clones (Fig. 5d).

The *wts* downregulation was shown to play a key role in the stem cell-mediated regenerative response to tissue damage in the *Drosophila* intestine [39, 40]. The fast-growing super-competitive cells with blocked Wts function and activated Yki may cause secondary tissue damage. The observed effects of *AdoR* and *Ent2* on the frequency of cells which lost their *wts* tumor suppressor might be part of a normal tissue protection mechanism which limits the extent of regenerative growth.

A number of recent studies have examined the involvement of adenosine in tumor-protective mechanisms in mice, namely triggering immunosuppression via the A2A adenosine receptor (*A2AR*) on the surface of activated immune cells [16, 41]. While this mechanism probably evolved for the protection of normal tissues from collateral damage caused by the overactive immune cells, the cancer tissue would also be protected. The best evidence for this was provided by experiments using WT and *A2AR* receptor knockout (*A2AR*⁻) mice inoculated with established melanomas. These hyperplastic outgrowths were completely rejected in 60 % of *A2AR*⁻ hyperplastic outgrowth-bearing mice while there was no rejection in WT mice [42]. The effects of the A2A receptor were T cell autonomous. In contrast, our results in *Drosophila* showed that the effects of *AdoR*¹ on *wts*^{x1} and *dco*³ mosaic hyperplastic outgrowth are cell autonomous. Results in both experimental systems suggest the involvement of Ado in the interactions

between hyperplastic outgrowths and their neighboring cells in tissues.

Our report is the first to show the interaction between the adenosine and Hippo/Warts signaling pathways in *Drosophila*. We believe that further investigation of adenosine signaling and its impact on competitive cell interactions may clarify important aspects regarding the maintenance of tissue homeostasis at a cellular level.

Acknowledgments We thank Ruzenka Kuklova for the technical assistance. We also thank Dr. Dana Carroll, Dr. Gennady Belitsky, Dr. Elizabeth Khovanova and Dr. Tamas Szlanka for critical reading of the manuscript and Dr. Tian Xu for the *wts¹* *Drosophila* strain. We acknowledge the use of research infrastructure that has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 316304. This research was supported by the Czech Science Foundation, grant no. GA14-27816S.

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