Competing Activities of Heterotrimeric G Proteins in Drosophila Wing Maturation

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Abstract

Drosophila genome encodes six alpha-subunits of heterotrimeric G proteins. The Gαs alpha-subunit is involved in the post-eclosion wing maturation, which consists of the epithelial-mesenchymal transition and cell death, accompanied by unfolding of the pupal wing into the firm adult flight organ. Here we show that another alpha-subunit Gαo can specifically antagonize the Gαs activities by competing for the Gβ13F/Gγ1 subunits of the heterotrimeric Gs protein complex. Loss of Gβ13F, Gγ1, or Gαs, but not any other G protein subunit, results in prevention of post-eclosion cell death and failure of the wing expansion. However, cell death prevention alone is not sufficient to induce the expansion defect, suggesting that the failure of epithelial-mesenchymal transition activation is key to the folded wing phenotypes. Overactivation of Gαs with cholina toxin mimics expression of constitutively activated Gαs and promotes wing blistering due to precocious cell death. In contrast, co-overexpression of Gβ13F and Gγ1 does not produce wing blistering, revealing the passive role of the Gβγ in the Gαs-mediated activation of apoptosis, but hinting at the possible function of Gβγ in the epithelial-mesenchymal transition. Our results provide a comprehensive functional analysis of the heterotrimeric G protein proteome in the late stages of Drosophila wing development.

Introduction

G protein-coupled receptors (GPCRs) represent the most populous receptor family in metazoans. Approximately 380 non-olfactory GPCRs are encoded by the human genome [1], corroborated by ca. 250 GPCRs in insect genomes [2,3], making 1–1.5% of the total gene number dedicated to this receptor superfamily in invertebrates and mammals. GPCRs transmit their signals by activating heterotrimeric G proteins in cells. A heterotrimeric G protein consists of a GDP-bound α-subunit and a βγ-heterodimer. Ligand-stimulated GPCR serves as a guanine nucleotide-exchange factor, activating the GDP-to-GTP exchange on the Gα-subunit. This leads to dissociation of the heterotrimeric complex into Gα-GTP and βγ, which transmit the signal further inside the cell [4].

The β- and γ-subunit repertoire of the Drosophila genome is reduced as compared with that of mammals: only two Gγ and three Gβ genes are present in flies (Table 1). Gγ30A and Gβ76C are components of the fly phototransduction cascade and are mostly expressed in the visual system [5,6]. Gγ1 and Gβ13F have been implicated in the asymmetric cell divisions and gastrulation [7,8], while the function of Gβ5 is as yet unknown.

Despite the fact that βγ can activate signal effectors [9], the main selectivity in GPCR coupling and effector activation is provided by the Gα-subunits [10]. Sixteen genes for the α-subunits are present in the human genome, and six in Drosophila. All human Gα-subunit subgroups are represented in Drosophila (Table 1): Gαi and Gαo belonging to the Gαi/o subgroup; Gαq belonging to the Gαq/11 subgroup; Gαs belonging to the Gαs subgroup, and concertina (cta) belonging to the Gαq/12/13 subgroup [10]. Additionally, Drosophila genome encodes for Gαi which probably represents an insect-specific subfamily of Gα-s subunits [11].

Multiple functions have been allocated to different heterotrimeric G proteins in humans and flies [12], see Table 1. For example, in Drosophila development cta is a crucial gastrulation regulator [13], Gαo is important for the transduction of the Wnt/Frizzled signaling cascade [14,15], and Gαq controls asymmetric cell divisions during generation of the central and peripheral nervous system [7] (the later in cooperation with Gαs [16,17]). Gαq is the Drosophila phototransduction Gα-s subunit, but probably has additional functions [18]. Pleiotropic effects arise from defects in Gαq function [19], while the function of Gαi has not yet been characterized.

Among the developmental processes ascribed to the control by Gαs are the latest stages of Drosophila wing development. Newly hatched flies have soft and folded wings, which during the 1–2 hours post-eclosion expand and harden through intensive synthesis of components of the extracellular matrix. These processes are accompanied by epithelial-mesenchymal transition and apoptosis of the wing epithelial cells, producing a strong but mostly dead adult wing structure [20,21,22]. Expression of the constitutively active form of Gαq leads to precocious cell death in the wing.

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epidermis, which results in failure of the closure of the dorsal and ventral wing sheets and accumulation of the hemolymph inside the wing, producing wing blistering [22,23]. Conversely, clonal elimination of Gαs leads to autonomous prevention of the cell death. Kimura and co-workers have performed an extensive analysis of the signaling pathway controlling apoptosis at late stages of wing development [22]. They provide evidence suggesting that the hormone bursicon, synthesized in the head of post-eclosion Drosophila and secreted in the hemolymph, activates a GPCR receptor on wing epithelial cells, which signals through Gαs to activate the cAMP-PKA pathway, culminating at the induction of apoptosis [22]. However, the identity and importance of the βγ subunits in bursicon signaling, as well as possible involvement of other Gα proteins remained outside of their investigation. There also remain some uncertainties as to the phenotypic consequences of elimination of wing development [22]. They provide evidence suggesting that the hormone bursicon, synthesized in the head of post-eclosion Drosophila and secreted in the hemolymph, activates a GPCR receptor on wing epithelial cells, which signals through Gαs to activate the cAMP-PKA pathway, culminating at the induction of apoptosis [22]. However, the identity and importance of the βγ subunits in bursicon signaling, as well as possible involvement of other Gα proteins remained outside of their investigation. There also remain some uncertainties as to the phenotypic consequences of elimination of wing development [22].

Here we describe a comprehensive functional analysis of the Drosophila heterotrimeric G protein proteome using loss-of-function and overexpression experiments. We show that loss of Gαs but not any other Gα subunit leads to the failure of wing expansion after fly hatching. We also show that Gαo, but not another Gα, can compete with Gαs and thus antagonize its function. Finally, we identify the Gβ13F and Gγ1 as the βγ subunits of the heterotrimeric Gs complex responding to the epithelial-mesenchymal transition and cell death-promoting signal.

**Results**

Gαo, but not other Gα subunits, in its GDP-loaded state prevented post-eclosion wing unfolding in Drosophila

In the course of our studies of the role of the Gαo subunit of heterotrimeric G proteins in the Wnt and PCP signaling in Drosophila wing development [15] we came across an observation that overexpression of Gαo in Drosophila wings often led to the failure of wing expansion after fly hatching from the pupal case. Using the X-chromosome-located MS1096-Gal4 driver line, we found that ca. 80% of the aged adult female flies and 90% of male flies had folded wings characteristic of the freshly eclosed flies - a phenomenon never observed with wild-type animals (Fig. 1A, B, C). MS1096-Gal4 drives strong expression in the dorsal domain and weaker expression in the ventral domain of the developing larval and pupal wing [25, 26, 27].

A similar overexpression of other Gα subunits, Gαs, Gαi, or Gαq, did not produce this effect (Fig. 1C–E), suggesting that Gαo was unique in its ability to prevent wing expansion post-eclosion. Interestingly, the activated Q205L mutant form of Gαo, which stays constantly bound to GTP [15, 16], could not induce the folded wing phenotype (Fig. 1F). These data suggest that the GDP-, but not the GTP-loaded, form of Gαo upon overexpression binds and sequesters a specific protein required for the proper post-eclosion wing development.

Proteomic analysis identifies very few proteins discriminatively interacting with Gαo in its GDP vs GTP form

In order to identify the protein(s) which might be sequestered by the overexpression of the wild-type (mostly GDP-loaded), but not the GTP-loaded form of Gαo during post-eclosion wing expansion, we performed a proteomic analysis of Gαo-binding partners which would bind specifically to its GDP- or its GTP-loaded states, but not to both forms. To this end, we bacterially expressed wild-type Gαo and immobilized it on CNBr-sepharose. These procedures resulted in Gαo which was approximately 50%}

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**Table 1. The list of Drosophila Gα, Gβ, and Gγ subunits, with the information on their function and human homologies.**

| G protein subunit | synonyms | number of isoforms | sub-group | human ortholog (% identity) | described function |
|-------------------|----------|--------------------|-----------|-----------------------------|-------------------|
| Gαo               | G protein α 47A, brokenheart (bkh) | 2         | Gαα/o     | Gαα (82%)                   | Frizzled receptor signal transduction in the Wnt and planar cell polarity pathways [15]; control of asymmetric cell divisions in the sensory organ lineage [17]; feeding behavior [42]; learning and memory [43]; heart development [44, 45]; axonal growth/guidance [44]; blood-brain barrier formation [46]; Hedgehog signal transduction [47] |
| Gαs               | G protein α60A | 2         | Gαs       | Gαs (72%)                   | Larval growth [19]; establishment of the neuro-muscular synapse [48]; post-eclosion wing maturation [22] |
| Gαf               | G protein α73B | 1         | Gαf       | none (40% to Gαs)           | None described    |
| Gαq               | G protein α49B | 2         | Gαq/11    | Gαq (77%)                   | Phototransduction [18]; olfaction [31] |
| concertina        | cta       | 1         | Gα12/13   | Gα13 (55%)                  | Gastrotrusion [13] |
| Gβ subunits       |           |           |           |                             |                   |
| Gβ13F             |          | 1         | Gβ11 (83%) | Control of the asymmetric cell divisions in the neuroblast and sensory organ lineages [7]; gastostrusion [7]; heart development [45] |
| Gβ13C             | Gβle     | 1         | none? (42% to Gβ11) | Phototransduction [5] |
| GβJS              |          | 1         | GβJS (68%) | None described              |                   |
| Gγ subunits       |           |           |           |                             |                   |
| Gγ1               |          | 1         | Gγ12 (44%) | Control of the asymmetric cell divisions in the neuroblast and sensory organ lineages [8]; gastostrusion [8]; heart formation [49] |
| Gγ30A             | Gγe      | 1         | Gγ13 (41%) | Phototransduction [6] |

doi:10.1371/journal.pone.0012331.t001
active as determined in the GTP-binding assays [16]. The matrix was then preloaded with GDP or GTP\(_\text{c}\) (a non-hydrolysable GTP analog) and used to apply Drosophila head extracts. After washing, proteins retained were eluted with Urea and resolved on SDS-PAGE (Fig. 2A). We could identify three bands which bound preferentially to either nucleotide form of G\(_\text{a}\)o: two in the GTP\(_\text{c}\)-matrix (ca. 53 kDa and 71 kDa), and one in the GDP-matrix (ca. 37 kDa). These findings could be confirmed by high resolution protein separation using 2D-PAGE with DIGE labeling [28]. The three proteins from Drosophila head extracts discriminatively bound to either nucleotide form of G\(_\text{a}\)o in our experiment (Fig. 2B–F), suggesting that the majority of G\(_\text{a}\)o target proteins interact equally well with GDP- and GTP-loaded G\(_\text{a}\)o. The 53 kDa and 71 kDa proteins migrated as several spots on 2D-gels (Fig. 2C, E, F), which might indicate post-translational modifications of the proteins. LC-MSMS after trypsin in-gel digestion was used to identify these three proteins. The 71 kDa protein was found to be the Heat-shock 70 kDa protein cognate 3 (gene name: Hsc70-3), the 53 kDa protein was identified as Tubulin \(\beta\)-chain (gene name: \(\beta\)-Tubulin at 56D), and the 37 kDa protein exclusively binding to G\(_\text{a}\)o-GDP - as the Guanine nucleotide-binding protein subunit \(\beta\)-1 (gene name: G\(\beta\)13F subunit). While tubulins have previously been found to physically bind G\(_\text{a}\)o-subunits [29,30], binding of Hsc70-3 to a G protein has not been reported before. As for the G\(\beta\)13F subunit, the interaction of GDP-loaded G\(_\text{a}\)o with the \(\beta\)\(\gamma\) heterodimers is expected. However, we initially did not suspect that sequestration of \(\beta\)\(\gamma\) by overexpressed G\(_\text{a}\)o could be the reason for the wing unfolding defects, as other G\(_\text{a}\)-subunits would also be expected to sequester \(\beta\)\(\gamma\), and yet were ineffective in preventing wing unfolding (Fig. 1).

| genotype          | % folded wings, females | \(n\) total wings analyzed, females | % folded wings, males | \(n\) total wings analyzed, males |
|-------------------|--------------------------|-------------------------------------|-----------------------|----------------------------------|
| UAS-G\(_\text{a}\)o | 79%                      | 180                                 | 93%                   | 41                               |
| UAS-G\(_\text{a}\)o; UAS-G\(\beta\)13F; UAS-G\(\gamma\)1 | 3%                       | 35                                  | 32%                   | 22                               |
| UAS-G\(_\text{a}\)o; UAS-G\(_\gamma\) | 18%                      | 50                                  | 50% (ns)              | 6 (ns)                           |
| UAS-G\(_\text{a}\)o; UAS-G\(_\gamma\) [GTP] | 50%                      | 30                                  | 100% (ns)             | 9 (ns)                           |

\(ns\)- the number of flies available for analysis is not significant. Note that the viability of the male flies expressing G\(_\text{a}\)o under the control of the X-linked MS1096-Gal4 driver is reduced.

doi:10.1371/journal.pone.0012331.t002
Gb13F and Gγ1, but not other Gβ/γ subunits, are required for the post-eclosion wing unfolding

To test whether the post-eclosion Gαo-overexpression phenotype was due to sequestration of Gβγ, we first aimed at rescuing the Gαo phenotype by providing more βγ. To this end, we co-expressed Gαo, Gb13F, and Gγ1 by the MS1096-Gal4 driver line. Indeed, we found an overwhelming rescue of the wing expansion defect if Gb13F/Gγ1 were co-overexpressed: only 3% of aged female wings and 32% of the male wings now remained folded, as compared to 79% and 93% of female and male flies, respectively, overexpressing Gαo alone (Table 2).

Next, to address the question whether Gβγ heterodimers were necessary for the post-eclosion wing development, we expressed RNAi lines targeting Gb13F, Gb5, Gb76C, Gγ1, or Gγ30A by MS1096-Gal4. As shown on Fig. 3A–C, RNAi against Gγ1, but not Gγ30A, prevented wing expansion similarly to that induced by Gαo overexpression (Fig. 1B). When RNAi lines targeting the three Gβ-subunits were expressed, RNAi against Gb13F, but not Gb5 or Gb76C, was found to prevent wing expansion (Fig. 3D–F).
Flies homozygous mutant for the Gβ76C gene also showed no defects in wing development (data not shown). Other phenotypes of the downregulation of Gβ13F and Gγ1 suggested the role of this Gβγ heterodimer in the process of asymmetric cell divisions [17], Wnt signaling [14], and planar cell polarity (not shown). Altogether, our results point to a simple model in which overexpression of the wild-type Gαo, but not Gαi, Gαs, or Gαq, sequestered Gβ13F/Gγ1 required for the post-eclosion wing expansion in Drosophila.

Gβ13F/Gγ1 constitute with Gαs the heterotrimeric G protein complex required for the post-eclosion wing expansion

We supposed that Gαo competed for Gβ13F/Gγ1 with another Gα-subunit, thus inactivating a heterotrimeric G protein complex required for the proper wing expansion. To investigate the nature of this Gα-subunit outcompeted by Gαo, we systematically removed all other Gα-proteins by using loss-of-function mutations or targeted RNAi expression. RNAi-targeted downregulation was employed to target Gαi, Gαq, Gαf, and Gαs (Fig. 4A–F); of these constructs, those targeting Gαq and Gαi were previously shown efficient in downregulating target gene expression [16,31]. Concertina was removed using the null allele [13]. Similar elimination of Gαo is not possible due to the requirement of this G protein for cell viability in the wing [15]. Gαo can be specifically uncoupled from GPCRs using the expression of pertussis toxin [29]; such whole wing expression of pertussis toxin does not result in any visible defects in wing expansion [16].

Out of all Gα tested, elimination of Gαs from the wing produced the wing unfolding defect similar to that induced by overexpression of Gαo or downregulation of Gβ13F/Gγ1 (Fig. 4E). In contrast, elimination of other Gα proteins in the wings did not produce visible defects (Fig. 4). Thus, we concluded that among different Gα-subunits only elimination of Gαs led to the wing unfolding defect. In agreement with this, we found that co-overexpression of Gαo together with Gαs strongly suppressed the ability of the latter to produce the folded wing phenotype (Table 2). Thus, the heterotrimeric G protein complex, consisting of the Gαs, Gβ13F, and Gγ1 subunits is required for the proper signaling regulating wing expansion post-eclosion, and can be antagonized by Gαo.

The wing expansion defect is associated with, but is not caused by, prevention of cell death

Clonal elimination of Gαs results in failure of the cell death in the wing [22]. Indeed, while aged flies retained live GFP- and rhodamine phalloidin-stained cells only along the veins and wing margin (Fig. 5A, B), we found that the MS1096-Gal4-driven expression of Gαs or RNAi constructs targeting Gβ13F, Gγ1, or Gαs all similarly resulted in maintenance of live cells within the wing blade of well-aged flies (Fig. 5C–G). To better resolve the remaining live cells, we performed the nuclear staining with DAPI [22,24]. Young (ca. 1h-old) wild-type wings contain many DAPI-positive living cells (Fig. 5H), but aged wild-type wings showed DAPI staining only alone the veins (Fig. 5I). In contrast, wings of the Gαo-overexpressing flies up to six days old were still filled with DAPI-positive living cells (Fig. 5J). These data clearly show that the wing expansion failure is associated with the failure of cell death. However, is prevention of the cell death sufficient to cause the folded wing phenotype? To investigate this possibility, we expressed the baculovirus apoptosis inhibitor p35 in the entire wing under the MS1096-Gal4 control. While apoptosis was efficiently prevented, wing expansion was normal in these wings (Fig. 5K). This data agrees with the similar observations obtained

Figure 3. Downregulation of Gγ1 or Gβ13F, but not of any other Gβ or Gγ subunit, leads to the failure of wing expansion.

Representative wings expressing the RNAi constructs targeting Gγ30A (B), Gγ1 (C), Gβ5 (D), Gβ76C (E), or Gβ13F (F) are shown along with the MS1096-Gal4 driver line alone (A). doi:10.1371/journal.pone.0012331.g003
when $p35$ was expressed using other $Gal4$ drivers [21,22]. Cumulatively, our data suggest that apoptosis, being an important process during post-eclosion wing maturation, is unlikely to be the sole driving force behind wing expansion. Wing expansion seems more dependent on the epithelial-mesenchymal transition [21,24], or perhaps requires both processes to act in concert. Elimination of the components of the heterotrimeric Gs proteins apparently leads to both the failure of epithelial-mesenchymal transition and apoptosis, leading cumulatively to the wing expansion defect.

Overactivation of Gs by cholera toxin mimics expression of the constitutively active mutant form of Gs, not reproduced by overexpression of Gβ13F/Gγ1

Expression of the GTPase-deficient point mutant of Gs induces precocious cell death, which results in hemolymph accumulation between the two epithelial wing sheets and wing blistering [22,23], Fig. 6A. In mammalian systems Gs can be overactivated by cholera toxin, which covalently ADP-ribosylates a conserved arginine residue of the GTPase active center [32]. To test whether cholera toxin was also active against Drosophila Gs, we expressed the toxin in developing Drosophila wings, and found wing blistering induced by the toxin (Fig. 6B) similar to that induced by the constitutively activated Gs (Fig. 6A). These data not only extend the known similarity between mammalian and fly Gs, but they also demonstrate that targeted activation of the endogenous, not overexpressed, Gs is sufficient to overactivate the pathway and produce wing blistering.

Cholera toxin-mediated activation of Gs mimics that achieved by GPCR-mediated activation and results in production of GTP-loaded Gs and free Gβγ subunits. As the latter can induce signal transduction in some instances [9], we investigated the effects of direct co-overexpression of Gβ13F/Gγ1 in Drosophila wings using a number of $Gal4$ drivers lines. Gβ13F or Gγ1 subunits expressed alone were ineffective in inducing phenotypes (Fig. 6C, D). Despite the fact that co-overexpression of Gβ13F and Gγ1 could affect asymmetric cell divisions [17], Wnt/Frizzled signaling [14], planar cell polarity (data not shown), and venation (Fig. 6E), Gβ13F/Gγ1 was in no condition able to mimic the wing blistering phenotype induced by activation of Gs (Fig. 6E). We also boosted Gβ13F/Gγ1 overexpression by combining two copies of the $UAS$-$G\beta13F$, $UAS$-$G\gamma1$ transgenes, as well as by providing two copies of the $Gal4$ driver lines; these attempts also failed to produce the wing blistering phenotype. These results demonstrate that the Gβγ heterodimer is required for the proper Gs signaling, but by itself plays only the passive, permissive role in the signal transduction leading to apoptosis.

To further prove that Gβγ is not necessary for the execution of the apoptosis program once the activated Gs is released, we co-expressed Gs[GTP] with the wild-type Gs sequestering the Gβγ subunits. We found that the potency of Gs[GTP] to induce wing blistering was not at all affected by such Gβγ sequestration (Fig. 6F). However, Gβγ might have a separate function in the Gs signaling, namely the induction of the epithelial-mesenchymal transition sub-pathway. Indeed, while co-overexpression of Gs is capable of rescuing the folded wing phenotype induced by overexpression of Gs, the constitutively activated form of Gs is much less potent in performing such a rescue (Table 2). These data suggest that it is not the GTP-loaded Gs, but the free Gβγ heterodimer, released from the heterotrimeric Gs complex upon receptor activation, which is required for the epithelial-mesenchymal transition and wing expansion. This issue is further discussed in the next section.

**Figure 4. Downregulation of Gαs, but not other Gα-subunits, leads to the failure of wing expansion.** Representative wings expressing the RNAi constructs targeting Gαi (B), Gαq (C), Gαf (D), or Gαs (E) are shown along with the MS1096-Gal4 driver line alone (A) and the wing of the concertina homozygous mutant fly (cta $^{-}$, F). doi:10.1371/journal.pone.0012331.g004
**Discussion**

The soft folded wings of the young insect freshly hatched from the pupal case within 1–2 hours expand and harden, becoming a robust flight organ. This process is accompanied by epithelial-mesenchymal transition and cell death of the wing epithelial cells [20,21]. Genetic dissection has revealed the function of the neurohormone bursicon and its wing epithelial receptor rickets in initiation of these processes [21,22,24]. The GPCR rickets couples to the heterotrimeric G protein Gs; the Gzs-activated cAMP-PKA pathway culminates at the induction of apoptosis [22]. However, the overall phenotypic consequences of the loss of the Gs signaling pathway in post-eclosion wings were unknown, as well as the nature of the Gbc subunits of the heterotrimeric Gs complex responding to the bursicon-rickets signaling.

Here we have performed an extensive analysis of the heterotrimeric G protein subunits in these post-eclosion stages of wing maturation. We find that the whole-wing down-regulation of Gzs results in the failure of wing expansion, demonstrating that this change in the shape of the wing is the major morphological outcome of the bursicon-rickets-Gs signaling. We also identify the Gβ13F and Gγ1 subunits as the other two constituents of the heterotrimeric Gs complex, as downregulation of Gzs, Gβ13F, or Gγ1, but not any other Gs, Gβ, or Gγ subunits encoded by the Drosophila genome, each leads to the same folded wing phenotype.

We also show that Gαo, but not any other Gα-subunits, can inhibit the wing expansion program through sequestration of the Gβγ heterodimer. The reason for the specificity of Gαo over other Gα-subunits in antagonizing the Gs signaling is unclear. It is unlikely that differences in expression levels of the tested Gα-subunits may account for the selective activity of Gαo. Indeed, most overexpression experiments were done with the X-chromosome-inserted MS1096-Gal4 UAS-GFP flies which are ≥1 day-old (except for the wing of panel (H)).

**Figure 5. Prevention of apoptosis is associated with, but is not sufficient to induce, the failure of wing expansion.** A–B. Wild-type wings are fully expanded and show GFP (A) or F-actin (B) staining only on the margin and along the veins, demonstrating that the adult wings are mostly dead structures. C–G. Downregulation of the Gs pathway by overexpression of Gαo (C, D) or by expression of RNAi constructs targeting Gβ13F (E), Gγ1 (F), or Gzs (G) leads to both failure of wing expansion and prevention of apoptosis, as visualized by persistence of F-actin- (D) and GFP-positive cells (C, E–G). H–J. DAPI nuclear staining. Overexpression of Gαo in aged wings leads to the DAPI staining pattern (J) characteristic of the young (ca. 1h-old, H) wild-type wings; aged wild-type wing only shows DAPI staining along the veins (I). K. Expression of the apoptosis inhibitor p35 prevents cell death throughout the wing as seen by persistence of GFP-positive cells, but does not cause the failure of wing expansion. All wings presented here are from MS1096-Gal4 UAS-GFP flies which are ≥1 day-old (except for the wing of panel (H)).

doi:10.1371/journal.pone.0012331.g005
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providing an explanation for a specific ability of Gzo to antagonize the Gs-mediated post-mitosis pathway. We are thus tempted to propose that a previously uncharacterized biochemical mechanism may allow for a specific antagonism physiologically existing between the Gs- and Gzo-mediated signaling pathways. As liberation of high amounts of GDP-loaded Gzo is expected to be a consequence of activation of multiple GPCRs [33], and as Gs is a greatly expressed protein representing the major G protein species e.g. in the brain of flies and mammals [35,36], this specific ability of Gzo to antagonize the Gs-mediated signaling may have physiological implications in other tissues and organs than Drosophila wing. However, we would like to add that these speculations are based on the analysis of the overexpression data and must be treated with caution when translating them into physiological situations.

Only the GDP-loaded, but not the activated GTP-loaded form of Gzo is effective in antagonizing Gs. We have performed a proteomics analysis of the Drosophila proteins which would discriminate between the two nucleotide forms of Gzo, and revealed surprisingly few targets of this kind. While the chaperone Hsc70-3 and β-tubulin preferentially interacted with the GTP-loaded Gβ13F, Gβ13F was found to specifically interact with Gzo-GDP. These data suggest that many Gzo-interaction partners do not discriminate between the two guanine forms of Gzo. These findings are in agreement with our other experimental findings [16], as well as our mathematical modeling predicting that high concentrations of free (monomeric) signaling-competent Gzo-GDP are produced upon activation of Go-coupled GPCRs [33].

Gzo-mediated sequestration of Gβ13F/Gγ1 depletes the pool of the heterotrimeric Gs complexes. As only heterotrimeric Gβγ, but not monomeric Gγ proteins can efficiently bind and be activated by their cognate GPCRs [4,34], overexpression of Gzo abrogates the 

nickel-Gs signaling. Phenotypic consequences of this abrogation are the failures of apoptosis and wing expansion. In contrast, expression of the constitutively activated form of Gzs induces premature cell death and wing blistering [22,23]. We find that this phenotype can be also induced by expression of cholera toxin, revealing that the ability of cholera toxin to specifically overactivate Gzs reported in mammalian systems [32] is reproduced with Drosophila proteins. These data also confirm that not only exogenously overexpressed, but also the endogenous Gzs can induce the precocious cell death upon overactivation.

However, prevention of apoptosis is not sufficient to produce the folded wing phenotype (Fig. 5). Together with the observation that the constitutively active form of Gzs is ineffective in rescuing the wing expansion defects produced by Gzo overexpression (Table 2), these data suggest that the Gzs-cAMP-PKA pathway culminating at apoptosis is not the sole signaling branch emanating from the hiruscin-nickel GPCR activation. We propose that the second signaling branch initiated by the nickel-mediated dissociation of the heterotrimeric Gs complex is represented by the free Gβγ subunits, signaling to epithelial-mesenchymal transition (Fig. 7). Such a double signaling impact mediated by the two components of the heterotrimeric G protein complex leads to initiation of two cellular programs - apoptosis and epithelial-mesenchymal transition - which cumulatively result in wing expansion and solidification (Fig. 7), producing the adult flight organ. This two-fold response of the Drosophila wing to the maturation signal, mediated by the two components of the heterotrimeric G protein complex activated by the single hormone-responsive GPCR, provides an elegant paradigm for the coordination of signaling and developmental programs.

Materials and Methods

Fly stocks and crosses

The following Drosophila lines were used: MS1096-Gal4 [25] and OK10-Gal4 [23]; Vg-Gal4, UAS-flp [37]; UAS-Gzo and UAS-Gzo(GTP) [15]; UAS-Gγ1 [7]; UAS-Gsf and UAS-Gsf(GTP) [23]; UAS-Gαq [38]; UAS-Gβ13F [17]; UAS-Gγ1 [8]; cfa [13]; UAS-p35 [39]; and UAS-GFP and Gβ76C (Bloomington Stock Center). The UAS-RX4 lines were from VDRC [40]; UAS-GαqRNAi was additionally from [31]. All crosses were performed at 25°C. The UAS>Gal4→cholera toxin [13] stock cannot be maintained without the fly-cut cassette and thus must be crossed with a flipase-expressing line for Gal4-mediated expression of the toxin.

Histology

All wings were prepared from ≥1 day-old flies and mounted in GMM as described [15]. For GFP, as well as for rhodamine phalloidin (Molecular Probes) visualization after treatment as described for imaginal discs [15], wings were mounted in Moviol. Whole young flies (≥1 day-old) were photographed through a Zeiss Steci 2000 binocular using the Canon PowerShot G10 camera to visualize wing blistering. DAPI staining was performed

Figure 6. Overactivation of Gs, but not Gβγ, leads to wing blistering due to precocious apoptosis. A. Expression of the constitutively active form of Gs by multiple Gal4 drivers produces the characteristic wing blistering. The picture shown represents an OK10-Gal4/UAS-Gs[GTP] fly. B. Activation of the endogenous Gs by expression of cholera toxin with multiple drivers also produces wing blistering (arrows). The picture shown represents a Vg-Gal4, UAS-flp; UAS->w+>cholera toxin fly. C-E. Expression of Gβ13F alone (C), Gγ1 alone (D), or both (E) by multiple drivers including OK10-Gal4 never produces the wing blistering. The pictures shown represent wings of MS1096-Gal4; UAS-Gs[GTP]; fly. F. Sequestration of Gβγ by Gzo does not prevent wing blistering induced by the constitutively active form of Gs. The picture shown represents an MS1096-Gal4, UAS-Gzo; OK10-Gal4/UAS-Gs[GTP] fly.

doi:10.1371/journal.pone.0012331.g006
Expression of Gαo inhibits this signaling through sequestration of the Gβγ-subunits. Proteomic analysis of Gαo-interacting partners

*Drosophila* head extracts were applied to the CNBr-immobilized Gαo preloaded with GDP or GTPγS as described [16]. The incubation slurry was packed into a 1 ml polypropylene column (Qiagen) and washed 3 times with 10 bed volumes of the binding buffer (50 mM Hepes pH 7.5, 100 mM KCl, 10 mM NaCl, 5% glycerol, 2 mM EGTA, 1× complete EDTA-free protease inhibitor cocktail (Roche), 0.5% Nonidet P-40, 0.1% Tween20) at 4°C. Retained proteins were eluted by 8 M Urea, separated by SDS-PAGE, and silver-stained. Alternatively, the eluted proteins were precipitated by methanol/chloroform [41] for mass spectrometry analysis.

Fifty μg of the precipitated proteins were labeled with CyDye DIGE Fluor minimal dyes according to the manufacturer recommendations (GE Healthcare Life Sciences). The samples were cup-loaded onto 24 cm pH 3–11 IEF strips and electro-focused with a total of 45’000 Vh using an Etan IPGphor II (both GE Healthcare Life Sciences). The strips were reduced and alkylated according to the manufacturer recommendations. The second dimension separation was performed on 10–15% linear gradient gels automatically casted using a2DEoipimerizer (NextGen Sciences) and the gels were run in the Etan Dalt II (GE Healthcare Life Sciences) at 25°C. The gels were scanned using a Typhoon 9200 scanner (GE Healthcare Life Sciences). The gel images were analyzed using SameSpots (Nonlinear Dynamics) involving automatic normalization and automatic background subtraction.

After subsequent Coomassie staining, spots of interest were picked using GelPal (Genetix) and digested overnight at 37°C (19 ng trypsin (Promega) in 47 mM Tris pH 9.0). The peptides were analyzed using LC-MSMS (4000 Q TRAP, Applied Biosystems) and proteins were identified using Mascot (Matrix Science) searching the protein sequence database UNIPROT-15.6.

**Acknowledgments**

We are thankful to Gary Struhl, Juergen Knoblich, Michael Forte, Gaiti Hasan, Fumio Matsuzaki, Dean Smith, and the Bloomington and the Vienna *Drosophila* Research Centers for sharing fly stocks.

**Author Contributions**

Conceived and designed the experiments: DH VLK. Performed the experiments: NK DK RP VLK. Analyzed the data: DH VLK. Contributed reagents/materials/analysis tools: DH. Wrote the paper: VLK.

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