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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

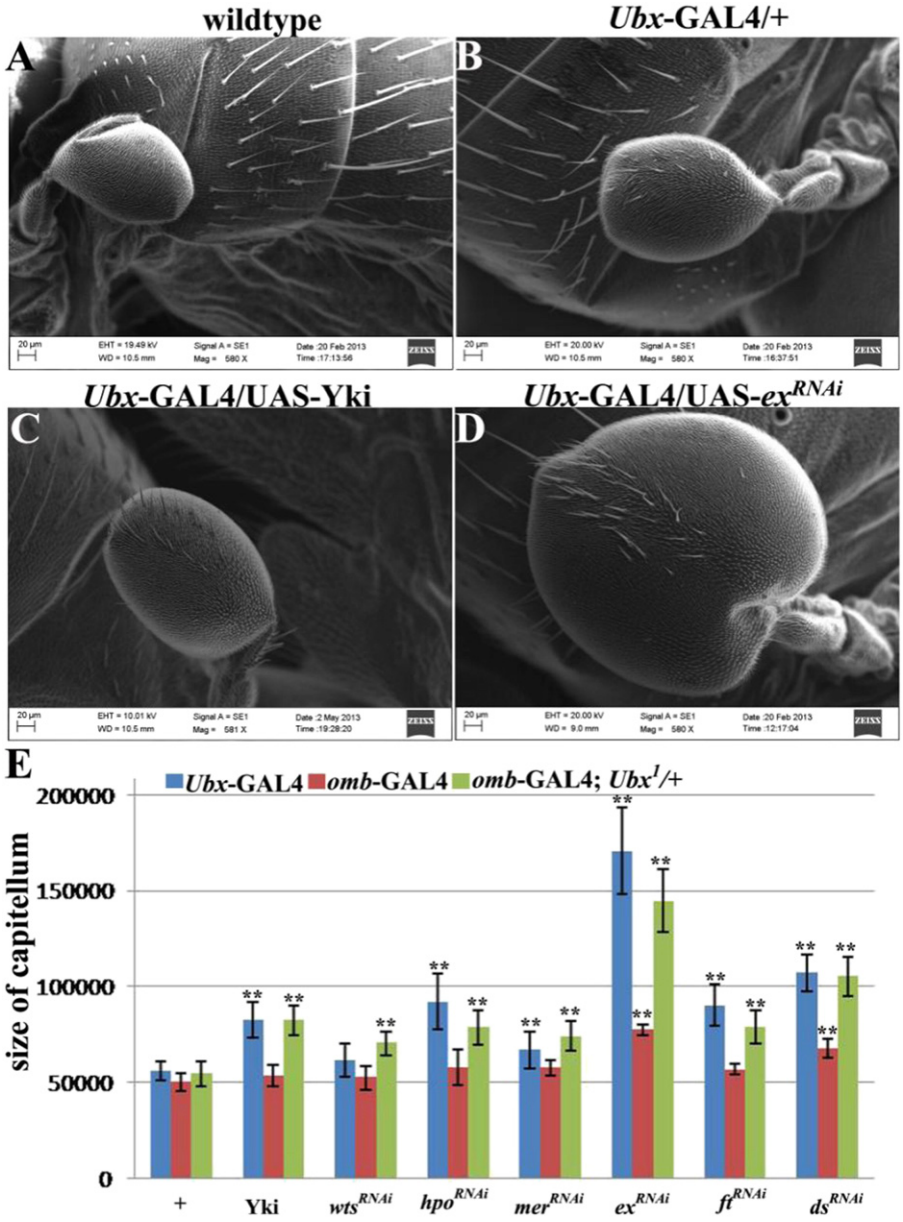

Fig. 1. Effect of modulating Fat/Hippo pathway components on haltere growth. (A–D) Scanning electron microscopy (SEM) images of wildtype (A), *Ubx*-GAL4/+ (B), *Ubx*-GAL4/UAS-Yki (C) and *Ubx*-GAL4/UAS-ex^{RNAi} (D) halteres. Please note considerable increase in haltere size when *ex* is down regulated. (E) Bar diagram showing the relative haltere size of different genotypes as shown in the diagram. Please note that *Ubx*-GAL4 driver is more effective than *omb*-GAL4; Ubx^1, perhaps due to differences in the expression patterns. Knock-down of *ex* showed strongest phenotype with both *Ubx*-GAL4 and *omb*-GAL4; Ubx^1/+. Number of halteres measured for various crosses are given in the Suppl. Text. $^{**}p = <0.001$.

suggesting that changes in the Fat/Hippo pathway alone is sufficient to induce homeotic transformation (Suppl. Fig. 3F). We compared the fold change in the size of the haltere capitellum to the increase in number of sensory bristles, by comparing the capitellum size and the number of sensory bristles on *Ubx*-GAL4 halteres. UAS-ex^{RNAi}, UAS-ds^{RNAi} and UAS-Yki had approximately 3.04, 1.77 and 1.47 fold increase in capitellum size, and 4.8, 2.4 and 2.58 fold increase in the number of ectopic sensory bristles, respectively (Fig. 1E; Suppl. Fig. 3G). Thus, appearance of sensory bristles in these experiments may not be due to increased proliferation of precursor cells already present on *Ubx*-GAL4 halteres, and may represent a change in cell fate.

2.4. Higher levels of nuclear Yki in haltere cells

Yki is the downstream effector of the Fat/Hippo pathway and functions as a transcriptional co-factor in concert with certain other nuclear proteins such as Scalloped (Sd) in *Drosophila* wing disc cells. Various components of the pathway regulate the function of Yki by blocking its nuclear entry by mediating its phosphorylation (Oh and Irvine, 2010). Results presented in the previous section, suggested increased activity of Ex and decreased activity of Yki in wildtype haltere discs. We therefore examined differences, if any, between wing and haltere discs at the level of Yki expression. Intriguingly, when compared to wing discs, haltere discs showed higher levels of nuclear Yki (Fig. 2A). $Ubx^{6.28}/Ubx^{6.28}$ clones in haltere discs showed lower levels of Yki expression compared to their wild type counterparts, particularly nuclear Yki was significantly reduced in Ubx^- cells (Fig. 2B). We, however, did not observe much difference in the levels of *ex* between wing and haltere discs (Suppl. Fig. 4A).

2.5. Yki is not functional in haltere cells

We then examined the expression levels of targets of Yki to directly test its activity. Targets of Yki such as *vein* (*vn*), *wingless* (*wg*), *dally-like*

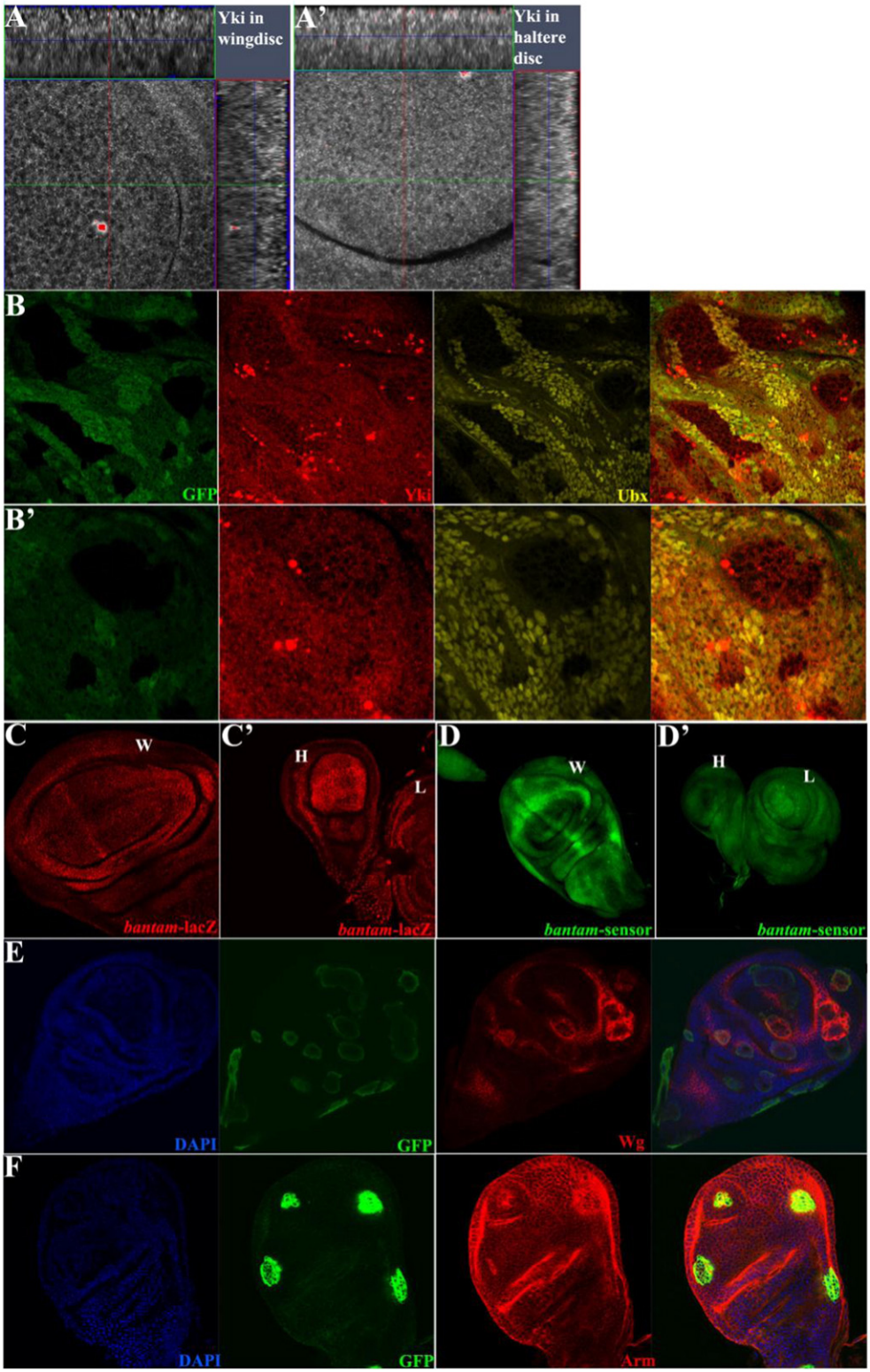

Fig. 2. Yki levels are higher in the nuclei of haltere discs compared to wing discs. (A, A′) Confocal images of wildtype wing (A) and haltere (A′) discs stained for Yki. Top panel in each image is the Z-section of the disc showing nuclei at higher magnification. In wing discs, Yki is mostly seen in the cytoplasm. In the image, regions of nuclei are mostly blank. However, in haltere cells, the Yki staining is uniform, although cytoplasmic levels are comparable to that of wing cells. Thus, only when compared amongst the nuclei, the staining is stronger in haltere discs than in wing discs. (B, B′) Haltere discs with $Ubx^{6.28}/Ubx^{6.28}$ clones stained for GFP (absence of GFP marks the clones), Ubx and Yki. Please note levels of nuclear Yki are lower in clones compared to surrounding cells. (C, C′) *bantam*-lacZ wing (C) and haltere (C′) discs. Note expression of the LacZ in much stronger in the haltere disc compared to the wing disc. (D, D′) *bantam*-sensor wing (D) and haltere (D′) discs. Note expression of the sensor in much weaker in the haltere disc compared to the wing disc. This is indicative of higher levels of Bantam activity in haltere discs compared to wing discs. Wing, haltere and leg discs in C and D are marked as W, H and L, respectively. (E, F) Wing (E) and haltere (F) discs with clones over-expressing yki^{S168A}, a constitutively activated form of Yki. Clones are marked with GFP. Please note over-growth associated with these clones in both wing and haltere discs.

(*dly*), and *vestigial* (*vg*; its quadrant enhancer) are also directly regulated by Ubx (Makhijani et al., 2007; Agrawal et al., 2011). We examined the expression patterns of a few more additional targets of Yki: Cyclin E (CycE), Death-Associated Inhibitor of Apoptosis 1 (DIAP1) and Bantam (Ban) micro RNA (using a *bantam*-lacZ and a *bantam*-sensor) and observed that all these genes are differentially regulated between wing and haltere discs. While CycE, DIAP1, *wg* and *vg*-quadrant enhancer (Suppl. Fig. 4) are down regulated, *ban* miRNA (as detected by *ban*-lacZ) levels are much higher in the haltere pouch compared to wing discs (Fig. 2C). Consistent with the increased levels of *ban*-lacZ, the *ban* sensor, which reflects Ban activity, is down regulated in haltere discs (Fig. 2D). This suggests a more complex pattern of regulation of the Fat/Hippo pathway by Ubx, perhaps by regulating multiple components at different levels of hierarchy of the pathway.

Upstream component of Fat/Hippo pathway phosphorylates Yki, which blocks its nuclear entry and thereby repression of Yki activity. Although significant levels of nuclear Yki were observed in haltere discs, many, if not all, of the targets of Yki are down regulated in haltere discs, suggesting that it is not in the activated form. It is, therefore, possible that the nuclear Yki observed in haltere discs is still in phosphorylated state. Consistent with this, over-expression of a constitutively activated form of Yki (yki^{S168A}; Ren et al., 2010), which is not phosphorylated, resulted in the activation of *wg* and *arm* in the pouch region of these discs (Fig. 2F).

We further examined if over-expression of any of the downstream effectors of Yki caused similar phenotypes. We over-expressed Cyc-E, DIAP1 and Bantam in developing halteres. No phenotype was observed with Cyc-E or DIAP1 with either *omb*-GAL4 or *Ubx*-GAL4 driver (Suppl. Fig. 5B–C, E), while over-expression of Bantam using *omb*-GAL4 caused increase in the size of the capitellum and in the number of sensory bristles (Suppl. Fig. 5D, E). Over-expression of Bantam with *Ubx*-GAL4, however, was early larval lethal.

Ubx and Yki have many common targets that are differentially regulated between wing and haltere. In addition, Yki itself is regulated by Ubx. To determine the genetic relationship between Ubx and Yki in specifying haltere fate, they were expressed either alone or together in developing wing using the *omb*-GAL4 driver. Ectopic expression of Ubx in wing imaginal disc caused reduction in the *omb*-GAL4 domain (Suppl. Fig. 6A, B) reduced adult wing blade (Suppl. Fig. 6E), and altered trichome arrangement and morphology that reflects wing-to-haltere transformations (Suppl. Fig. 6I). Over-expression of Yki alone had no such phenotypes (Suppl. Fig. 6F, J). Contrary to our expectations, we observed enhanced phenotype when Ubx and Yki were co-expressed (Suppl. Fig. 6C, G, K). These results suggest that Yki may cooperate with Ubx to induce haltere fate. Higher levels of nuclear Yki in haltere discs compared to wing discs could be an indication of this requirement, although precise function of Yki in this context remains to be investigated. Nonetheless, all the observations described here suggest that Ubx specifies both growth and pattern in the haltere, at least partially, by regulating the components of the Fat/Hippo pathway.

2.6. IIS/dAkt pathway is down regulated in developing haltere

dAkt is the central component of IIS signalling pathway that positively regulates tissue growth in *Drosophila* (Verdu et al., 1999). Genome-wide studies have suggested that dAkt is a target of Ubx (Mohit et al., 2006; Choo et al., 2011; Agrawal et al., 2011). However, antibody staining for dAkt (phosphorylated form) demonstrated only a subtle difference in its expression pattern between wing and haltere imaginal discs (Fig. 3A, C). As phospho-dAkt expression is highly dynamic, we examined its expression within the same imaginal disc by comparing anterior and posterior compartments of $pbx/DfUbx^{109}$ haltere, in which the posterior compartment is transformed to that of wing-type. The transformed posterior compartment of haltere imaginal disc showed increased levels of dAkt expression in the pouch region compared to the non-transformed *Ubx*-expressing anterior compartment (Fig. 3B, D).

We next examined the expression levels/patterns of targets of the IIS/dAkt pathway. None of the targets examined (4EBP, S6K and Rheb) showed any differential expression between wing and haltere imaginal discs (data not shown). We also carefully examined differences in their expression in $Ubx^{6.28}/Ubx^{6.28}$ null clones and the neighbouring wildtype twin clones. We did not observe any change in their expression levels in Ubx^{-} cells (Suppl. Fig. 7A–C). This suggests that marginal differences observed in dAkt expression between wing and haltere cells may not have any functional significance.

Over-expression of dAkt with *Ubx*-GAL4 did not cause any growth phenotype in the haltere capitellum (Fig. 4B; Suppl. Fig. 8A, B). Over-expression of Rheb or down regulation of *Tsc1* in haltere using *Ubx*-GAL4, however, resulted in moderate increase in the size of the adult haltere capitellum (Suppl. Fig. 8C, D). As *Ubx* appears to regulate dAkt pathway at multiple levels, we modulated the expression of more than one gene at a time. UAS-dAkt; UAS-Rheb and UAS-dAkt; UAS-$tsc1^{RNAi}$ with *Ubx*-GAL4 showed phenotypes similar to driving Rheb or $tsc1^{RNAi}$ alone (Suppl. Fig. 8E, F). In all these experiments, we did not observe any change in the number of sensory bristles (Suppl. Fig. 8).

Expression of upstream components of IIS/dAkt pathway such as UAS-*PI3K* and UAS-Dp110 too did not cause any increase in the size of the adult haltere capitellum (data not shown). Thus, unlike the Fat/Hippo pathway, the IIS/dAkt pathway appears to be a minor target of Ubx during haltere specification (however, see below).

2.7. Simultaneous activation of Fat/Hippo and IIS/dAkt pathways cause strong homeotic transformations in haltere

As most signalling pathways, including IIS/dAkt, are integrated through the Fat/Hippo pathway, we examined the effect of simultaneously manipulating both. Since Yki activates dAkt in wing discs (Ye et al., 2012), we first examined if over-expression of Yki can override the effect of Ubx and activate dAkt in haltere discs. We observed increased levels of dAkt in haltere discs, to the same extent as in wing discs, in response to the over-expression of a constitutively active form of Yki, yki^{S168A} (Fig. 3E, F).

We then examined the effect of expression of dAkt along with either ex^{RNAi} or Yki using the *Ubx*-GAL4 driver. While there was no haltere phenotype when dAkt alone is over-expressed (Fig. 4B), its co-expression with Yki (Fig. 4D) or ex^{RNAi} (Fig. 4F, G) caused significant increase in haltere size. Particularly, UAS-dAkt with UAS-ex^{RNAi} using *Ubx*-GAL4 driver resulted in dramatic increase in the haltere size; most severe amongst all the genetic combinations examined so far (here and published elsewhere). Interestingly, the effect of co-expression of UAS-dAkt with UAS-ex^{RNAi} was more prominent than the co-expression of UAS-Yki and UAS-ex^{RNAi} (data not shown). This is reflective of Ubx regulating multiple pathways to control size of the haltere.

2.8. Regulation of cell number and size during haltere specification

The Fat/Hippo pathway is known to regulate growth by controlling cell number and the IIS/dAkt pathway by controlling cell size, although the two pathways interact with each other. Thus, in the above-mentioned experiments, the combined effect of deregulating Fat/Hippo and IIS/dAkt pathways on haltere size could be due to their individual effects on cell number and cell size. We further explored possibility of decoupling cell number and cell size and quantifying their relative effects during haltere specification.

Over-expression of dAkt resulted in considerable increase in cell size only in wing discs, and its effect on haltere discs was marginal (Fig. 5C, D, I). This is consistent with the observation that over-expression of dAkt did not cause any change in haltere size. Expression of UAS-ex^{RNAi}, which caused significant increase in the size of the haltere, did not affect

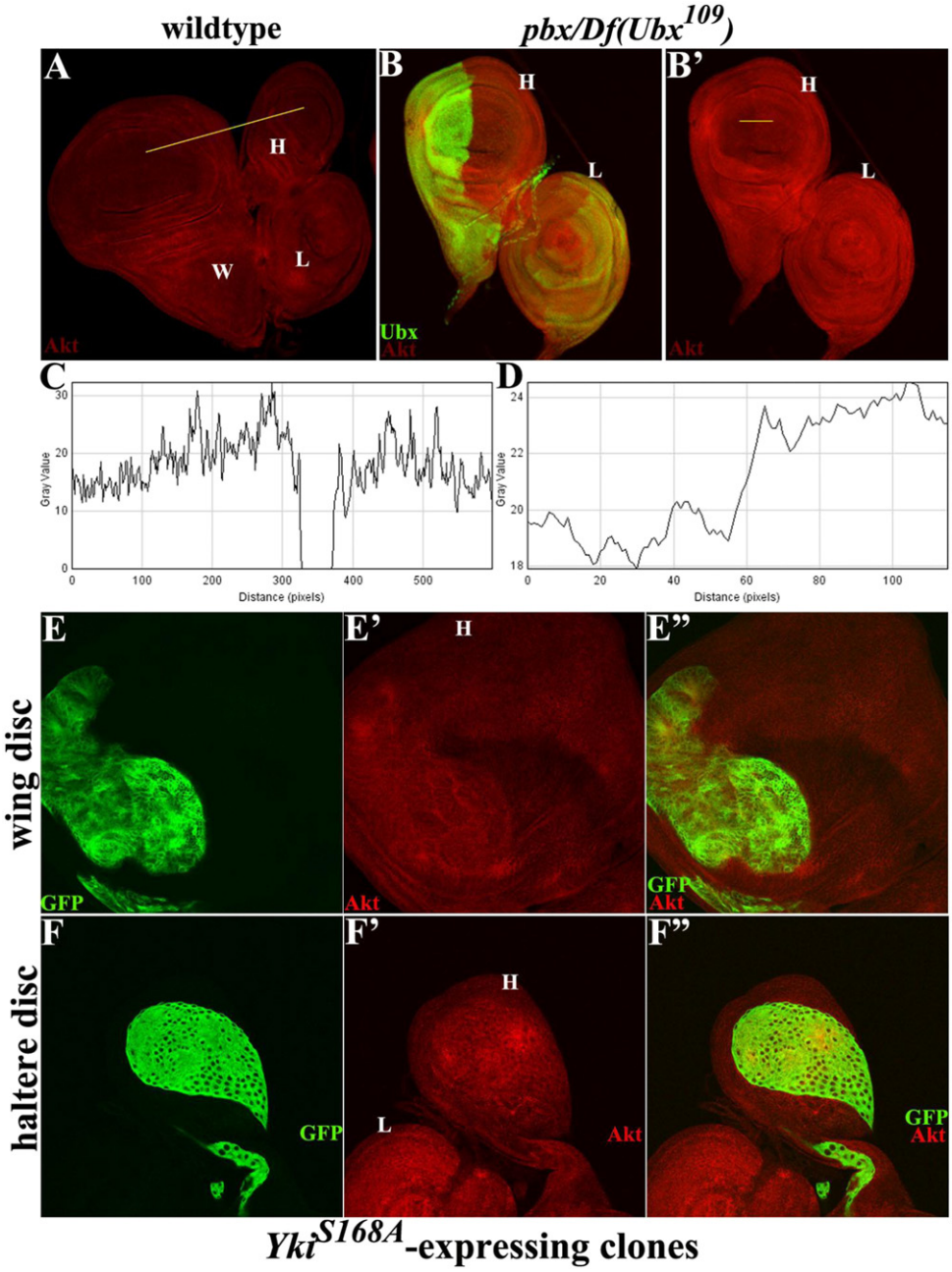

Fig. 3. dAkt is down regulated in haltere discs. (A) Wing (marked W) and haltere (marked H) discs stained for dAkt. As dAkt levels are not very high even in wing discs, the differences, if any, between wing and haltere discs are not apparent. Wing, haltere and leg discs in this and other images are marked as W, H and L, respectively. (B, B′) $pbx^{1}/DfUbx^{109}$ haltere disc stained for Ubx and dAkt. Note Ubx is not expressed in the posterior compartment, allowing us to compare dAkt levels between haltere (anterior compartment) and wing (posterior compartment) cells in the same disc. dAkt levels are marginally higher in the transformed posterior compartment. (C, D) intensity plot of dAkt staining levels in the regions marked in A and B′, respectively. Please note, higher levels of dAkt in the transformed posterior compartment of $pbx^{1}/DfUbx^{109}$ haltere disc. (E, F) wing (E) and haltere (F) discs with clones over-expressing yki^{S168A}, the constitutively activated form of Yki. The discs are stained for dAkt and clones are marked with GFP. Note increased levels of dAkt within the clones in both wing and haltere discs. Large clone in the haltere appears to be more than one clone fused as it crosses the A/P boundary.

cell size either (Fig. 5F, I). This suggests that the increased growth in these experiments is because of change in cell number. Interestingly, although UAS-dAkt alone did not have any effect on cell size, co-expression of UAS-dAkt and UAS-ex^{RNAi} resulted in the increase in cell size in haltere discs, which was comparable to the changes observed in wing cdiscs (Fig. 5G, H, I). As activation of the Fat/Hippo pathway affects dAkt signalling, the increase in cell size observed could be due to the compounded effect of manipulating both the pathways simultaneously. Thus, the dramatic increase in the size of UAS-dAkt; UAS-ex^{RNAi} halteres is attributable to changes in both cell size and cell number.

2.9. Combined effect of Fat/Hippo and IIS/dAkt pathways on cellular organization

The distinct morphology of adult wing and haltere is attributable to the differential nature of organization of their constituent cells. Trichomes that are present on the surface of wing and halteres are indicators of cellular organization leading to distinct structure (Fig. 6A, B). In *Ubx*-GAL4;UAS-ex^{RNAi} halteres, trichomes are less densely arranged and their base is flatter compared to that in *Ubx*-GAL4 (Fig. 6E, compare with Fig. 6C). This suggests partial transformation of haltere cells to wing type. We observed similar, but to a lesser degree, phenotypes

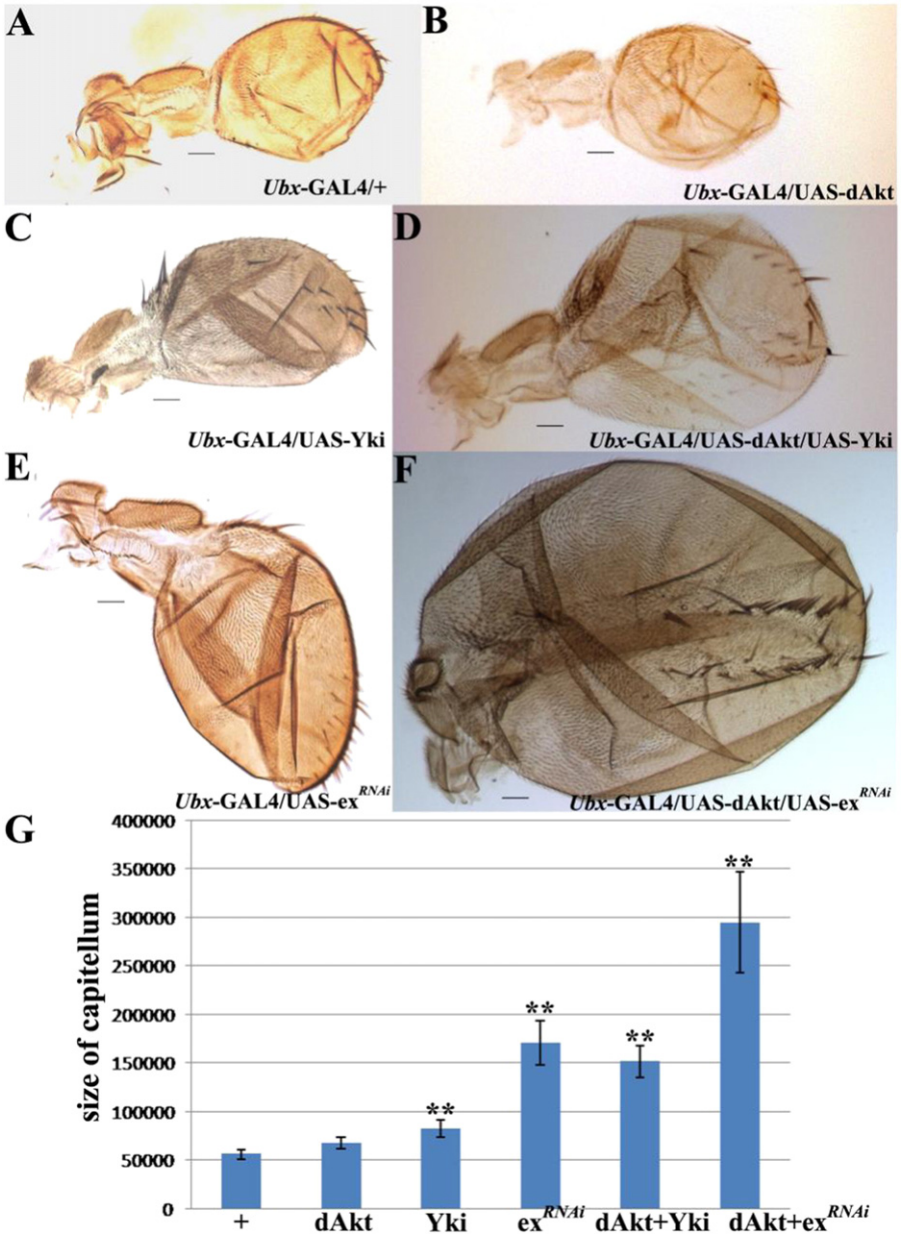

Fig. 4. Simultaneous modulation of IIS/dAkt and Fat/Hippo pathways has synergistic effect on haltere growth. (A–F) Halteres of genotypes as shown in the images. Please note that over-expressing dAkt in the background of down regulation of *ex* has a dramatic effect on the haltere size. Also note that the haltere appears flatter and shows large number of mechano-sensory bristles that are normally seen along the anterior margin of the wing. (G) Bar diagram to show quantitatively the effect of various genetic manipulations on haltere size. Number of halteres measured for various crosses are given in the Suppl. Text. $^{**}p = <0.001$.

with *Ubx*-GAL4;UAS-ds^{RNAi} (data not shown). *Ubx*-GAL4;UAS-ft^{RNAi} and *Ubx*-GAL4;UAS-Yki halteres did not show any such change in trichome morphology or density, while *Ubx*-GAL4;UAS-hpo^{RNAi} halteres had severely disorganised trichomes (data not shown). Over-expression of dAkt had no effect of on the trichome morphology or their arrangement (Fig. 6F).

We investigated the effect of modulating both cell number (Fat/Hippo pathway) and cell size (IIS/dAkt pathway) on the trichome morphology and arrangement. We observed marginal changes to trichome morphology and arrangement when Yki and dAkt were co-expressed (Fig. 6G). UAS-dAkt;*Ubx*-GAL4/UAS-ex^{RNAi} halteres, however, exhibited near wing-like morphology and arrangement of trichomes (Fig. 6H, I). The dramatic increase in the size of UAS-dAkt;*Ubx*-GAL4/UAS-ex^{RNAi} haltere could be due to an increase in cell size, particularly in post-larval stages, and in further flattening of cells during morphogenesis. Thus, it is likely that Ubx-mediated regulation of Fat/Hippo and IIS/dAkt pathways plays a major role in regulating both growth and differentiation during haltere development.

2.10. Effect on shape of haltere via compartment size

In the wing pouch, anterior and posterior compartments are of similar size, A/P ratio being ~1.2:1. In haltere pouch, the posterior compartment is highly reduced in size compared to the anterior compartment, displaying an A/P ratio of ~2.5:1. This skewed compartment ratio is thought to be an important determinant of the haltere shape. Modulation of Dpp expression and its pathway components by Ubx is reported to be responsible for this phenomenon (Crickmore and Mann, 2006; de Navas et al., 2006; Makhijani et al., 2007; Crickmore and Mann, 2007). Mis-expression of *dally* or *master of thick-veins*, downstream components of the Dpp pathway, using posterior-specific GAL4 drivers induces moderate increase in size of the posterior compartment (de Navas et al.,

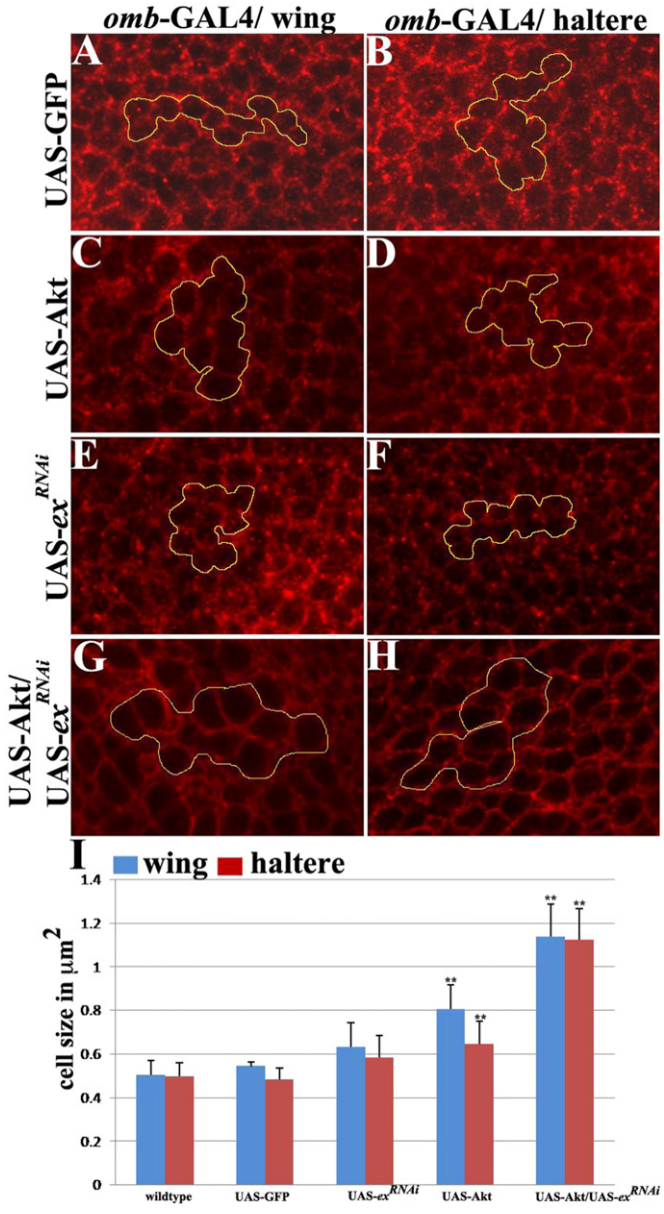

Fig. 5. Upregulation of IIS/dAkt and Fat/Hippo pathways are associated with increase in cell size in haltere discs. (A–H) Wing and haltere discs of genotypes as shown on the images. They are all stained for Armadillo to outline the cells. In each image a region of 10 cells is marked, which is used for estimation of average cell size (see the Methods section for more information). (I) Bar diagram showing quantitative analysis of cell-size change in various experimental conditions. Note, over-expressing dAkt alone is sufficient to cause considerable increase in cell size in wing discs. Its effect on cell size in haltere discs is marginal. However, over-expression of dAkt in the background of down regulation of *ex* caused considerable increase in haltere cell size, equivalent to the degree of change in the size of wing disc cells. $^{**}p = <0.005$.

2006; Makhijani et al., 2007; Crickmore and Mann, 2007), but neither of them is able to restore the wing-type ratio of A/P compartment size.

Over-expression of dAkt, dRheb, $tsc1^{RNAi}$ and ex^{RNAi} in third instar wing and haltere imaginal discs using posterior compartment-specific GAL4 drivers (*en*- and *hh*-GAL4) caused marginal (dAkt) to strong (dRheb, $tsc1^{RNAi}$ and ex^{RNAi}) decrease in the ratio of A/P compartment size in haltere discs, but not in wing discs (Fig. 7A–C). It is likely that there are many compensatory mechanisms to maintain organ size and shape in wing discs. Such dynamically interacting mechanisms may not be operational in haltere discs due to the repression of large number of growth control systems by Ubx. The inability of over-expressed *dally* or *mtv* to restore the size of the posterior compartment to that of the anterior one in earlier reports could be due to regulation of Fat/Hippo and IIS/dAkt pathways by Ubx, which are essential to execute the growth programme set by D/V and A/P morphogens.

Ubx-GAL4 is expressed in the anterior compartment of haltere imaginal discs. We examined the effect of expressing UAS-dAkt, UAS-ex^{RNAi} and UAS-dAkt;UAS-ex^{RNAi} in the anterior compartment using *Ubx*-GAL4 driver on the ratio of A/P compartment size. Expression of dAkt or ex^{RNAi} alone in haltere discs had no effect on the ratio of A/P compartment size (Fig. 7D). These observations suggest that the posterior compartment is more sensitive to changes in the expression levels of Fat/Hippo and IIS/dAkt pathway components than the anterior compartment. Their combined expression, however, did cause increase in A/P compartment ratio (Fig. 7D).

Taken together, the observations reported here suggest that downstream of Ubx, the Fat/Hippo and IIS/Akt pathways are critical to restrict the number, size and shape of haltere cells, which in turn determine the size and shape of the haltere.

3. Discussion

Elucidating the mechanisms that regulate growth to confer an organ a specific size and shape is a fundamental question in developmental biology. Differential development of wing and haltere in *Drosophila* provides an excellent experimental system to study this phenomenon. Wing and haltere are homologous organs originating from identical groups of epithelial cells, but differ greatly in organ size and shape. This differential development is dependent on the function of Ubx (Lewis, 1978), a member of the Hox family of transcription factors. Although Ubx is expressed in the third thoracic segment of all insects studied so far, the function of Ubx to specify haltere by suppressing wing development appears to have evolved only in the dipteran lineage. The precise mechanism of Ubx function, however, is still largely unknown. This is attributable to the fact that *Ubx* regulates a large number of signalling pathways at different levels of their hierarchy.

Our findings reported here suggest that, downstream of Ubx, the Fat/Hippo pathway is critical for haltere specification. It is required for Ubx-mediated specification of organ size, sensory bristle repression, trichome morphology and arrangement. The Fat/Hippo pathway cooperates with the IIS/dAkt pathway, which is also a target of Ubx, in specifying cell size and compartment size in developing haltere. The fact that over-expression of Yki or downregulation of *ex* show haltere-to-wing transformations at the levels of organ size and shape, and trichome morphology and arrangement, suggest that regulation of the Fat/Hippo pathway by Ubx is central to the modification of wing identity to that of the haltere.

The observations made here pose new questions and suggest various interesting possibilities to study the Fat/Hippo pathway with a new perspective.

(i) We have observed that while Yki is nuclear in haltere discs, it appears to be non-functional. Yki is a transcriptional co-activator protein, which requires other DNA-binding partners for its activity. In this context, understanding the precise relationship between Yki and Ubx may provide an insight into mechanism of haltere specification.

(ii) We have observed that the Fat/Hippo pathway (along with the IIS/dAkt pathway) may be involved in the specification of cell size, trichome morphology and their arrangement, all of which are important parameters in determining organ morphology. Recent studies indicate that the Fat/Hippo pathway regulates cellular architecture and the mechanical properties of cells in response to the environment (reviewed in Schroeder and Halder, 2012). It would be interesting to study the role of the Fat/Hippo pathway in regulating the cytoskeleton of epithelial cells during development. Haltere cells at pupal stages exhibit higher levels of F-actin than wing cells (Roch and Akam, 2000). One possible

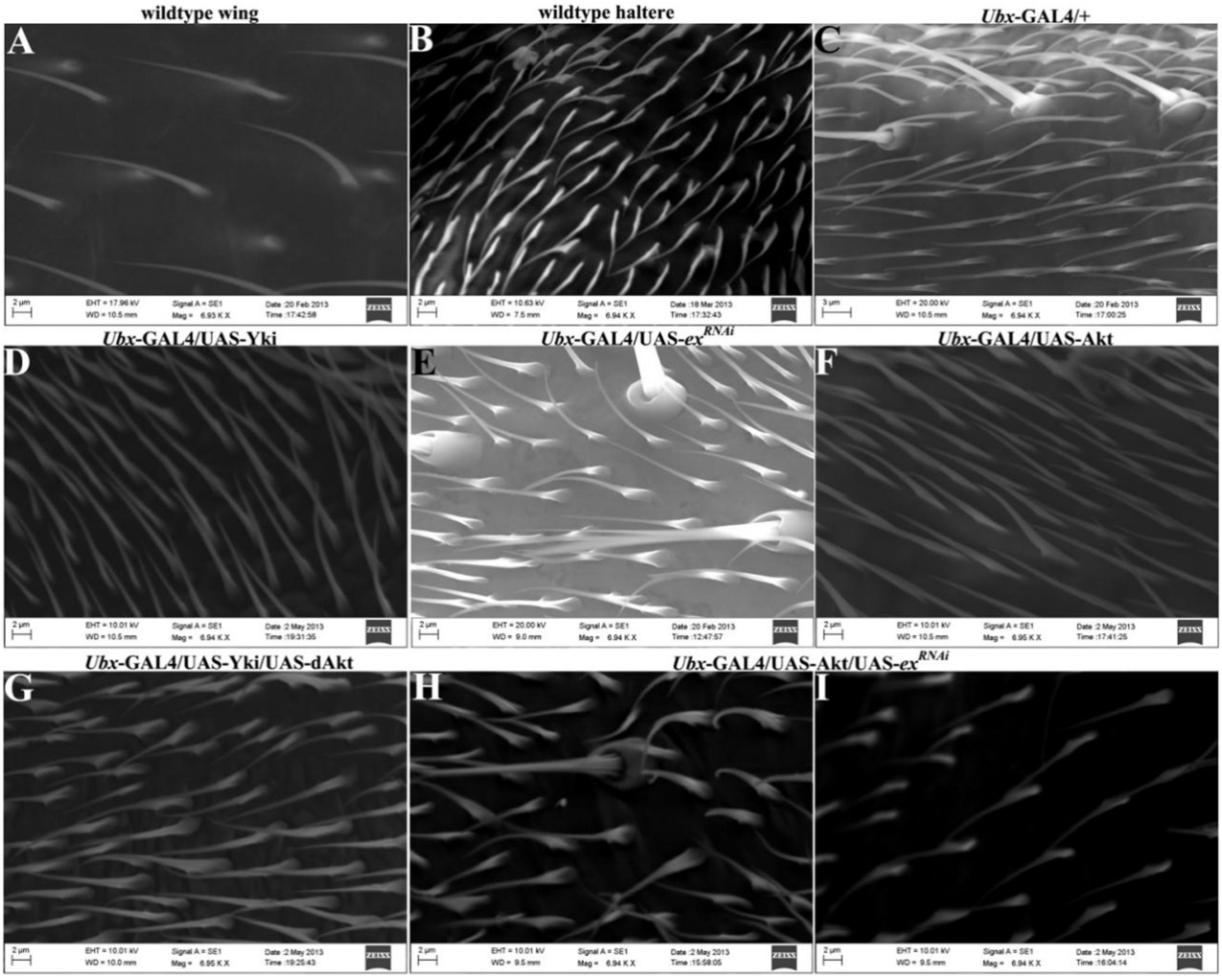

Fig. 6. Upregulation of IIS/dAkt and Fat/Hippo pathways causes haltere-to-wing homeotic transformations at the level of organization of the epithelial cells. (A) SEM image of a representative area of wildtype wing blade. Note, long thin trichomes, which are sparsely arranged (B–I) SEM images of representative halteres of various genotypes as shown on the images. Note near complete transformation of trichome arrangement in halteres over-expressing dAkt in the background of down regulation of *ex*.

mechanism that is currently being investigated is lowering of F-actin levels in transformed haltere cells due to over-expression of Yki or down regulation of *ex*. This may cause flattening of cells during morphogenesis leading to larger organ size.

(iii) Reversing cell size and number was sufficient to induce homeotic transformations at the level of haltere morphology. This suggests the importance of negative regulation of genetic mechanisms that determine cell size and number, in specifying an organ size and shape. As a corollary, Ubx-mediated regulation of Fat/Hippo and IIS/dAkt pathways provides an opportunity to study cooperative repression of cell number and cell size during organ specification.

(iv) Certain genetic backgrounds investigated here showed severe effect on cell proliferation in haltere discs than in wing discs. This could be due to the fact that, the wing disc has already attained a specific size by the third instar larval stage (the developmental stage examined here), which is controlled by several pathways. Any change to this size may need more drastic alteration to the controlling mechanisms. As Ubx specifies haltere by modulating various wing-patterning events, there may still exist a certain degree of plasticity in mechanisms that determine the size of the haltere. However, in absolute terms, the haltere is also resistant to changes in growth control due to regulation by Ubx at multiple levels. Thus, differential development of wing and haltere provides a very good assay system to study not only growth control, but also to dissect out function of important growth regulators (tumour suppressor pathways) such as the Fat/Hippo pathway using various genome-wide approaches.

4. Methods

4.1. General fly maintenance

The required fly stocks for crosses were grown on standard cornmeal–sugar–agar media and were maintained at 25 °C. The wild type strain used during study is Canton-S. All the crosses were set up at 25 °C, unless specified otherwise.

4.2. Fly strains

$Ubx^{6.28}$ is a null allele of *Ubx* and the *Df109* deletion eliminates the *Ubx* gene (Lewis, 1978; Beachy et al., 1985). *pbx* allele causes loss of Ubx expression in the posterior compartment of the haltere disc. We employed the GAL–UAS system (Brand and Perrimon, 1993) for over-expression or knock-down of gene expression.

4.3. GAL4 drivers used in this study

Ubx-GAL4 (Pallavi and Shashidhara, 2003), *omb*-GAL4 (Lecuit et al., 1996), *MS1096*-GAL4 (Capdevila and Guerrero, 1994), *en*-GAL4 (Brand

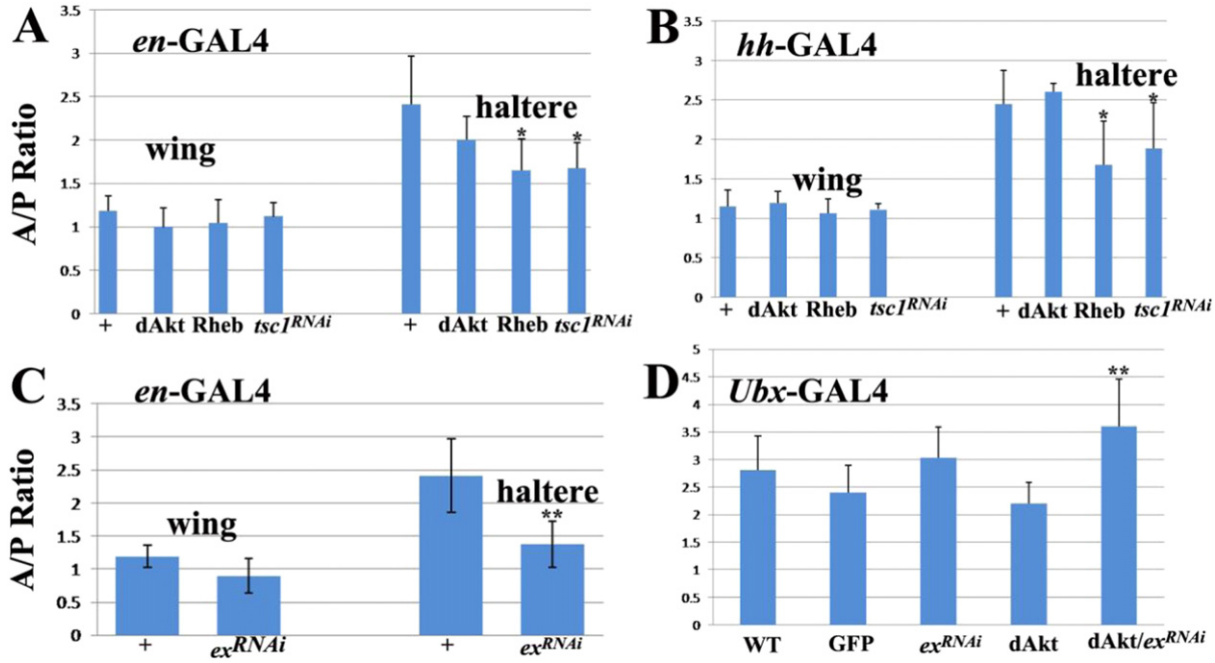

Fig. 7. Growth in the posterior compartment is more sensitive to the changes in the expression of IIS/dAkt and Fat/Hippo pathways. (A–B) Various components of the IIS/dAkt pathway were over-expressed using either *en*-GAL4 (A) or *hh*-GAL4 (B), both of which are specific to the posterior compartment. Bar diagrams show ratio of the size of anterior and posterior compartments in wing and haltere discs of different genotypes as shown on the images. Note reduction in the ratio (means increase in the size of the posterior compartment) when either Rheb is over-expressed or *tsc1* is down regulated. (C) Down regulation of *ex* using *en*-GAL4 also causes similar reduction in the A/P compartment ratio. (D) Neither over-expression of dAkt or down regulation of *ex* in the anterior compartment of the haltere using *Ubx*-GAL4 has no significant effect on the A/P compartment ratio. Only when dAkt is over-expressed in a background of down regulation of *ex*, there was an effect on A/P compartment ratio. $^{**}p = <0.005$.

and Perrimon, 1993), and *hh*-GAL4 (Bloomington stock list; originally developed by Andrea Brand).

4.4. UAS lines used in this study

UAS-Rheb, UAS-dAkt, UAS-4EBP (all from Exelixis), UAS-dAkt (Verdu et al., 1999), UAS-Dp110 (Leevers et al., 1996), UAS-dS6K (Tapon et al., 2001), UAS-Yki (Huang et al., 2005), UAS-Yki-GFP (Oh and Irvine, 2008), UAS-*yki*^{S168A} (Oh and Irvine, 2009), UAS-DIAP1 (Wang et al., 1999), UAS-CycE, UAS-Bantam (from K. Irvine lab), UAS-*dpp* (Bloomington), UAS-*vg* (Kim et al., 1996), UAS-Bantam::sponge (Herranz et al., 2012) and UAS-Ubx.

4.5. UAS-hairpin transgenes used for gene knock-down studies

UAS-*Tsc1*^{RNAi} (TRiP.JF01484)/(TRiP.JF01262), UAS-*ex*^{RNAi,} (TRiP.JF03120), UAS-*hpo*^{RNAi} (TRiP.JF02740), UAS-*wts*^{RNAi} (TRiP.JF02741), UAS-*ds*^{RNAi} (TRiP.JF02842), UAS-*mer*^{RNAi} (TRiP.JF02841), UAS-*ft*^{RNAi} (TRiP.JF03245), UAS *yki*^{RNAi} (TRiP.HMS00041)/(TRiP.JF03119) (KK109756), and UAS-*PI3K*^{RNAi} (TRiP.JF02270).

The *tub*-Gal80^{ts}/Gal4 system (McGuire et al., 2003) was used to temporally control the induction of transgenes with the Gal4-UAS method.

4.6. LacZ and GFP reporter transgenes used in this study

To monitor expression patterns/levels of a given gene, the following reporter transgenes were used: *cycE*-lacZ, *bantam*-lacZ (P{lacW}banL1170a) described in Flybase, *vg*-quadrant enhancer-lacZ (Kim et al., 1996), *expanded*-lacZ (Hamaratoglu et al., 2006), *diap1*-lacZ (Huang et al., 2005), UAS-Bantam-sensor-GFP (Brennecke et al., 2003) and UAS-Nuclear lacZ.

4.6.1. Generation of *Ubx*$^{-}$ clones

Mitotic clones of a null allele of *Ubx* were generated using FLP–FRT method (Xu and Rubin, 1993) using FRT82B *Ubx*$^{6.28}$ (Weatherbee et al., 1998). Clones were generated using hsFLP and Ubi-GFP was used as the clonal marker. Clones were induced by giving heat shock for 1 h at 37 °C during the larval period. Wandering third in-star larvae were dissected after clonal induction. The genotype of the larvae was: y hs-flp122; FRT82B *Ubx*$^{6.28}$/FRT82B Ubi-GFP.

4.6.2. Generation of Ay-Gal4 flip-out clones

Flip-out clones over-expressing a gene of interest (Ito et al., 1997) were generated by crossing UAS-transgene to hs-flp; AyGal4 UAS-GFP. Whenever this experiment was done for the purpose of measuring the influence of gene expression on growth of cells within a clone, hs-flp; AyGal4 UAS-GFP female flies were crossed to males of both UAS-transgene of interest and UAS-nuclear-LacZ in the same vial. The latter was used as control. They were allowed to mate for 48 h and then the females were transferred to a separate vial to lay eggs. Heat shock was given at 60–72 h after AEL for 15 min at 37 °C. Discs were stained with both anti-β-gal (red) and anti-GFP (green) to differentiate between the larval discs bearing test and control clones within the same set of experiments.

4.7. Immunohistochemistry

Immunohistochemistry in larval and pupal discs were essentially as described in Patel et al. (1989). The primary antibodies used were: monoclonal anti-Achaete (1:10; Developmental Studies Hybridoma Bank (DSHB)), anti-Armadillo (1:100; DSHB), anti-β-galactosidase (1:100, Sigma), anti-Engrailed 4D9 (1:200; DSHB), anti-Ultrabithorax (1:30; White and Wilcox, 1985), anti-Wingless (1:1000; DSHB); anti-GFP (1:10,000; Invitrogen), anti-p-Akt 473 (1:100; Cell signalling), antiphospho-4EBP (1:10; Cell signalling), anti-S6k (1:10; Cell signalling), anti-Rheb (1:1000; Abcam), anti-Yki (1:100; a gift from Ginés Morata) and polyclonal anti-Armadillo (1:100; Abcam) and anti-N-terminal Ubx (1:1000; Agrawal et al., 2011).

All secondary antibodies used during this study were all obtained from Invitrogen.

4.8. Fluorescence and confocal microscopy

Fluorescence images were obtained on Zeiss 780 LSM confocal/multiphoton microscope, Zeiss LSM 710 confocal microscope or Zeiss Apotome upright microscope. Images were processed using Zen light software, Axiovision 4.8.2 software or NIH image J. Bright field images of haltere capitellum were obtained with Zeiss Apotome upright microscope.

4.9. Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out on Carl Zeiss EVO LS10 Scanning Electron Microscope Zeiss using Axiovision 4.8.2 software to operate the microscope and for image analysis. Fresh samples of flies were cleaned with 70% ethanol and were directly used for imaging.

4.10. Measurements of anterior::posterior compartment ratio

The posterior compartment of imaginal disc was marked by staining it with Engrailed and the whole disc with armadillo or DAPI. The discs were mounted taking care that they do not lose their morphology. Discs were imaged using a confocal microscope. Surface areas of anterior and posterior compartments were measured using NIH image J. The surface area of compartment was calculated in pixels and then ratio of anterior to posterior compartment was measured. Identical microscope settings were used for all images.

4.11. Measuring surface area of cell

To measure cell size, wing and haltere imaginal disc were stained with Armadillo (Arm) and then the surface area occupied by 10 cells was calculated as the total number of pixels constituting the marked area. Identical microscope settings were used for all images.

4.12. Measuring size of AyGAL4 clones in wing and haltere

Z-stacks of wing and haltere imaginal disc were processed to 3D reconstructions in software of respective confocal microscope. The surface area of clones was outlined using NIH image J programme and the area was estimated as total number of pixels. Identical microscope settings were used for all images.

4.13. Measuring size of haltere capitellum

Bright field Images of adult haltere cuticle were taken using Zeiss Apotome microscope at 10× magnification. Outlines of just the capitellum (the bulbous portion, excluding the stalk) of halteres were traced by converting them to binary and the total pixels were estimated using Image J software.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mod.2015.07.017.

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