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The Dorsocross T-box transcription factors promote tissue morphogenesis in the *Drosophila* wing imaginal disc

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SUMMARY

The *Drosophila* wing imaginal disc is subdivided into notum, hinge and blade territories during the third larval instar by formation of several deep apical folds. The molecular mechanisms of these subdivisions and the subsequent initiation of morphogenic processes during metamorphosis are poorly understood. Here, we demonstrate that the Dorsocross (Doc) T-box genes promote the progression of epithelial folds that not only separate the hinge and blade regions of the wing disc but also contribute to metamorphic development by changing cell shapes and bending the wing disc. We found that Doc expression was restricted by two inhibitors, Vestigial and Homothorax, leading to two narrow Doc stripes where the folds separating hinge and blade are forming. Doc mutant clones prevented the lateral extension and deepening of these folds at the larval stage and delayed wing disc bending in the early pupal stage. Ectopic Doc expression was sufficient to generate deep apical folds by causing a basolateral redistribution of the apical microtubule web and a shortening of cells. Cells of both the endogenous blade/hinge folds and of folds elicited by ectopic Doc expression expressed Matrix metalloproteinase 2 (Mmp2). In these folds, integrins and extracellular matrix proteins were depleted. Overexpression of Doc along the blade/hinge folds caused precocious wing disc bending, which could be suppressed by co-expressing *MMP2RNAi*.

KEY WORDS: *Drosophila* wing, Dorsocross, Fold formation, Metamorphosis, Extracellular matrix

INTRODUCTION

In *Drosophila*, eight T-box genes have been identified (Pflugfelder et al., 1992; Murakami et al., 1995; Brook and Cohen, 1996; Singer et al., 1996; Porsch et al., 1998; Reim et al., 2003; Hamaguchi et al., 2004; Ingham and Placzek, 2006). Dorsocross (Doc), the *Drosophila* Tbx6 subfamily gene cluster, encodes three closely related proteins: Doc1, Doc2 and Doc3, which are essential for proper development of amnioserosa, heart and hindgut (Lo and Frasch, 2001; Reim et al., 2003; Hamaguchi et al., 2004; Reim and Frasch, 2005). The three genes are arranged in a cluster and are expressed in identical patterns in the embryo. They appear to be functionally redundant. In the embryonic dorsal ectoderm, Doc is expressed in the amnioserosa, a specialized epithelium that is required for proper morphogenesis of the embryo during germ band retraction (Reim et al., 2003; Hamaguchi et al., 2004). In the embryonic Malpighian tubules, Doc is required for the execution of an epithelial restructuring process (Hatton-Ellis et al., 2007). Many vertebrate Tbx genes are involved in limb development (King et al., 2006). Doc genes are required for the specification of wing and haltere disc primordia (Hamaguchi et al., 2004). Ectopic *Doc2* expression in the Dpp domain inhibits *wingless* (*wg*) and results in the loss of distal structures of wing, leg and antenna of adult animals (Reim et al., 2003).

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The *Drosophila* wing imaginal disc is subdivided along the proximal-distal axis into the notum, hinge and blade territories (Bryant, 1970; Cohen, 1993; Casares and Mann, 2000; Grieder et al., 2009). Compared with the well-studied wing blade, development of the hinge has not been intensively investigated. The hinge region can be further subdivided into three regions: proximal, intermediate and distal (Klein, 2001). Previous studies have suggested that subdivision of the dorsal hinge is achieved through interactions between adjacent cell populations. The Iroquois complex (Iro-C) homeodomain proteins are expressed in the prospective notum and are required to specify body wall identity. Cells lacking these proteins convert to the hinge fate (Diez del Corral et al., 1999). Importantly, mutual repression between Iro-C and *muscle segment homeobox* (*msh*; *Drop* – FlyBase) has been found to be essential for establishing the boundary between body wall and wing (Villa-Cuesta and Modolell, 2005).

Wg signaling is crucial for intermediate and distal hinge specification, and for the subdivision between pouch and hinge. In the absence of Wg function, neither wing blade nor hinge is specified, leading to a duplication of the notum (Sharma and Chopra, 1976; Morata and Lawrence, 1977). Ectopic activation of the Wg pathway in the wing hinge leads to overgrowth, indicating a mitogenic role for Wg in this region (Neumann and Cohen, 1996). Wg signaling induces different responses in wing hinge and pouch. In response to Decapentaplegic (Dpp), Notch (N) and Wg signaling, Vestigial (Vg) functions as a selector protein, determining wing blade and repressing hinge fate (Klein et al., 1998; Klein and Arias, 1999; Liu et al., 2000). Subsequently, a mutually antagonistic loop between *vg*, *homothorax* (*hth*) and *teashirt* (*tsh*) is crucial for the subdivision into wing blade and hinge. As a hinge Wg target gene, *hth* collaborates with its co-effector *tsh* to specify hinge fate and to repress wing blade fate by repressing *vg* (Azpiazu and Morata, 2000; Casares and Mann, 2000; Soanes et al., 2001; Wu and Cohen, 2002).

Specified cell populations (notum, hinge and blade) are separated by epithelial folds, which are initiated by the apical shortening of cells at the early to mid-L3 stage (illustrated in Fig. 1A). The morphology of the wing disc epithelium is developmentally regulated. During larval development, main disc cells undergo an elongation process caused by restructuring of the cytoskeleton (Widmann and Dahmann, 2009a). At the onset of metamorphosis, main disc cells shorten again and stretch. This, in combination with cell rearrangement and cell division leads to the evaginated form of the pupal wing disc (Fristrom and Fristrom, 1993; Taylor and Adler, 2008).

Here, we found that the distal Doc expression domains in the wing imaginal disc are restricted by two inhibitors, Vg and Hth, and thus are set up as two narrow stripes along the Vg/Hth borders, where two folds form to separate proximal blade from distal hinge [dorsal and ventral blade/hinge (B/H) folds]. Doc is required for B/H fold progression during the third larval instar (L3) and promotes the subsequent wing disc bending during the early pupal stage. Doc causes changes in cell shape with reorganization of the microtubule web and affects the abundance of integrins and extracellular matrix (ECM) components. Mmp2 expression correlates with wild-type wing fold formation and is required for Doc-promoted precocious wing disc bending.

MATERIALS AND METHODS

Drosophila stocks

The mutant allele eliminating all three Doc genes was *Df(3L)DocA* (Reim et al., 2003). The transgenes used were as follows: *tubP-Gal80^{ts}* (McGuire et al., 2003), *ap-Gal4* (Calleja et al., 1996), *dpp-Gal4* (Shen and Mardon, 1997), *30A-Gal4* (Brand and Perrimon, 1993), *UAS-Doc1#F2*, *UAS-Doc2#I2*, *#K3* and *#M2*, *UAS-Doc3#C2* (Reim et al., 2003), *UAS-CD8-GFP* (Lee and Luo, 1999), *UAS-Mmp2* (Page-McCaw et al., 2003), *UAS-Timp*, *UAS-MMP1RNAi*, *UAS-MMP2RNAi* (Uhlirova and Bohmann, 2006), *UAS-vg* (Kim et al., 1996), *UAS-vgRNAi* (Dietzl et al., 2007), *UAS-GFP-hth* (Casares and Mann, 1998), *UAS-hthRNAi* (Brockmann et al., 2010), *UAS-Doc1RNAi*, *UAS-Doc2RNAi* (from VDRC), *UAS-Timp* (Page-McCaw et al., 2003) and flip-in *AYGal4* (Pignoni and Zipursky, 1997). Enhancer trap lines were hth-lacZ:hthP6 (Rieckhof et al., 1997) and vgQE-lacZ (Kim et al., 1996) and the protein trap GFP fusion construct was Viking-GFP (Collagen IV; G00454, http://flytrap.med.yale.edu).

Transgene expression and clone generation

Larvae were raised at 25°C. For efficient expression of RNAi and UAS transgenes driven by the weaker 30A-Gal4, larvae were raised at 29°C. Larvae of genotype *UAS-Doc2/tubP-Gal80^{ts}; dpp-Gal4/TM6B* were raised at 18°C and were then shifted to 29°C for the indicated duration before dissection.

Mitotic recombination was induced using the FLP/FRT system (Lee and Luo, 1999; Xu and Rubin, 1993). Larvae of the genotype *y w hs-flp/y w; FRT2A Ubi-GFP/FRT2A DocA* were subjected to heat shock for 1.5 hours at 38.8°C for the generation of Doc mutant clones. To generate *UAS-Doc2* clones, larvae of genotype *y hs-flp; UAS-GFP; AYGal4/UAS-Doc2* were subjected to 35.5°C for 30 minutes. For *vg* misexpression clones, we subjected larvae of genotype *y w hs-flp; UAS-GFP; AYGal4/UAS-vg* to the same heat shock treatment. To generate *UAS-hth* clones, larvae of genotype *y w hs-flp; AYGal4 /UAS-GFP-hth* (Casares and Mann, 1998) were treated as above.

Immunohistochemistry

Dissected wing imaginal discs were fixed and stained with antibodies according to standard procedures. The primary antibodies used were: rabbit anti-Doc1/2, 1:1000 (Reim et al., 2003); mouse anti-α-Tubulin, 1:2000 (Sigma); mouse anti-α-Integrin, 1:200 (DSHB); mouse anti-β-Integrin, 1:200 (DSHB); mouse anti-Wg, 1:200 (DSHB); mouse anti-Mmp1, 1:200 (DSHB); rabbit anti-Mmp2, 1:400 (Abcam); rabbit anti-Laminin, 1:200 (DSHB); mouse anti-β-galactosidase, 1:2000 (Promega); mouse anti-BrdU, 1:200 (MBL); and rabbit anti-GFP, 1:2000 (MBL). Secondary antibodies used were goat anti-mouse DyLight 488, goat anti-rabbit DyLight 488, goat anti-mouse DyLight 549 and goat anti-rabbit DyLight 549, all 1:200 (Agrisera). Nuclei were stained with DAPI, 1:500 (Sigma). Actin was visualized with Rhodamine-phalloidin, 1:2000 (Sigma). BrdU incorporation was performed according to the manufacturer's specification (AppliChem). Images were collected using a Leica TCS SP2 AOBS confocal microscope.

Wing disc cryosectioning

After secondary antibody staining, discs were refixed for 30 minutes in 4% paraformaldehyde, washed, and stored in 30% sucrose solution at 4°C overnight. Discs were oriented in Tissue-Tek (Sakura Finetek), frozen and cut into 20 μm sections on a cryostat (YD-1900, YIDI, China).

RESULTS

The spatiotemporal expression pattern of Doc genes in the wing disc

The expression pattern of the *Doc1*, *Doc2* and *Doc3* (collectively Doc) genes appears indistinguishable during embryogenesis (Hamaguchi et al., 2004; Reim et al., 2003) and in the wing imaginal disc (Butler et al., 2003). In the late third larval wing disc, Doc is expressed in four distinct areas: two large stripes close to the B/H folds in the wing pouch; one smaller domain in the proximal dorsal hinge close to the anterior-posterior compartment boundary; and one stripe in the posterior lateral notum (Butler et al., 2003; Reim et al., 2003). When we analyzed Doc expression in cryo-embedded wing discs in their natural shape by performing confocal microscopy on *x-z* cryosections, we found that in late third larval discs the two Doc stripes were located in the dorsal and ventral B/H folds (Fig. 1D,D′, arrows). In order to determine whether there was a correlation between Doc expression and B/H fold formation, we examined the expression pattern of Doc at several larval and early pupal stages by anti-Doc and phalloidin or DAPI staining to visualize the folds. Doc was not detectable at 80 hours after egg laying (AEL). The dorsal hinge-internal fold (dorsal H/H) was formed at this stage (Fig. 1B,B′). Doc was detectable from ~85 hours AEL. Doc expression appeared as two narrow stripes at the position where the B/H folds were simultaneously initiated (Fig. 1C,C′, arrows). Subsequently, both Doc stripes extended laterally along with the B/H folds (Fig. 1D, arrows). At the end of larval development, the wing disc started to bend back on itself, the ventral B/H fold moving towards the dorsal B/H fold. Half an hour after puparium formation (APF) (Fig. 1E,E′) and 2 hours APF (Fig. 1F,F′), the ventral B/H fold had progressed further towards the basal side of the dorsal compartment. Wing disc bending was completed at 4 hours APF. The two B/H folds and Doc stripes were now juxtaposed at their basal side (Fig. 1G,G′, arrows). The process of wing bending and the cell shape changes from columnar to cuboidal were also visualized by phalloidin staining (supplementary material Fig. S1). The data show that Doc expression is spatiotemporally correlated with B/H fold progression. This indicates that Doc might play a role in fold progression in L3 and in the subsequent wing disc bending at the early pupal stage.

Doc expression is confined by mutual antagonism between Doc/Vg and Doc/Hth

Wg signaling controls the fate of both blade and hinge via its downstream targets *vg* and *hth* (Klein et al., 1998; Klein and Arias, 1999; Liu et al., 2000). When marking the wing blade by *vg* and the hinge by *hth* expression we observed that the Doc expression domains bordered on the *vg* and *hth* domains (Fig. 2A-B′).

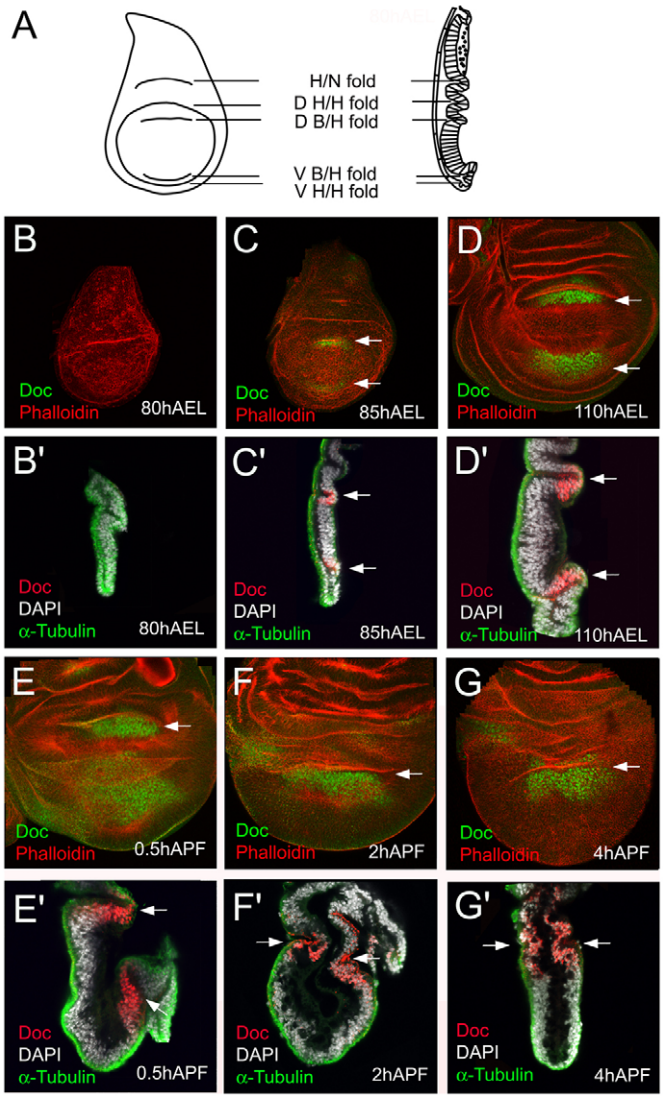

Fig. 1. Spatiotemporal expression pattern of Doc in the *Drosophila* wing disc. In this and subsequent figures, late L3 wing discs of the indicated developmental stage were imaged with a confocal microscope. *x-y* views are oriented with dorsal up and anterior left; cryosections (*x-z* views) along the anterior-posterior (A/P) boundary are oriented with dorsal up and apical left. (B-G) *x-y* views, (B′-G′) *x-z* views of the wing disc cryosections. Phalloidin (red), DAPI (white) and α-Tubulin (green) staining were used to reveal the outline and folds of the wing disc. (**A**) Schematic to illustrate folds in *x-y* and *x-z* views. H/N, hinge/notum; H/H, hinge-internal; B/H, blade/hinge. (**B**,**B′**) Doc was not detectable 80 hours after egg laying (AEL). The dorsal hinge-internal (D H/H) fold was visualized by phalloidin and DAPI staining. (**C**,**C′**) Doc became detectable as two narrow stripes at 85 hours AEL (arrows). The dorsal blade/hinge (D B/H) fold had already formed. The ventral blade/hinge (V B/H) fold was just initiating. There was a spatiotemporal correlation between Doc expression and the formation of the two B/H folds. (**D**,**D′**) Doc stripes are expanded along with a deepening of the B/H folds (arrows) at late L3 (110 hours AEL). (**E**,**E′**) At the early pupal stage [0.5 hours after puparium formation (APF)], the ventral compartment bent towards the basal side of the dorsal compartment. This accounts for the apparent approach of the two Doc crescents seen in the *x-y* view (E). (**F**,**F′**) The ventral B/H fold moved further towards the dorsal B/H fold (arrows) at 2 hours APF. In the *x-y* view the Doc domains appear to have fused (F). (**G**,**G′**) Wing disc bending was complete at 4 hours APF. The two B/H folds and Doc stripes are juxtaposed at their basal sides. See supplementary material Fig. S1 for a whole-disc view of the bending process.

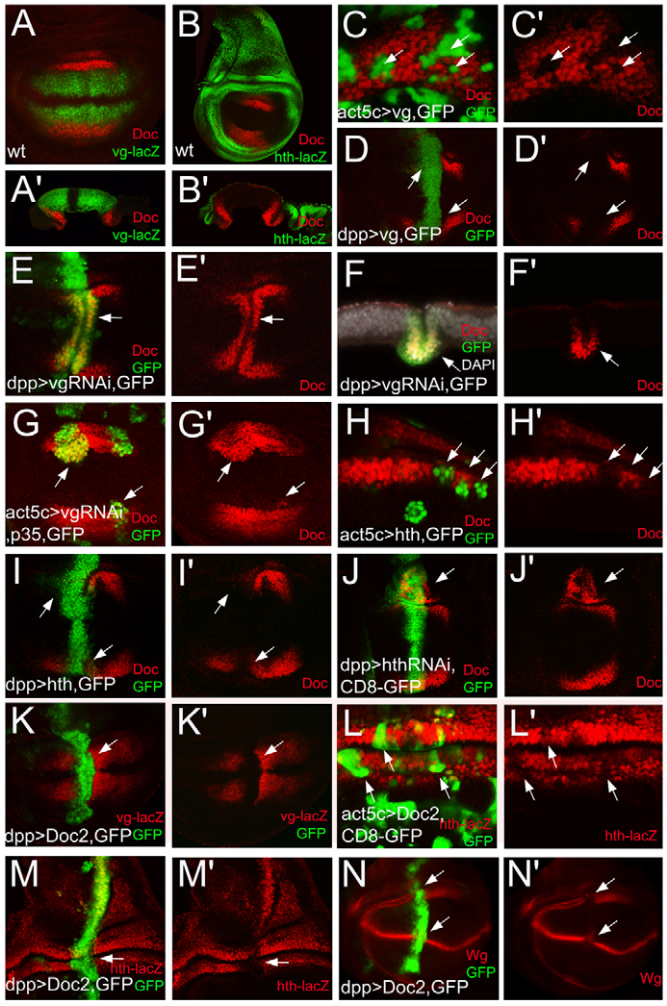

Fig. 2. Doc expression is confined by mutual antagonism between Vg/Doc and Hth/Doc. (A′,B′) Cryosections along the A/P boundary and are oriented with dorsal right and apical up; (F,F′) cryosections along the dorsal-ventral (D/V) boundary and are oriented with apical up and posterior right. (**A**,**A′**) Doc stripes ran along the dorsal and ventral borders of *vgQE-lacZ*. (**B**,**B′**) Doc stripes bordered on the *hth-lacZ* hinge expression domain. (**C**,**C′**) Doc was repressed in clones overexpressing *UAS-vg* (arrows). (**D**,**D′**) Doc was repressed by *UAS-vg* in the dpp-Gal4 domain (arrows). (**E**,**E′**) Doc was activated in the dpp-Gal4 domain when *vg* was repressed by *UAS-vgRNAi* (arrows). (**F**,**F′**) An *x-z* cryosection of dpp>vgRNAi wing disc. (**G**,**G′**) Doc was activated in clones expressing *UAS-vgRNAi*. (**H**,**H′**) Doc was repressed in clones overexpressing *UAS-hth* (arrows). (**I**,**I′**) Doc was repressed by *UAS-hth* in the dpp-Gal4 domain (arrows). (**J**,**J′**) Doc was ectopically activated in the proximal hinge region when *hth* was repressed by dpp>hthRNAi (arrow). (**K**,**K′**) *vgQE-lacZ* was repressed by *UAS-Doc* in the dpp-Gal4 domain (arrow). (**L**,**L′**) *hth-lacZ* was downregulated in *UAS-Doc* clones (arrows). (**M**,**M′**) *hth-lacZ* was repressed by *UAS-Doc* in the dpp-Gal4 domain (arrow). (**N**,**N′**) Wg expression was repressed both in the hinge and the blade stripe by *UAS-Doc* in the dpp-Gal4 domain (arrows).

Therefore, we examined whether Doc is repressed by Vg and Hth. Doc was repressed in cells expressing *UAS-vg* either in clones or in the dpp-Gal4 domain (Fig. 2C-D′). When Vg expression was knocked down by *UAS-vgRNAi* in clones or in the dpp-Gal4 domain, Doc was derepressed (Fig. 2E-G′). These results suggest

that Doc is repressed by Vg in the wing blade region. Ectopic expression of *UAS-hth* either in clones or in the dpp-Gal4 domain caused cell-autonomous Doc repression (Fig. 2H-I′). Hth knockdown by expressing *UAS-hthRNAi* in the dpp-Gal4 domain caused Doc derepression in the hinge (Fig. 2J,J′). These data suggest that *hth* restricts Doc expression in the wing hinge region.

The repression between Doc and Vg/Hth was mutual. When overexpressing *UAS-Doc* in the dpp-Gal4 domain or clonally, both *vg* and *hth* were repressed (Fig. 2K-M′, arrows). Since dpp>UAS-Doc inhibits both blade and hinge *wg* expression (Reim et al., 2003) (Fig. 2N,N′), repression of *vg* and *hth* by Doc could be caused by a decrease in Wg signaling. However, co-expression of *wg* and Doc in the dpp-Gal4 domain still repressed *vg* expression (supplementary material Fig. S2A,A′). Therefore, repression of *vg* by ectopic Doc is a direct action. Repression of *hth* by ectopic Doc was stronger in the distal than in the proximal hinge, but was generally less effective than the repression of *vg* (Fig. 2K-M′, arrows). This might be taken to indicate that repression of *hth* by Doc is indirect.

In conclusion, Doc is restricted to two stripes adjacent to the Vg and Hth expression domains by mutual repression between Doc and Vg and between Doc and Hth. However, Doc loss-of-function clones did not cause ectopic expression of either *vg* or *wg* (supplementary material Fig. S2B-C′), indicating that loss of Doc is not sufficient for the activation of these genes.

Doc is necessary for B/H fold progression

Because of the spatiotemporal correlation between Doc expression and the B/H folds, we analyzed the Doc requirement for fold progression. We reduced overall Doc activity by knockdown of two of the three Doc genes in the dorsal compartment (*ap>Doc1RNAi + Doc2RNAi*). The dorsal B/H fold appeared shallower than in the control (Fig. 3A-B′). Some cases also showed malformation of the hinge, which was not due to the overgrowth effect revealed by BrdU incorporation (Fig. 3B,B′, arrow). The adult wings showed a held-up phenotype with severe hinge defects (supplementary material Fig. S3C,D).

In order to determine Doc requirement for fold progression, we generated Doc null mutant clones in L1 and analyzed the effect at the mid to late L3 stage. Doc mutant clones in a lateral position of the presumed B/H fold prevented lateral fold extension (Fig. 3C,C′). The fold in the area of the clone was shallower than in the adjacent control tissue. This was apparent by a comparison of apical (Fig. 3D) and basal (Fig. 3D′) confocal sections and when discs were inspected in the *x-z* plane, the B/H fold in the Doc mutant clone being shallower than in the neighboring control (Fig. 3E,E′). At 2 hours APF, wing discs with Doc mutant clones covering the B/H fold showed a delay in bending (Fig. 3G; 83% penetrance, *n*=18) compared with the control wing disc without Doc mutant clones (Fig. 3F). The shape of the delayed bending wing discs was similar to that of the 0.5-hour APF wild-type disc (Fig. 1E′). The adult wings showed severe hinge defects with a held-up phenotype (supplementary material Fig. S3E,F). These results suggest that Doc is not required for B/H fold initiation but for their progression, i.e. for lateral extension and basal deepening at the larval stage and for normal wing disc bending progression at the early pupal stage.

In order to determine whether Doc is sufficient to induce fold formation and wing disc bending, we investigated the effects of Doc gain-of-function. When *UAS-Doc2* was ectopically expressed in the dpp-Gal4 domain, it induced a long and deep apical fold in the wing pouch (Fig. 3H-I′). Ectopic co-expression of the

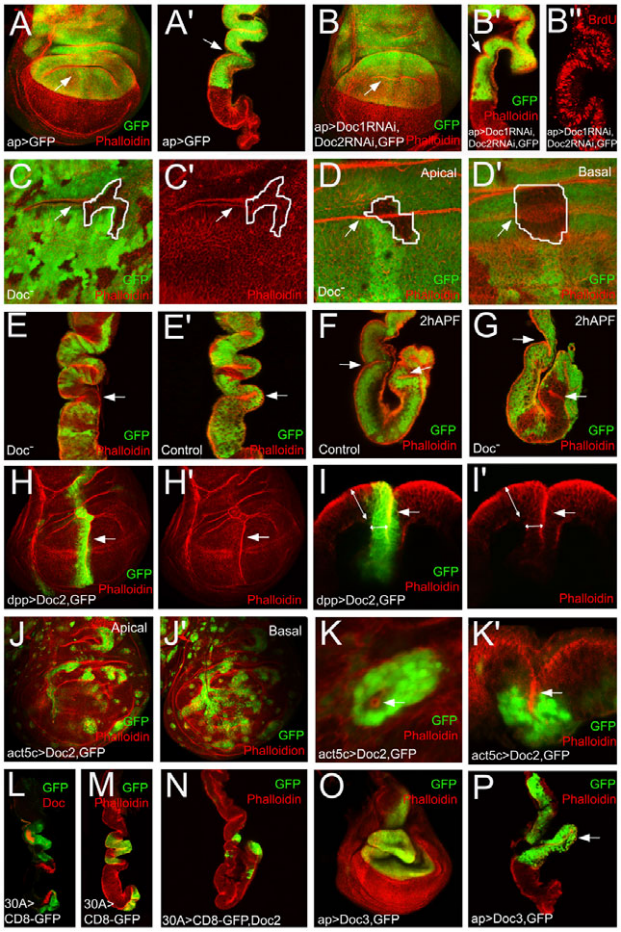

Fig. 3. Doc is necessary for fold progression. (A′,B′,B″,E-G,L-N,P) Cryosections along the A/P boundary of the wing disc; (I,I′,K′) cryosections parallel to the D/V boundary of the wing disc. (**A**,**A′**) ap>GFP wing disc. The arrow points to the dorsal B/H fold. (**B**,**B′**) The dorsal B/H fold was smaller than normal (arrow) when Doc was partly repressed by co-expressing *UAS-Doc1RNAi* and *UAS-Doc2RNAi* in the ap-Gal4 domain. (**B″**) Cryosection of a wing disc stained for BrdU showing uniform proliferation along the proximodistal axis. (**C**,**C′**) The dorsal B/H fold (arrow) failed to extend into a lateral Doc mutant clone (white outline). (**D**) An apical section of a Doc mutant clone located in the central region showed normal fold formation. (**D′**) A basal section of the same clone as in D showing the shallow base of the mutant fold. (**E**) Cryosection through the Doc mutant clone in D revealing the shallowness of the mutant fold (arrow). (**E′**) Parallel *x-z* cryosection through the adjoining wild-type fold. (**F**) Cryosection of a 2-hour APF wing disc without Doc mutant clones. (**G**) Cryosection of a 2-hour APF wing disc with Doc mutant clone covering the B/H fold. (**H**,**H′**) Overexpressing Doc induced a deep fold in the dpp-Gal4 domain (arrow). (**I**,**I′**) Cryosection across the dpp>Doc2-induced fold (arrow) showing that Doc-overexpressing cells are significantly shorter than wild-type columnar cells (double-headed arrows). (**J**,**J′**) Sections close to the apical (J) and basal (J′) side of a wing disc show a predominantly basal localization of Doc-expressing clones (green). (**K**) High magnification of a Doc-overexpressing clone with central enrichment of F-actin (arrow). (**K′**) Cryosection of a Doc-overexpressing clone showing retraction towards the basal membrane (arrow). (**L**,**M**) Cryosection of 30A>GFP late L3 wing discs. Doc and GFP double staining showed that the 30A-Gal4 expression domain lay proximal to the two Doc stripes. (**N**) *Doc2* overexpression in the 30A-Gal4 domain caused precocious wing disc bending already in late L3. (**O**,**P**) Overexpressing *Doc3* within the ap-Gal4 domain induced fusion of the dorsal hinge folds and extremely deep fold formation (arrow).

functionally redundant weaker UAS constructs of *Doc1* and *Doc3* induced similar ectopic folds (supplementary material Fig. S4). When *UAS-Doc2* was ectopically expressed in clones, these clones were localized predominantly in the basal region of the epithelium (Fig. 3J,J′). The clone center formed a ring-like structure marked by F-actin enrichment (Fig. 3K, arrow). When sectioned in an *x-z* plane, the basal retraction of central clone cells was apparent (Fig. 3K′, arrow). In order to test whether Doc-induced fold formation/extrusion is due to loss of Vg, Wg or Dpp signaling activity in the blade region, *vg*, *wg* or tkv^{QD} were co-overexpressed together with Doc. None rescued the Doc-induced phenotype (supplementary material Fig. S5). These data suggest that Doc is sufficient for fold generation.

Ectopic expression of *UAS-Doc2* in the hinge domain using the 30A-Gal4 driver, the expression domain of which lies largely outside of the B/H fold (Fig. 3L,M), caused wing discs to bend already in L3 and to reach a conformation that wild-type discs only attained 2 hours APF (Fig. 3N). Adult wings of 30A>Doc3 flies were fixed in a held-out position, showed a malformation of the proximal hinge and frequently failed to drain hemolymph after wing inflation (supplementary material Fig. S3G,H). Expression of Doc (*UAS-Doc3* or *UAS-Doc1*) in the ap-Gal4 domain caused an extreme invagination (Fig. 3O,P, arrows). The adult wings had a strongly aberrant hinge structure and failed to appose the dorsal and ventral leaflets (supplementary material Fig. S3I-L). Larvae expressing the stronger UAS construct *UAS-Doc2* in the 30A-Gal4 or ap-Gal4 domain already died at an earlier stage. These results show that Doc is required for normal hinge development and is able to promote wing disc bending at the B/H folds.

High Doc levels induce cell shape changes along with a reorganization of the microtubule network

During larval wing disc development, the elongation of main disc cells correlates with the asymmetric distribution of microtubules to an apical position. At the late L3 stage, medial columnar cells had apically enriched microtubule arrays, whereas lateral cells, i.e. the fold cells and the cuboidal cells, had an inverted microtubule distribution. In these cells, microtubules were enriched basolaterally (Fig. 4A,A′). Loss of the apical microtubule web is a common feature of cells undergoing retraction or extrusion caused by inappropriate Dpp or Wg signaling (Gibson and Perrimon, 2005; Shen and Dahmann, 2005; Shen et al., 2008; Widmann and Dahmann, 2009a; Widmann and Dahmann, 2009b).

To explore the process of cell shape change and the cytoskeleton dynamics of Doc-induced fold formation, we used the temperature-sensitive $Gal80^{ts}$ system (McGuire et al., 2003) to temporally control ectopic Doc expression in the dpp-Gal4 domain. After 12 hours of Doc switch on, Doc-overexpressing cells expanded their apical cell diameter and showed loss of apical microtubule enrichment (Fig. 4B-C″). The basal cell diameter at this time had not yet changed (supplementary material Fig. S6A-A″). After 24 hours of Doc switch on, the microtubule web was largely redistributed to the basolateral membrane along with a shortening in cell height, which caused a shallow fold (Fig. 4D-E″, double-headed arrows) (most of the remaining apical α-Tubulin staining is in the overlying peripodial epithelium). Doc-expressing cells widened their basal diameter (supplementary material Fig. S6B-B″). After 48 hours of Doc switch on, cell height was further reduced. Basal cell diameters had increased further, resulting in a deep fold with a similar microtubule distribution as in wild-type B/H folds (Fig. 4F-G″, double-headed arrows; supplementary material Fig. S6C-C″; compare with Fig. 4A′).

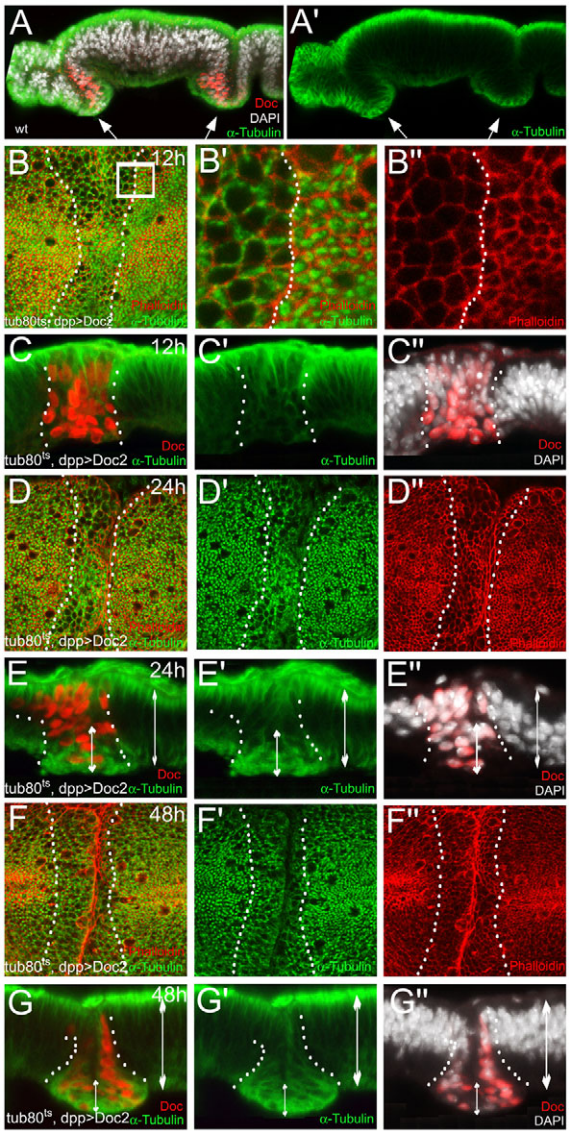

Fig. 4. Doc induces cell shape changes with reorganization of the microtubule network. (A,A′) Cryosections along the A/P boundary of a wing disc oriented with apical up and dorsal right; (C-C″,E-E″,G-G″) cryosections along the D/V boundary of wing disc oriented with apical up and posterior right. (**A**,**A′**) Wild-type B/H fold cells were shortened in cell height and showed a redistribution of the apical microtubule web towards the basolateral side (arrows). (**B-G″**) Conditional Doc expression in the dpp-Gal4 domain by tubP-$Gal80^{ts}$ control 12 (B-C″), 24 (D-E″) or 48 (F-G″) hours after temperature upshift. Dotted lines demarcate the ectopic Doc expression domain. (B-C″) Twelve hours after Doc switch on, Doc cells were apically enlarged and the central enrichment of the apical microtubule web (green) was redistributed to the cellular cortex. The boxed region in B is shown at high magnification in B′-B″. (D-E″) Twenty-four hours after Doc switch on, cells began to shorten and initiated fold formation (double-headed arrows). The microtubule web (green) was redistributed towards the basolateral side. (F-G″) Forty-eight hours after Doc switch on, cell height was significantly reduced resulting in a deep fold (double-headed arrows). The microtubule distribution now appeared similar to that in normal B/H folds (see Fig. 4A′).

These data suggest that the cell shape changes along with the apicobasal redistribution of microtubules are an early event in fold formation induced by ectopic Doc. The microtubule web

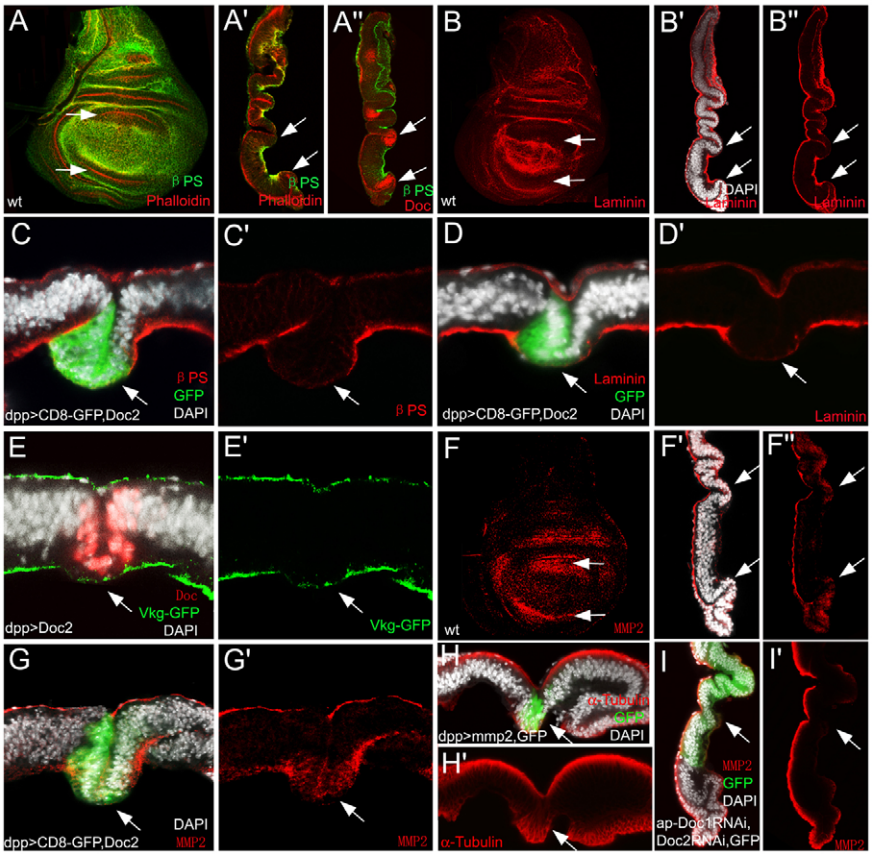

Fig. 5. Doc-overexpressing cells degrade integrin and ECM components. (A,B,F) *x-y* views of whole wing discs; (A′,A″,B′,B″,F′,F″,I,I′) cryosections along the A/P boundary oriented with dorsal up and apical left; (C-E′,G-H′) cryosections along the D/V boundary oriented with apical up. (**A**,**A′**) The β-Integrin level (green) was reduced in wild-type B/H folds (arrows). (**A″**) Doc and integrin double staining showed that the reduction of integrin levels coincided with Doc expression. (**B-B″**) The Laminin level (red) was reduced in wild-type B/H folds (arrows). (**C**,**C′**) The β-Integrin level was reduced in Doc-overexpressing cells (arrows). (**D**,**D′**) The Laminin level was reduced in Doc-overexpressing cells (arrows). (**E**,**E′**) The Vkg-GFP level was reduced in Doc-overexpressing cells (arrows). (**F-F″**) The Mmp2 level was increased in wild-type B/H folds (arrows). (**G**,**G′**) Overexpressing *Doc2* in the dpp-Gal4 domain induced accumulation of Mmp2 (arrow), similar to the situation in wild-type B/H folds. (**H**,**H′**) Overexpressing Mmp2 in the dpp-Gal4 domain induced fold formation and microtubule web redistribution to the basolateral side (arrow). (**I**,**I′**) Repressing Doc in the ap-Gal4 domain reduced the Mmp2 level in the B/H fold (arrow).

redistribution might be required to generate the necessary mechanical force. Microtubules, in addition to their structural role, are required for cellular transport of organelles and proteins and for membrane trafficking (Giannakakou et al., 2000; Caviston and Holzbaur, 2006). Our experiments do not address the mechanism by which the altered microtubule distribution affects cellular shape.

Strong Doc expression causes precocious wing disc bending through degradation of the ECM

Cell migration within the plane of an epithelium normally requires degradation of, or detachment from, the ECM (Chen et al., 2003). Integrins are heterodimeric transmembrane receptors consisting of an α-subunit non-covalently associated with a β-subunit. They link the ECM to the actin cytoskeleton (Gumbiner, 1996; Moser et al., 2009). Integrin-mediated cell-ECM adhesions and an intact ECM are crucial for the maintenance of columnar cell shape (Poodry and Schneiderman, 1971; Chen and Gumbiner, 2006). Disturbing integrin activity or cleaving ECM components induces cells to prematurely adopt a cuboidal morphology in the wing imaginal disc (Dominguez-Gimenez et al., 2007). Overexpression of Doc induced a similar cell shape change. Thus, an altered interaction between Doc-expressing cells and the ECM might be part of the mechanism for fold extension and deepening.

To test this possibility, we analyzed the distribution of ECM components and integrins in the wild-type B/H fold. Staining against the integrin subunits αPS (which is expressed in the dorsal compartment) and βPS (which is ubiquitously expressed) (Brower et al., 1984) showed that integrin was depleted from the basal cell membrane of wild-type folds. In the B/H folds this coincided with Doc expression (Fig. 5A,A″, arrows; supplementary material Fig. S7A,A′). By visualizing one of the main ECM components, Laminin (Fristrom et al., 1993), we found that the concentration of this protein was likewise reduced in the fold compared with the flanking epithelium (Fig. 5B,B″, arrows). Ectopic *Doc2* expression in the dpp-Gal4 domain, which induced a deep ectopic fold, caused a reduction of integrin and of the ECM components Laminin and Collagen IV (vkg-GFP) (Fessler et al., 1993; Morin et al., 2001) (Fig. 5C-E′; supplementary material Fig. S7B,B′). This suggests that remodeling or partial degradation of the ECM is involved in efficient Doc-induced fold formation.

The matrix metalloproteinases (MMPs) are highly conserved enzymes that are able to degrade the ECM (Wojtowicz-Praga et al., 1997; Coussens et al., 2002; Lynch and Matrisian, 2002; Shim et al., 2007; Page-McCaw, 2008). Their activity promotes cellular migration in two- and three-dimensional environments (Brook and Cohen, 1996; Sheetz et al., 1998; Wolf and Friedl, 2009). *Drosophila* has two MMPs: Mmp1 and Mmp2 (Page-McCaw et al., 2003). It has been reported that Mmp1 is expressed in peripodial and stalk cells and both enzymes are required for basement membrane degradation during disc eversion (Srivastava et al., 2007). We examined the expression of MMPs during fold progression. Antibody staining showed that Mmp2 is expressed in wild-type fold cells (Fig. 5F-F″) and in ectopic Doc-induced folds (Fig. 5G,G′, arrows). Although Mmp1 was not detectable in wild-type fold cells, it was expressed in Doc-overexpressing cells (supplementary material Fig. S7C-D′). Upon decreasing the level of Doc in the ap-Gal4 domain, the Mmp2 level was downregulated in the dorsal B/H fold cells (Fig. 5I,I′). Overexpressing Mmp2 in the dpp-Gal4 expression domain induced fold formation along with a

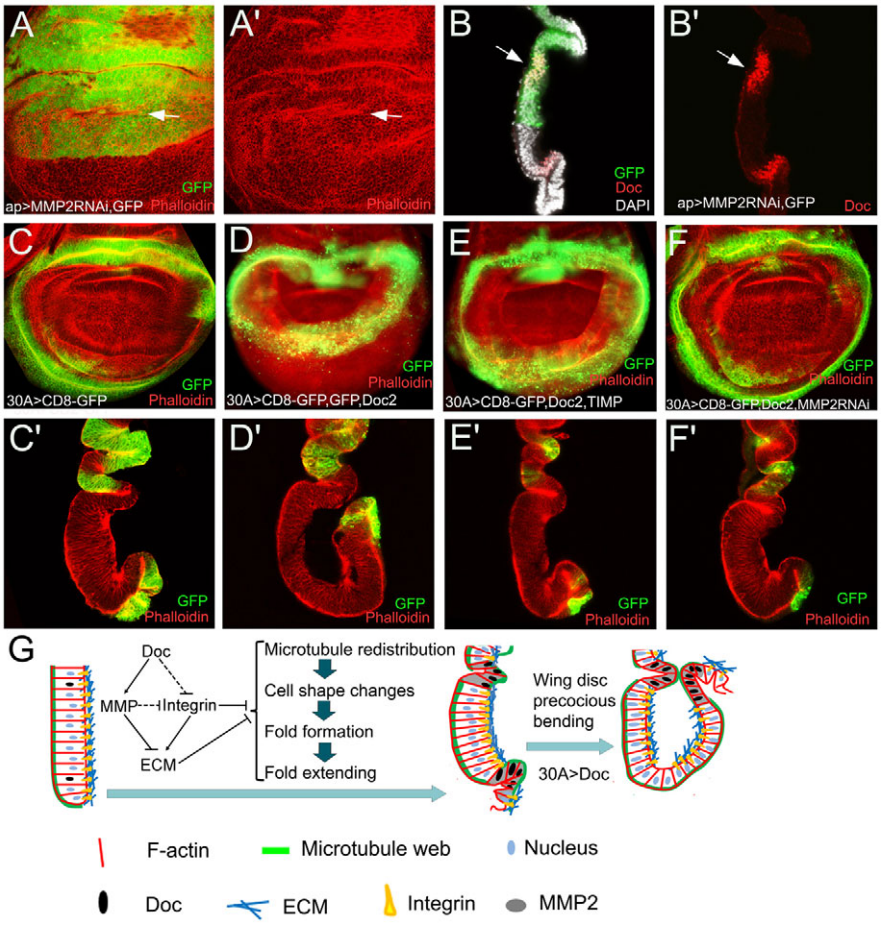

Fig. 6. Overexpressing Doc along the B/H folds promotes wing disc bending through Mmp2-dependent ECM degradation. (B,B′,C′-F′) Cryosections of wing discs along the A/P boundary oriented with dorsal up and apical left. (**A-B′**) Overexpression of *MMP2RNAi* under the control of ap-Gal4 strongly attenuated dorsal hinge fold formation (arrow). (**C**,**C′**) 30A>CD8-GFP wing discs. (**D**,**D′**) Overexpressing *Doc*2 in the 30A-Gal4 domain promoted an early onset of wing disc bending to the pupal shape. (**E**,**E′**) Co-overexpressing Timp with Doc blocked precocious wing disc bending. (**F**,**F′**) This was also observed upon co-overexpression of *MMP2RNAi* with *Doc*2. (**G**) Model of the molecular and cellular dynamics that occur during Doc-promoted B/H fold progression and wing disc bending. Doc-expressing cells enhance MMP activity thereby loosening their contacts with the underlying ECM. Changes in integrin and ECM promote cell shape changes and fold progression, which facilitate subsequent tissue remodeling. Overexpressing Doc along the B/H fold by 30A>Doc is sufficient to induce precocious wing disc bending.

reorganization of the microtubule network (Fig. 5H,H′), mimicking the effect of overexpressing Doc (Fig. 4). When *MMP2RNAi* was expressed in the ap-Gal4 domain to reduce the level of Mmp2, the formation of the dorsal B/H fold was largely suppressed (Fig. 6A), similar to the effect of Doc loss-of-function (Fig. 3B and Fig. 5I). Doc expression was unaffected when Mmp2 was repressed (Fig. 6B,B′, arrow). Thus, Mmp2 is downstream of Doc in mediating cell shape changes and proper B/H fold progression.

To determine whether enhanced MMP function contributed to the precocious wing disc bending in 30A>Doc2 animals, we repressed MMP activity by co-overexpressing the MMP repressor *Timp* (Stetler-Stevenson et al., 1992; Godenschwege et al., 2000), together with Doc under 30A-Gal4 driver control. The Doc-dependent precocious disc bending was suppressed by Timp (Fig. 6E,E′). A similar rescue was achieved by *MMP2RNAi* co-expression (Fig. 6F,F′). Taken together, these data suggest that Doc promotes B/H fold progression and disc bending by MMP-dependent degradation of the ECM.

DISCUSSION

Although the morphogenesis of the *Drosophila* wing disc epithelium has been studied intensively, the molecular mechanisms that tie wing disc subdivision to different fates and subsequent morphogenetic processes remain poorly understood. Here, we demonstrate that the T-box Doc genes take part in fold formation and promote the metamorphic development of the wing disc by controlling cell shape changes and tissue remodeling.

The dynamic Doc expression pattern is regulated by mutual antagonism between Doc /Vg and Doc/Hth

To study the role of Doc in wing disc development, we first analyzed its spatiotemporal expression pattern by antibody staining. By simultaneous recording of Doc expression and fold formation, we found that there was a correlation between Doc expression and B/H fold formation and progression. Doc was not activated until the initiation of the B/H folds, which appeared later than the hinge-internal fold in early L3 (Fig. 1B,C′). During early pupal development, the ventral compartment of the wing disc folds underneath the dorsal compartment, leading to a basal apposition of the ventral and dorsal B/H folds (Fig. 1E-G′). This suggests that Doc plays an important role during these morphogenetic changes.

As in the embryo (Reim et al., 2003; Hamaguchi et al., 2004; Hatton-Ellis et al., 2007), Doc in the peripheral wing pouch is activated by Dpp (Szuperák et al., 2011). The formation of the double-crescent pattern requires, in addition, Doc repression. Previous studies established that the subdivision of the wing disc into notum, hinge and blade regions is attained by the action of Iro-C, Hth/Tsh and Vg in these territories, respectively (Villa-Cuesta and Modolell, 2005). The subdivision of hinge and blade cell fates requires mutual repression between Hth and Vg (Azpiazu and Morata, 2000). Our data revealed that Doc is expressed in the proximal region of low *vg* expression adjacent to the *hth* expression domain (Fig. 2A,B). Ectopic expression of either *vg* or *hth* was sufficient to repress Doc (Fig. 2C-J′). As with the mutual repression between *vg* and *hth*, there was feedback repression

between Doc and *vg* and between Doc and *hth*, ectopic Doc being sufficient to repress both *vg* and *hth* (Fig. 2K-M′). Therefore, the mutual antagonism between Doc/Vg and Doc/Hth defines Doc expression at the two B/H folds.

Doc promotes B/H fold extension by changing cell shape in the wing disc

Doc was not detectable in the wing imaginal disc until the initiation of the B/H folds at ~85 hours AEL. Doc expression coincided in time and space with B/H fold formation (Fig. 1C-D′). Lack of Doc function inhibited B/H fold extension at the larval stage and caused a delay in wing disc bending at the early pupal stage (Fig. 3A-G). When Doc was ectopically expressed in the dpp-Gal4 domain, it was sufficient to generate an apical fold with the same characteristics as the endogenous B/H folds (Fig. 3H-I′)

To explore the mechanism of Doc-controlled cell shape changes and collective cell movement, we examined the distribution of cell adhesion, cytoskeletal and basal membrane proteins. The cell adhesion molecule DE-cadherin is localized at adherens junctions, which maintain the polarized architecture of epithelial cells but limit their movement. Remodeling adhesion to neighboring cells contributes to cell shape changes and cell movement (Lecuit, 2005; Pilot and Lecuit, 2005). We found no obvious effect of Doc overexpression on the distribution of the cell adhesion protein E-cadherin (data not shown). Similarly, the ectopic fold formed at the anterior-posterior boundary of the wing pouch when expression of the Tbx gene *omb* (*bifid* – FlyBase) is reduced, is not associated with an altered distribution of DE-cadherin (Shen et al., 2008). The actin and microtubule cytoskeletons coordinately control cell shape. The elongation of columnar epithelial cells requires the assembly of aligned microtubules that form a diffuse microtubule-organizing center at the apical surface (Meads and Schroer, 1995). Failure of proper microtubule organization causes columnar cells to round up and shorten along their apicobasal axis (Gibson and Perrimon, 2005; Lee et al., 2007). Both in endogenous folds and in folds triggered by ectopic Doc expression, the apical microtubule web was redistributed to a basolateral position, along with an expanded cell diameter and shortened cell height (Fig. 4B-G″). We propose that one mechanism by which Doc causes cell shape changes is by reorganizing the microtubule web.

Mmp2-dependent ECM degradation is crucial for Doc-induced wing fold progression and disc bending

During development, morphogenesis requires the coordination of cell-cell and cell-ECM adhesions, and coordination between molecules involved in these processes is essential for tissue formation and morphogenesis (Chen and Gumbiner, 2006). The degradation of basement membrane barriers is an essential step in cancer invasion (Srivastava et al., 2007; Lukaszewicz-Zajac et al., 2011). Basement membrane modulation also plays an important role during development. A role for extracellular proteolysis in imaginal disc eversion has long been recognized (Fessler et al., 1993). Recently, it was shown that integrin-ECM interactions are necessary to maintain the columnar shape of wing disc epithelial cells (Dominguez-Gimenez et al., 2007). We have shown that overexpression of *Doc2* also causes cell shortening and widening of the cell diameter. The levels of integrin and main ECM components were downregulated in Doc-overexpressing cells (Fig. 5C-E′) and MMP expression was ectopically activated in these cells (Fig. 5G,G′). The same changes were observed in wild-type B/H folds (Fig. 5A-B″,F,F′).

Members of the MMP family are able to degrade most ECM proteins (Rowe and Weiss, 2008). Therefore, the changes in the distribution of ECM proteins and integrins might be secondary to increased MMP activity (Fig. 5G,G′). Mmp2 is downstream of Doc in cell shape control, as overexpressing Doc was sufficient to induce Mmp2 (Fig. 5G) and repressing Doc induced a reduction of Mmp2 in B/H fold cells (Fig. 5I). Manipulation of the Mmp2 level mimicked the effects of Doc on microtubule web redistribution and fold progression (Fig. 5H,H′ and Fig. 6A-B′). Repression of Mmp2 by expressing its inhibitor *Timp* or *MMP2RNAi* efficiently rescued the abnormal wing disc bending induced by 30A>Doc expression (Fig. 6E-F′). Taken together, Doc-expressing cells loosen their contacts with the underlying ECM owing to enhanced MMP activity, and changes in integrin and the ECM promote cell shape changes and facilitate subsequent tissue remodeling.

In the wing pouch bending process, cells at the dorsal-ventral boundary detach from the basal membrane, shorten and acquire a wedge-shaped morphology (Dominguez-Gimenez et al., 2007). This process is likely to contribute to the force that causes the doubling-up of the flat pouch epithelium. Our data show that Doc is required for proper hinge development by deepening the B/H folds of the wing disc (Fig. 3A-F; supplementary material Fig. S3C,D). Doc mutant clones covering the B/H fold cells lead to the delay in wing disc bending at the early pupal stage (Fig. 3G). Overexpression of Doc along the B/H folds elicits precocious wing pouch bending (Fig. 3N). These data indicate that the B/H folds contribute to the bending process in either a passive or active way. The deepening of the B/H folds could provide more pliability during wing disc bending.

Members of the MMP family are involved in tissue remodeling and contribute to cell migration by destroying the ECM and the basement membrane barrier (Rowe and Weiss, 2008). Elevated MMP levels cause cell shape changes and promote cell mobility by disorganizing the normal tissue architecture (Bourboulia and Stetler-Stevenson, 2010). Doc-expressing B/H fold cells have increased MMP levels, causing enhanced ECM degradation. This might facilitate the concerted migration of fold cells. Ectopic Doc expression in the central wing pouch induced abnormal cell migration, both in the plane and out of the plane of the epithelium (data not shown). Doc could contribute to the wing disc bending process by promoting the movement of B/H fold cells, possibly emulating its role in amnioserosa development.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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