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Critical role for Fat/Hippo and IIS/Akt pathways downstream of Ultrabithorax during haltere specification in *Drosophila*

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ABSTRACT

In *Drosophila*, differential development of wing and haltere, which differ in cell size, number and morphology, is dependent on the function of Hox gene *Ultrabithorax* (*Ubx*). Here we report our studies on Ubx-mediated regulation of the Fat/Hippo and IIS/dAkt pathways, which control cell number and cell size during development. Over-expression of Yki or down regulation of negative components of the Fat/Hippo pathway, such as *expanded*, caused considerable increase in haltere size, mainly due to increase in cell number. These phenotypes were also associated with the activation of Akt pathways in developing haltere. Although activation of Akt alone did not affect the cell size or the organ size, we observed dramatic increase in haltere size when Akt was activated in the background where *expanded* is down regulated. This was associated with the increase in both cell size and cell number. The organ appeared flatter than wildtype haltere and the trichome morphology and spacing resembled that of wing suggesting homeotic transformations. Thus, our results suggest a link between cellular growth and pattern formation and the final differentiated state of the organ.

1. Introduction

Wing and haltere are the dorsal appendages of second and third thoracic segments, respectively, of adult *Drosophila*. They are homologous structures, although differ greatly in their morphology. The homeotic gene *Ultrabithorax* (*Ubx*), which is required and sufficient to confer haltere fate to epithelial cells (Lewis, 1978), is known to regulate many wing patterning genes to specify haltere, but the mechanism is still poorly understood.

There are a number of differences between wing and haltere at the cellular and organ levels. Wing is a large, flat and thin structure, while haltere is a small globular structure (Suppl. Fig. 1A–D), although both are made up of 2-layered sheet of epithelial cells. Space between the two layers of cells in haltere is filled with haemocytes (Roch and Akam, 2000). Cuticle area of each wing cell is 8 fold more than a haltere cell (Roch and Akam, 2000). Haltere has smaller and fewer cells than the wing. Trichomes of wing cells are long and thin, while haltere trichomes are short and stout in morphology. The ratio of anterior to posterior compartment size in the haltere (~2.5:1) is much different from that in the wing (~1.2:1). Haltere also lacks wing-type vein and sensory bristles. Haltere cells are more cuboidal compared to flatter wing cells (Roch and Akam, 2000). Thus, cell number, size and shape all add to the differences in the size and shape of the two organs.

However, cells of the third instar larval wing and haltere discs are similar in size and shape (Makhijani et al., 2007). The difference between cell size and shape becomes evident at late pupal stages (Roch and Akam, 2000; Suppl. Fig. 1G–J). Wing cells become much larger, compared to haltere cells (Suppl. Fig. 1I, J). At pupal stages, they also exhibit differences in the organization of actin cytoskeleton elements viz. F-actin levels are much higher in haltere cells compared to wing cells (Roch and Akam, 2000).

In the context of final shape of wings and halteres, one needs to understand the mechanism by which Ubx influences cell size, shape and arrangement. It is possible that Ubx regulates overall shape of the haltere by regulating either cell size and/or shape. The current understanding of mechanisms by which wing and haltere differ at cellular, tissue and organ level is ambiguous (Sánchez-Herrero, 2013). For example, while removal of *Ubx* from the entire haltere, or at least from one entire compartment, leads to haltere to wing transformation with increased growth of Ubx^{-} tissues (Lewis, 1978), mitotic clones of *Ubx* (using the null allele $Ubx^{6.28}$) show similar sized twin spot in small clones (Crickmore and Mann, 2006; de Navas et al., 2006; Makhijani et al., 2007; Suppl. Fig. 1E). Only when very large clones of $Ubx^{6.28}/Ubx^{6.28}$ are generated, one can see increased growth compared to their twin spots (Crickmore and Mann, 2006) (Suppl. Fig. 1F). This suggests that

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unless a certain threshold level of growth factors is de-repressed, the haltere does not show any overgrowth phenotype.

There have been several efforts to identify functional and molecular mechanisms by which Ubx regulates genes/pathways to provide haltere its distinct morphology. Various approaches have been used to identify targets of Ubx that are expected to differentially express between wing and haltere, e.g., loss-of-function genetics, deficiency screens, enhancer-trap screening and genome wide approaches such as microarray analysis and chromatin immunoprecipitation (ChIP). Targets include genes involved in diverse cellular functions like components of the cuticle and extracellular matrix, genes involved in cell specification, cell proliferation, cell survival, cell adhesion, or cell differentiation, structural components of actin and microtubule filaments, and accessory proteins controlling filament dynamics (reviewed in Sánchez-Herrero, 2013).

Decapentaplegic (Dpp), Wingless (Wg), and Epidermal growth factor receptor (EGFR) are some of the major growth and pattern regulating pathways that are repressed by Ubx in the haltere (Weatherbee et al., 1998; Shashidhara et al., 1999; Prasad et al., 2003; Mohit et al., 2006; Crickmore and Mann, 2006; Pallavi et al., 2006; de Navas et al., 2006; Makhijani et al., 2007). However, over-expression of Dpp, Wg, Vestigial (Vg) or Vein (Vn) provides only marginal growth advantage to haltere compared to the wildtype. In this context, we studied additional growth regulating pathways amongst the targets of Ubx. Genome wide studies have identified many components of Fat/Hippo and Insulin–insulin like/dAkt signalling (IIS/dAkt) pathways as potential targets of Ubx (Mohit et al., 2006; Hersh et al., 2007; Pavlopoulos and Akam, 2011; Slattery et al., 2011; Choo et al., 2011; Agrawal et al., 2011). The Fat/Hippo pathway is a crucial determinant of organ size in both *Drosophila* and mammals (reviewed by Halder and Johnson, 2011). It regulates cell proliferation, cell death, and cell fate decisions and coordinates these events to specify organ size. In contrast, the IIS/dAkt pathway is known to regulate cell size (Verdu et al., 1999).

Recent studies have revealed that the Fat/Hippo pathway networks with other signalling pathways (Irvine, 2012; Kwon et al., 2013). For example, during wing development, Fat/Hippo pathway activities are dependent on Four-jointed (Fj) and Dachous (Ds) gradients, which are influenced by Dpp, Notch, Wg and Vg (Rogulja et al., 2008; Zecca and Struhl, 2010). Glypicans, which play a prominent role in morphogen signalling, are regulated by Fat/Hippo signalling (Baena-Lopez et al., 2008). EGFR activates Yorkie (Yki; effector of Fat/Hippo pathway) through its EGFR-RAS-MAPK signalling by promoting the phosphorylation of Ajuba family protein WTIP (Reddy and Irvine, 2013). However, EGFR negatively regulates events downstream of Yki (Herranz et al., 2012). The Fat/Hippo pathway is also known to inhibit EGFR signalling (Yi and Kissil, 2010), which makes the interaction between the two pathways very complex and context-dependent. IIS/dAkt pathway is also known to activate Yki signalling and vice-versa (Straßburger et al., 2012). Thus, Fat/Hippo pathway may specify organ size by regulating both cell number (directly) and cell size (via regulating IIS/dAkt pathway).

Here we report our studies on the functional implication of regulation of Fat/Hippo and IIS/dAkt pathways by Ubx in specifying haltere development. Over-expression of Yki or down regulation of negative components of the Fat/Hippo pathway, such as *expanded* (*ex*), induced considerable increase in haltere size, mainly due to increase in cell number. Although activation of dAkt alone did not affect the organ size or the cell size, activation of Yki or down regulation of *ex* in the background of over-expressed dAkt caused dramatic increase in haltere size, much severe than Yki or *ex* alone. In this background, we observed increase in both cell size and cell number. The resulted haltere appeared flatter than wildtype haltere and the morphology of trichomes and their spacing resembled that of wing suggesting homeotic transformations. Thus, our results suggest a link between cellular growth and pattern formation and the final differentiated state of the organ.

2. Results

2.1. Modulation of Fat/Hippo pathway in developing haltere results in increased growth

Components of the Fat/Hippo pathway such as Ex, Ft, Ds, and Hpo are primarily tumour suppressors, which control organ growth by inhibiting the nuclear function of Yki. To understand to what extent they are involved in Ubx-mediated specification of haltere size, we down regulated the expression of *ex*, *ft*, *ds*, and *hpo* and over-expressed Yki in developing haltere using two pouch-specific GAL4 drivers, *omb*-GAL4 and *Ubx*-GAL4. RNAi-mediated down regulation of *ex*, *ft*, *ds* or *hpo* or over-expression of Yki, they all resulted in increase in the size of haltere capitellum (Fig. 1; Suppl Fig. 2). As *Ubx*-GAL4 is also a null allele of *Ubx*, we observed, as expected, significantly enhanced haltere size when this GAL4 was used compared to when *omb*-GAL4 was used (Fig. 1E). This is further validated as comparable enhanced growth was also observed when the UAS lines were crossed to *omb*-GAL4 driver in a genetic background that is heterozygous for Ubx^{1}, a null allele of *Ubx* (Fig. 1E; Suppl. Fig. 3D). In all these experiments, down regulation of *ex* caused maximum increase in haltere size.

We examined the developmental stage at which activation of the Fat/Hippo pathway is critical for haltere specification. We temporally controlled the expression of UAS-ex^{RNAi} using *Ubx*-GAL4/*tub*-Gal80^{ts}. *Ubx*-GAL4 expression starts at early stages of development and remains throughout the pupal stage (Pallavi and Shashidhara, 2003). We restricted the activity of GAL4 by incubating embryos at 19 °C, and transferring them to 29 °C at different stages of development to activate the GAL4 protein. The flies showed transformation only when *ex* was down regulated in embryonic to early third instar larval stages. Down regulation of *ex* at subsequent stages did not show any phenotype (data not shown).

2.2. Cell-autonomy in growth response

Smaller Ubx^{-} clones do not show any growth advantage over their wildtype twin clones, suggesting that there is no difference in the proliferation rates between wing and haltere discs, at least, at larval stages (Suppl. Fig. 1E). However, larger Ubx^{-} clones show increased growth rate compared to their wildtype counterparts (Suppl. Fig. 1F), suggesting activation of growth promoting signalling pathways, which reinforce the cell-autonomous effect of Ubx. We examined the effect of activation of the Fat/Hippo pathway in this phenomenon. We generated clones by crossing the flp-out driver *Ay-GAL4* to UAS-*lacZ*, UAS-ex^{RNAi} and UAS-Yki. We observed increased growth in Yki or ex^{RNAi}-expressing clones compared to control LacZ (control) clones in both wing and haltere discs (Suppl. Fig. 3E). Interestingly, haltere discs showed higher fold increase in the size of UAS-ex^{RNAi} clones than wing discs (Suppl. Fig. 3E). This further indicates that the Fat/Hippo pathway is an important target of Ubx during haltere specification.

2.3. Haltere-to-wing homeotic transformations at the level of sensory bristles

Flies heterozygous for *Ubx*-GAL4 show few sensory bristles on the haltere capitellum (Fig. 1B), which is an indication of homeotic transformation, albeit partial and mild. Expression of UAS-ex^{RNAi} and UAS-Yki with *Ubx*-GAL4 resulted in increased number of sensory bristles on the capitellum (Fig. 1C–D; Suppl. Fig. 3G). Although we did not observe appearance of bristles when *omb*-GAL4 driver was used, we observed similar increase in bristle number when UAS-ex^{RNAi} was expressed using *omb*-GAL4 driver in a genetic background that is heterozygous for Ubx^{1} (Suppl. Fig. 3D, G). These sensory bristles were arranged in two rows in the same way as seen on the wing margin. Finally, we observed bristle development when *ex* was down regulated using *MS1096*-GAL4 driver, albeit a single one, in wildtype background

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