Cell death and selective adhesion reorganize the dorsoventral boundary for zigzag patterning of Drosophila wing margin hairs

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A B S T R A C T

Animal tissues and organs are comprised of several types of cells, which are often arranged in a well-ordered pattern. The posterior part of the Drosophila wing margin is covered with a double row of long hairs, which are equally and alternately derived from the dorsal and ventral sides of the wing, exhibiting a zigzag pattern in the lateral view. How this geometrically regular pattern is formed has not been fully understood. In this study, we show that this zigzag pattern is created by rearrangement of wing margin cells along the dorsoventral boundary flanked by the double row of hair cells during metamorphosis. This cell rearrangement is induced by selective apoptosis of wing margin cells that are spatially separated from hair cells. As a result of apoptosis, the remaining wing margin cells are rearranged in a well-ordered manner, which shapes corrugated lateral sides of both dorsal and ventral edges to interlock them for zigzag patterning. We further show that the corrugated topology of the wing edges is achieved by cell-type specific expression and localization of four kinds of NEPH1/nephrin family proteins through heterophilic adhesion between wing margin cells and hair cells. Homophilic E-cadherin adhesion is also required for attachment of the corrugated dorsoventral edges. Taken together, our results demonstrate that sequential coordination of apoptosis and epithelial architecture with selective adhesion creates the zigzag hair alignment. This may be a common mechanism for geometrically ordered repetitive packing of several types of cells in similarly patterned developmental fields such as the mammalian organ of Corti.

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Introduction

Morphogenesis and patterning of tissues and organs are regulated by the combination of cell proliferation, cell death, cell shape change, and cell rearrangement. Understanding how these cellular events are coordinated is a goal of developmental biology.

Drosophila is a genetically tractable model system well suited for studying the underlying molecular mechanisms of morphogenesis and patterning. The Drosophila wing margin is covered with hundreds of sensory bristles and non-innervated hairs, both of which are located in a reproducible pattern (Hartenstein and Posakony, 1989; Palka et al., 1979). In the proximal region of the anterior wing margin (anterior to wing vein L2), a triple row of bristles comprising several chemosensory and mechanosensory bristles aligns. The rest of the wing margin is covered with a double row comprising chemosensory and mechanosensory bristles in the L2–L3 region, and non-innervated hairs in the region posterior to L3 (Figs. 1A, B). These posterior wing margin hairs are aligned in two rows equally and alternately derived from the dorsal and ventral sides to form an elaborate zigzag pattern in the lateral view, which probably affects airflow over the surface of the wing during flight.

Specification and development of the bristle precursors on the Drosophila thorax have been well studied (Lai and Orgogozo, 2004). Within an epithelial field, groups of adjacent cells called proneural clusters, which express low levels of proneural genes such as acute and scute, acquire neural potential. Subsequently, a single sensory organ precursor (SOP) in each proneural cluster is selected. The underlying mechanism of this process is Notch-mediated lateral inhibition, in which prospective bristle precursors prevent neighboring cells from differentiating into bristle precursors by activating Notch signaling in those cells. This lateral inhibition controls the number and spacing of the SOP. The SOP then undergoes a series of asymmetric cell divisions, producing the components of sensory bristles including a shaft, socket, sheath, glial cell, and neuron.

In the larval wing disc, the expression of proneural genes is induced by Wingless (Wg) signaling along the dorsoventral (DV) boundary, which is the prospective wing margin (Couso et al., 1994; Phillips and Whittle, 1993). During metamorphosis, the wing disc evaginates, and the dorsal and ventral parts of the wing adhere to

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alignment. In *Drosophila* change could be involved in the zigzag alignment of hair cells in the margin cells. Wing cells are irregularly packed during the larval and wing has not been fully investigated.

Different perspectives. (D) Lateral view of the pupal wing margin at 30 h APF, showing hair cells (neur-GAL4/UAS-GFP, magenta) and inter-hair cells (wg-lacZ, green). Scale bars: 500 μm in A; 50 μm in B; and 10 μm in D.

Fig. 1. The *Drosophila* posterior wing margin consisted of long hairs and wing margin cells. (A) An adult wing showing longitudinal veins L2 and L3. Anterior is to top. A red dashed line indicates the anteroposterior compartment boundary. (B) Higher magnification view of the rectangular region in A. (C) Transverse section reconstructed from stacks of images of the pupal wing at 30 h APF. Phalloidin staining revealed dorsal and ventral wing blade epithelia and the protrusion of hair shaft cells (arrowheads). Wing margin cells (wg-GAL4/UAS-GFP) are shown in green. Yellow arrows show two different perspectives. (D) Lateral view of the pupal wing margin at 30 h APF, showing hair cells (neur-GAL4/UAS-GFP, magenta) and inter-hair cells (wg-lacZ, green). Scale bars: 500 μm in A; 50 μm in B; and 10 μm in D.

each other, forming an epithelial bilayer (Fristrom et al., 1993) (Fig. 1C). In the posterior wing margin, all the hair precursors, which is the equivalents of SOPs, are specified by 18 h after puparium formation (APF) (Hartenstein and Posakony, 1989). However, how differentiated hair cells (shaft and socket cells), which are daughters of hair precursors, establish the zigzag pattern observed in the adult wing has not been fully investigated.

One possible mechanism is a change in shape of pupal wing margin cells. Wing cells are irregularly packed during the larval and prepupal stages but their ordered hexagonal packing becomes established by 30 h APF (Clasen et al., 2005). A similar cell shape change could be involved in the zigzag alignment of hair cells in the pupal wing margin.

Apoptosis is another plausible cellular event refining the hair alignment. In *Drosophila*, apoptosis is involved in many processes of morphogenesis and patterning during metamorphosis. In the compound eye comprising 750–800 ommatidia, unnecessary interommatidial cells are removed by apoptosis, resulting in well-ordered hexagonal packing of the ommatidia (Brachmann and Cagan, 2003). In addition, apoptosis eliminates the ommatidia with incomplete sets of photoreceptor cells around the edge of the retina (Lin et al., 2004). Apoptosis is also involved in morphogenesis of the terminal segment of the antenna (He and Adler, 2001) and the leg joint (Manjon et al., 2007). In the pupal wing, apoptotic cells are observed along the anterior wing margin from 20 to 26 h APF (Aigouy et al., 2004), although their spatial pattern and role have not been firmly established. Since pupal wing cells are postmitotic by 24 h APF (Milan et al., 1996; Schubiger and Palka, 1987), the occurrence of apoptosis could affect the patterning of wing margin hairs.

In this study, we investigated the underlying mechanisms of the zigzag positioning of posterior wing margin hairs and found that a subset of wing margin cells, except for the cells neighboring the hair cells, undergo apoptosis. This apoptosis induces a rearrangement of the remaining wing margin cells, resulting in the zigzag hair alignment. We also found that EGFR signaling is involved in the selection of the wing margin cells destined to survive. Blocking EGFR signaling results in an extra apoptosis, while ectopic activation of EGFR signaling inhibits normal apoptosis, indicating that EGFR signaling is both necessary and sufficient for survival of wing margin cells. Moreover, several adhesion molecules such as *Drosophila* E-cadherin and NEPH1/nephrin homologs are involved in the elaborate cell patterning after apoptosis through well-designed selective adhesion between cell types.

Materials and methods

Fly strains

The following lines were used: UAS-GFP<sup>CGS7</sup>, UAS-mCD8::GFP<sup>L5</sup>, UAS-p35, UAS–E-cad<sup>DN</sup>, UAS–Ras85D<sup>V12</sup>, UAS–EGFR<sup>DN</sup>, Tubulin–GAL80<sup>ts</sup>, neur-GAL4<sup>1011</sup>, ap–GAL4<sup>md44</sup>, wg–lacZ<sup>2657</sup>, vn–lacZ<sup>10567</sup>, and UAS–tcf<sup>DN</sup> were obtained from the Bloomington *Drosophila* Stock Center; sd–GAL4, NP2736–GAL4 and NP2044–GAL4 were obtained from the *Drosophila* Genetic Resource Center (Kyoto, Japan); rp298–lacZ was obtained from Akina Nose; sns–lacZ was a gift from Susan Abmayr; hbs–lacZ was provided by Mary Baylies; wg–Gal4<sup>5556</sup> is a newly isolated GAL4 enhancer-trap allele of wg, which shows an authentic expression pattern as seen in other enhancer-trap alleles of wg; and UAS-RNAi lines (*Egfr* #107130, DE-cad #103962, kirre #109585, rst #27223, sns #109442, hbs #105913) were obtained from the Vienna *Drosophila* RNAi Center (Vienna, Austria).

Ectopic gene expression with GAL80<sup>ts</sup>

A forced and temporal expression system in the wg-expressing wing margin cells, which is referred to here as wg–GAL4<sup>ts</sup>, was composed of wg–GAL4, UAS–mCD8::GFP, Tubulin–GAL80<sup>ts</sup>, and any of the UAS transgenes. The animals were raised at 18 °C (permissive temperature) and shifted to 29 °C (restrictive temperature) at puparium formation. At 29 °C, constitutively expressed GAL80<sup>ts</sup> was inactivated, thereby activating GAL4, which mediated the UAS-transgene expression (McGuire et al., 2003). The shape of the cells where the gene expression was induced was visualized by simultaneous induction of a membrane-targeted GFP (mCD8::GFP) (Lee and Luo, 1999). The pupal developmental stages are expressed here in hours APF with white pre-pupae defined as 0 h APF. The pupae were dissected 25 h later, the time point equivalent to 30 h APF at 25 °C. Here, pupal wing age (developmental stage) will be expressed in hour equivalents at 25 °C.

Immunohistochemistry

Pupal wings were fixed in 4% paraformaldehyde for 15 min at room temperature and washed in PBT. The following antibodies (and dilution) were used: mouse anti-Hnt (1:20), mouse anti-Wg (1:20), and rat anti-DE-cad (1:50) were obtained from the Developmental Studies Hybridoma Bank (Iowa, USA); mouse anti-Ig-Galactosidase (1:100; Promega); Rabbit anti-Kirre (1:400), mouse anti-Rst (1:400), rabbit anti-Sns (1:400), and rabbit anti-Hbs (1:400) were kindly provided by Karl-Friedrich Fischbach. The secondary antibodies used were anti-mouse IgG with Alexa Fluor 555 (1:200; Molecular Probes), anti-rabbit IgG with Alexa Fluor 555 (1:200; Molecular Probes), anti-mouse IgG with Cy5 (1:200; Jackson ImmunoResearch), and anti-rat IgG with Cy5 (1:200; Jackson ImmunoResearch). F-actin was labeled by TRITC-conjugated phalloidin (1:500, Sigma). Pupal wings were mounted in VECTASHIELD mounting medium (Vector Laboratories). Images were collected with Nikon Digital Eclipse C1 and C1Si confocal microscopes (Nikon), Z-stacks of images were processed with ImageJ (NIH) and FluoRender software (Wan et al., 2009).

TUNEL assay

Apoptotic cells were labeled by the TUNEL method using the ApopTag Red kit (Chemicon) following the manufacturer’s instructions.

Adult wing preparation

Adult wings were dissected and mounted in Canada balsam/ methyl salicylate (1:1). Bright-field images were collected with a Keyence VHX-100 digital microscope.
Results

Apoptosis occurs along the pupal wing margin during early metamorphosis

First, to examine the spatiotemporal pattern of apoptosis in the pupal wing, we labeled apoptotic cells at several developmental stages using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay, which detects the fragmentation of nuclear DNA. At 20 h APF, TUNEL-positive pyknotic nuclei were observed along the wing margin (Fig. 2A). At 24 h APF, TUNEL-positive nuclei were similarly observed (Fig. 2B) although the apoptotic cell populations were different between the two stages. The details of this will be described in a later section. The number of TUNEL-positive nuclei decreased between 26 and 28 h APF (data not shown). At 30 h APF, few TUNEL-positive cells were detected along the wing margin (Fig. 2C). Forced expression of anti-apoptotic protein p35 (Hay et al., 1994) under the control of scalloped (sd)-GAL4strong driver (Adachi-Yamada et al., 2005) in the pupal wing resulted in loss of TUNEL-positive nuclei at 24 h APF (Fig. S1 in Supplementary material), indicating that the TUNEL signal is specific to apoptotic cells. These results show that apoptosis in the wing margin occurs at least from 20 h APF and has ceased by 30 h APF.

Two different types of cells undergo apoptosis at different stages

Next we identified the types of cells that underwent apoptosis in the posterior wing margin. There are two main types of cells in the posterior wing margin. One is a double row of hair cells (Fig. 1D), which are labeled by neuralized (neur)-GAL4 expression (Huang et al., 1991; Jhaveri et al., 2000). The other is wg-expressing cells along the DV boundary, which we refer to here as wing margin cells (Fig. 1D). At 20 h APF, the majority of TUNEL-positive pyknotic nuclei, which were observed on the basal side of the wing epithelium, were marked by the neur-GAL4 expression (Fig. 2D). This indicates that some cells in hair precursor lineage undergo apoptosis and basally extruded. Because posterior wing margin hairs are composed of only shaft and socket cells (Hartenstein and Posakony, 1989), the remaining components have been thought to undergo apoptosis during development (Lai and Orgogozo, 2004). In fact, blocking apoptosis in the wing resulted in ectopic appearance of neuron, sheath, and glial cells (Jafar-Nejad et al., 2006; data not shown). These results suggest that these cells are removed by apoptosis at around 20 h APF.

We next examined the apoptosis observed at 24 h APF. At this point, TUNEL signal was observed between the double row of hair cells in the lateral view (Fig. 2E), where wg-expressing wing margin cells are located (Figs. 1C, D). We then investigated whether the wing margin cells, which were marked by wg-GAL4 expression, underwent apoptosis. We found that all the TUNEL-positive nuclei expressed wg-GAL4 (Fig. 2F). As in the apoptosis of cells in hair precursor lineage at 20 h APF, TUNEL-positive nuclei were observed on the basal side of the epithelium (Fig. 2G). Taken together, these results demonstrate that two different populations of cells undergo apoptosis with just 4 h difference. First, some of the hair precursor lineage cells are selected to die. Second, shortly after that apoptosis, some wing margin cells undergo apoptosis. Since the partial loss of wing margin cells seems to affect the patterning of the remaining ones, we further investigated the apoptosis of wing margin cells in later sections.

Selective removal of wing margin cells leads to cell rearrangement

At 30 h APF, when apoptosis was no longer detected in the wing margin (Fig. 2C), the wg-positive wing margin cells were still...
observed (Fig. 3B), indicating that not all wing margin cells undergo apoptosis. We then identified the subset of wing margin cells eliminated through apoptosis by comparing the states of the cells before and after apoptosis.

At 20 h APF, before apoptosis of the wing margin cells, we observed two morphologically distinct types of wing margin cells on the basal plane (Fig. 3H) in cross section: (i) dorsal and ventral single rows of triangular “inter-hair cells” (Fig. 3A, asterisks), which were aligned alternately with the hair cells marked by anti-Hindsight (Hnt; Pebbled — FlyBase) antibody, and (ii) two or three rows of “flat cells” between the two rows of the inter-hair cells and hair cells along the DV boundary (Figs. 3A, C). However, at 30 h APF, after apoptosis of wing margin cells, the flat cells were not observed (Fig. 3B). Interestingly, the surviving inter-hair cells were aligned in a geometrically ordered zigzag manner by alternate positioning of the dorsal and ventral cells (Figs. 3B, D). Blocking apoptosis by p35 expression using wg-GAL4 resulted in survival of the flat cells and no zigzag alignment of the wing margin cells (Fig. 3E). This means that apoptosis is necessary for cell rearrangement and the resultant well-ordered organization of the wing margin cells.

Apoptosis is required for correct alignment of wing margin hairs

This apoptosis-induced rearrangement of the wing margin cells simultaneously implies rearrangement of the hairs. We thus compared the alignment of the hair shafts between the wild-type and p35-expressing wings. We blocked apoptosis in the wing margin cells by expressing p35 under the control of wg-GAL4 driver. To visualize the position of the shafts in the pupal wing, we stained F-actin, which is concentrated in the extrusion of shaft cells, using TRITC-conjugated phalloidin. In the wild-type posterior wing margin at 30 h APF, the double row of hair shafts derived from the dorsal and ventral sides was aligned in a zigzag manner in the lateral view: each hair shaft was located midway between two hair shafts in the other row (Fig. 3G). In the pupal wing expressing p35, however, the relative positions of the hair shafts were not ordered: some were aligned in an almost side-by-side manner by chance (Fig. 3F). Consistent with these developmental features, in the adult wild-type posterior wing margin, the hairs showed an alternate projection from the dorsal and ventral wing sides with even intervals from the en face view (Fig. 3J). In contrast, in the adult wing expressing p35, the alternate projection of the hairs was

![Fig. 3. Selective removal of wing margin cells leads to cell rearrangement for zigzag hair alignment. (A–E) Close-up images of the posterior wing margin from the lateral view showing wing margin cells by the expression of membrane-tethered GFP (wg-GAL4/UAS-mCD8::GFP) (A, B, E) or wg-lacZ (C, D) in green. (A) At 20 h APF, before apoptosis of wing margin cells, there are two–three rows of morphologically flat cells and two rows of inter-hair cells (asterisks) between hair cells (anti-Hnt antibody, magenta). (B) At 30 h APF, after apoptosis of wing margin cells, flat cells are not observed. Surviving inter-hair cells (asterisks) are aligned in ordered zigzag pattern. (C) Flat cells straddled the DV boundary. The dorsal compartment is shown in magenta (ap-GAL4/UAS-GFP). (D) One row of inter-hair cells is derived from the dorsal compartment (ap-GAL4/UAS-GFP, magenta), and the other row is from the ventral compartment. (E) Blocking apoptosis by p35 expression rescues flat cells at 30 h APF. (F, G) Lateral views of the posterior wing margin at 30 h APF showing F-actin (TRITC-conjugated phalloidin, magenta) in hair shafts. (F) In the papal wing expressing p35 by wg-GAL4, two rows of hair shafts are often aligned in an almost side-by-side manner. (G) In the wild-type pupal wing, two rows of hair shafts are aligned in a zigzag manner. (H) Schematic drawing of the transverse section of the pupal wing margin at 30 h APF showing hair cells (shaft and socket cell, magenta), inter-hair cells (dark green), and tooth cells (light green). Dashed lines in blue and red indicate the apical and basal focal planes, respectively. (I–J) Close-up views of the posterior wing margin of adult wings from the en face view. (I) In the adult wing expressing p35 by wg-GAL4, two rows of hairs overlapped (asterisks). (J) In the control wing (wg-GAL4/+), hairs are positioned at even intervals. Scale bars: 10 μm in A–E; 20 μm in F, G; and 50 μm in I, J.](#)
disturbed, and hairs from the dorsal and ventral compartments overlapped (Fig. 3I, asterisks). Blocking apoptosis also affected the position of sensory bristles in the anterior wing margin (Fig. S2 in Supplementary material), although the phenotypic relationship between the anterior and posterior wing margins was unclear. Taken together, these results indicate that apoptosis in the wing margin cells is required for correct hair patterning.

**Ecdysone signaling is necessary for triggering apoptosis**

Ecdysone, an insect steroid hormone required for progression in most of the developmental stages, has been reported to trigger various apoptotic events associated with metamorphosis through its binding with ecdysone receptor (EcR) (Yin and Thummel, 2005). To investigate whether ecdysone is also responsible for inducing the apoptosis of flat cells, we expressed a dominant-negative form of EcR (EcR\textsuperscript{DN}) to block ecdysone signaling. Induction of EcR\textsuperscript{DN} during 0–30 h APF blocked apoptosis and rearrangement of the wing margin cells (Fig. S3A in Supplementary material). In addition, blocking ecdysone signaling resulted in overlap of the double-row hairs in the adult wing (Fig. S3B in Supplementary material, asterisks), similar to the result of apoptosis inhibition by p35 expression (Fig. 3I). These results indicate that ecdysone signaling is required for triggering apoptosis of wing margin cells, as it does for other types of morphogenesis-dependent apoptosis in *Drosophila* and other insects (Fujiwara and Oğuz, 2001).

**Spatial pattern of EGFR signaling activation is correlated with cell survival in wing margin cells**

We next examined how one subset of wing margin cells is removed by apoptosis while the other can survive, and found that EGFR signaling shows an interesting activation pattern. We observed that vein (vn), which encodes a secreted ligand of EGFR, was specifically expressed in the hair cells at 20 h APF, as revealed by the expression of lacZ enhancer-trap insertion, vn-lacZ (Fig. 4A). In addition, the expression of sprouty (sty), a target gene of EGFR signaling in the wing, was observed in cells neighboring the hair cells by using *NP2736-GAL4*, an enhancer-trap line of sty (Fig. 4B), indicating that EGFR signaling is activated in these cells. While autocrine activation of EGFR signaling was previously reported for wing vein cells (Blair, 2007), EGFR signaling in the wing margin acts in a paracrine manner, as observed in other tissues (Shilo, 2005). We further investigated the spatial activation pattern of EGFR signaling in wing margin cells. We found that the inter-hair cells were sty-positive on the basal plane (Fig. 4C, asterisks) while most of the flat cells that spatially separated from the hair cells were sty-negative (Fig. 4C). These results indicate that the activation pattern of EGFR signaling correlates with the cell survival pattern in wing margin cells.

**EGFR signaling affects survival of wing margin cells**

Accordingly, we investigated whether EGFR signaling is required for cell survival in wing margin cells. We inhibited the activity of EGFR signaling in wing margin cells by inducing RNAi-mediated knockdown of *Egr* using the wg-GAL4\textsuperscript{40}, an enhancer-trap line of *Egr* in wing margin cells resulting in an extra apoptosis even at 30 h APF (Figs. 4E, G), while few apoptotic cells were observed in the control wing at this stage (Figs. 4D, F). As a result of the apoptosis, some inter-hair cells were lost (Fig. 4I, arrows). Similar phenotypes were observed in a wing expressing a dominant negative form of EGFR (data not shown). These results indicate that EGFR signaling is required, at least in part, for cell survival in wing margin cells. We next examined whether EGFR activation is sufficient to prevent the flat cells from dying. To ectopically activate EGFR signaling, we induced Ras\textsuperscript{N17}, a constitutively active form of its downstream activator Ras (Ras85D—FlyBase) (Karim and Rubin, 1998). As a result, the flat cells remained without undergoing apoptosis (Fig. 4J), indicating that EGFR signaling is sufficient for cell survival in wing margin cells. Taken together, these results indicate that EGFR signaling affects cell survival in wing margin cells and is thus involved in selective removal of these cells (Fig. 4K).

**DE-cadherin is required for the epithelial architecture of the wing margin**

The results described above show that the removal of some of the wing margin cells induces cell rearrangement, which leads to zigzag hair alignment. To achieve such an ordered pattern during rearrangement, the cell shape and topology of the remaining wing margin cells must be regulated. The cell shape and topology are determined by the property of adhesion with neighboring cells at the adherens junctions. *Drosophila* E-cadherin (DE-cad) (Shotgun—FlyBase), which is a main component of adherens junctions, binds in a homophilic manner and is necessary for the distinctive hexagonal morphology of wing intervein cells and hollow structures of wing vein cells (Clasen et al., 2005; O’Keefe et al., 2007). Antibody staining of DE-cad showed that at 20 h APF, the inter-hair cells flanked by hair cells showed a shrunken apical surface (on apical plane depicted in Fig. 3H) compared with the rest of the wing margin cells (Figs. 5A, H). At 30 h APF, there was a strong accumulation of DE-cad in the wing margin (Fig. 5B). At this stage, there are apparently two distinct types of wing margin cells: one is previously described inter-hair cell (dark green in Fig. 5I) and the other is a single row of wing margin cells (light green in Fig. 5I). Given this positioning of the latter cells, which is similar to the interlocking teeth of a zip, we call this type of wing margin cells “tooth cells.” To determine whether DE-cad is necessary for this patterning of wing margin cells, we depleted DE-cad by RNAi. Knockdown of DE-cad in wing margin cells by using wg-GAL4\textsuperscript{40} resulted in an undetectable expression of DE-cad (Fig. 5C). This depletion of DE-cad severely disturbed the organization of the cells: the wing margin cell region became broader and necrotic (Fig. 5C) and the wing margin structure became flattened in the transverse section (Fig. 5F), compared with the cell organization in the control wing (Figs. 5B, E). Accordingly, the dorsal and ventral wing edges did not attach (Fig. 5F). In the adult wing with DE-cad depleted from the wing margin cells, the alignment and growth of wing margin hairs were severely disrupted, and the ectopic tanning and shrinking of the wing blade were found (Fig. 5J), probably due to necrotic disruption of the wing margin epithelial structure. These results indicate that DE-cad is necessary for normal morphology of wing margin cells.

Since Wg signaling regulates DE-cad levels in the larval wing disc (Jaiswal et al., 2006), we investigated whether Wg signaling is required for DE-cad accumulation in the pupal wing margin. To inhibit Wg signaling in wing margin cells, we expressed Tcf\textsuperscript{DN}, a dominant negative form of Tcf (Pangolin—FlyBase), which is a transcriptional effector of the Wg signaling pathway (Brunner et al., 1997; van de Wetering et al., 1997). Expression of Tcf\textsuperscript{DN} by wg-GAL4\textsuperscript{40} in the pupal wing margin resulted in a loss of accumulated DE-cad (Fig. 5D) and a collapsed wing margin (Figs. 5D, G), similar to the phenotypes of the DE-cad knockdown (Figs. 5C, F). This demonstrates that Wg signaling is necessary for the normal architecture of wing margin cells, at least in part, through DE-cad accumulation.

**NEPH1/neprhin homologs are necessary for zigzag alignment of wing margin hairs**

Another type of cell adhesion molecules we examined was *Drosophila* NEPH1/neprhin homologs, which are transmembrane proteins belonging to the immunoglobulin superfamily. These proteins, also called irr cell recognition module (IRM) proteins (Fischbach et al., 2009), can be classified into two subfamilies, NEPH1 and neprhin. The
heterophilic adhesion properties between these subfamilies have been shown to be involved in various cellular interactions, including myoblast fusion (Galletta et al., 2004; Shelton et al., 2009), cell sorting in eye morphogenesis (Bao and Cagan, 2005; Bao et al., 2010), axonal pathfinding in the visual system (Fischbach et al., 2009; Ramos et al., 1993; Schneider et al., 1995), retinotopic map formation (Sugie et al., 2010), and nephrocyte formation (Weavers et al., 2009; Zhuang et al., 2009). In *Drosophila*, there are two NEPH1 homologs, *roughest* (also known as *irreC*) and *kin of irreC* (*kirre*, also known as *dumbfounded*) (Artero et al., 2001; Bour et al., 2000; Dworak et al., 2001; Ramos et al., 1993; Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). Antibody staining of the NEPH1 homolog *Kirre* revealed that it accumulated at the contact surface between the hair cells and the surrounding cells on the apical plane at 30 h APF (Fig. 6A). Another NEPH1 homolog *Rst* accumulated specifically at the interface between the hair cells and inter-hair cells (Fig. 6B). We next examined the expression pattern of two *Drosophila* nephrin homologs, *Sticks and stones* (*Sns*) and *Hibiris* (*Hbs*). Antibody staining of *Sns* and *Hbs* revealed that both *Sns* and *Hbs* proteins accumulate at the border between the hair cells and the surrounding cells (Figs. 6C, D). Taken together, these results show that
all the IRM proteins accumulated at the contact surface between the hair cells and the surrounding cells.

To determine which cells express which IRM proteins, we investigated the expression of their enhancer-trapped reporter genes. With regard to the NEPH1 homologs, the expression of kirre reporter rP298-lacZ (Nose et al., 1998) was observed in the cells surrounding the hair cells (Fig. 6E). In contrast, rst reporter NP2044-GAL4 was strongly detected in the inter-hair cells but not in the tooth cells (Fig. 6F). This result is consistent with the accumulation pattern of Rst protein described above (Fig. 6B). With regard to the nephrin homologs, the expression of sns-lacZ (Kocherlakota et al., 2008) was observed in the hair cells (Fig. 6G), which is consistent with the heterophilic binding of Sns to Kirre and Rst (Bao et al., 2010; Galletta et al., 2004). In contrast, hbs-lacZ (Artero et al., 2001) was expressed not only in the hair cells but also in the surrounding wing margin cells (Fig. 6H). A previous finding that Hbs is a heterophilic binding partner of Kirre and Rst (Bao and Cagan, 2005; Bao et al., 2010) well accounts for the Hbs expression in hair cells since Kirre and Rst are expressed specifically in the surrounding cells (Figs. 6E, F). However, the expression of Hbs in the wing margin cells enables us to predict that it may homophilically bind to Hbs expressed in wing margin cells or heterophilically bind to Sns expressed in hair cells.

Finally, we investigated whether these IRM proteins contribute to the establishment of the zigzag alignment of wing margin hairs. We depleted each IRM protein by RNAi-mediated knockdown in the wing margin cells or hair cells. With regard to the NEPH1 homologs,
knockdown of *kirre* or *rst* in wing margin cells disrupted the normal hair alignment (Figs. 7A, C), but the same treatment in hair cells did not (Figs. 7B, D). These results are consistent with the gene expression patterns described above. With regard to the nephrin homologs, the loss of Sns in hair cells (Fig. 7F) but not in wing margin cells (Fig. 7E) also resulted in disruption of the normal hair alignment. In the case of Hbs, however, similar phenotypes were observed for its knockdown in both wing margin cells (Fig. 7G) and hair cells (Fig. 7H). The difference in the responsible cells between *sns* and *hbs* knockdowns can be explained by the difference in expression patterns between the two genes. Together, each IRM adhesion molecule is required for zigzag alignment of wing margin hairs (Fig. 7I).

**Discussion**

Our results showed that the zigzag alignment of the double row of hairs in the posterior wing margin results from the rearrangement of the surrounding wing margin cells (Fig. 7I). This cell rearrangement is triggered by apoptosis of the wing margin cells (flat cells) that are spatially separated from the hair cells. Survival of the wing margin cells (inter-hair cells) adjacent to the hair cells is affected by activation of EGFR signaling. We have also shown that the shapes and topology of wing margin cells, which are important in creating the interlocking arrangement of the dorsal and ventral wing edges, are governed by adhesion molecules, such as DE-cad and IRM proteins.
Apoptosis during pupal wing development

Previous studies on other insects have shown that apoptosis sculpts the peripheral region of the pupal wings in Lepidoptera including butterflies and moths (Dohrmann and Nihjout, 1988; Kodama et al., 1995) and that ecdysone signaling is involved in this process (Fujiwara and Ogai, 2001). In the case of Drosophila, although the apoptosis along the pupal wing margin has been described as in other insects, its purpose and underlying mechanism were not well understood (Aigouy et al., 2004; Jafar-Nejad et al., 2006). In this study, we showed that two distinct surges of apoptosis occur in Drosophila pupal wing development. In the earlier stage (~20 h APF), some of the hair precursor lineage cells undergo apoptosis (Figs. 2A, D), eliminating unnecessary cells including excess neuron, sheath, and glial cells (Jafar-Nejad et al., 2006; data not shown).

In the later stage, shortly after the apoptosis of hair precursor lineage cells, a subset of wing margin cells undergoes apoptosis (Fig. 2). While wing margin cells that surround hair cells can survive, ones that are spatially separated from hair cells are eliminated (Figs. 3A–D). This apoptosis is required for the zigzag alignment of wing margin hairs (Fig. 3) but does not seem to affect wing shape much, suggesting that the role of this apoptosis is different from that reported for lepidopteran wing apoptosis. Nevertheless, ecdysone signaling is required for triggering apoptosis in both groups of insects (Figs. 2A, D). Similar phenotypes are observed in the wing with depleted Sns in hair cells (Fig. S1) but not in wing margin cells (E). Knockdown of hbs in wing margin cells (G) and in hair cells (H) also disrupts the hair alignment. (I) Model for establishing interlocking pattern of the posterior wing margin. At 20 h APF, hair cells (magenta) are not yet positioned in a well-ordered zigzag manner (left panel). Selective removal of wing margin cells, except for inter-hair cells (dark green) and tooth cells (light green in right panel), induces cell rearrangement in the wing margin. Asterisks indicate presumably prospective tooth cells at 20 h APF. DE-cad (cyan line), which is necessary for forming normal epithelial structure of wing margin cells, becomes more accumulated in both wing margin cells and hair cells through 20 to 30 h APF (upper right panel). Blue line indicates the DV boundary. IRM proteins accumulate at the border between hair cells and the surrounding cells (lower right panel). Different sets of the IRM proteins contribute to the shapes and arrangements of inter-hair cells and tooth cells for zigzag alignment of hair cells. Scale bars: 50 μm in A–H.

Rearrangement for patterning

We also showed that the coordination of apoptosis and the epithelial architecture of wing margin cells induces the cell rearrangement necessary for well-ordered zigzag patterning. Similar mechanisms have been reported for Drosophila eye morphogenesis. In the pupal eye, 750–800 ommatidia are packed hexagonally. This patterning is established by cell sorting and apoptosis of interommatidial cells (Brachmann and Cagan, 2003). Cell sorting of interommatidial cells is mediated by heterophilic adhesion between IRM proteins (Bao and Cagan, 2005; Bao et al., 2010). Primary pigment cells secrete an EGFR ligand, Spitz, and activate its signaling only in the neighboring interommatidial cells for survival (Brachmann and Cagan, 2003). Interommatidial cells that do not receive Spitz undergo apoptosis. EGFR signaling downregulates the activity of Hid, a proapoptotic protein in the eye (Bergmann et al., 1998; Kurada and White, 1998). As a result of apoptosis, a single row of interommatidial cells forms an outline of each ommatidium, which is required for hexagonal packing of ommatidia.

Fig. 7. IRM proteins are required for normal zigzag alignment of wing margin hairs. (A–H) Close-up views of the posterior margin in adult wings. (A–D) Alternate projection of wing margin hairs from the dorsal and ventral sides with even intervals disrupted by knockdown of kirre and rst in wing margin cells (A, C) but not in hair cells (B, D). (E, F) Similar phenotypes are observed in the wing with depleted Sns in hair cells (F) but not in wing margin cells (E). Knockdown of hbs in wing margin cells (G) and in hair cells (H) also disrupts the hair alignment. (I) Model for establishing interlocking pattern of the posterior wing margin. At 20 h APF, hair cells (magenta) are not yet positioned in a well-ordered zigzag manner (left panel). Selective removal of wing margin cells, except for inter-hair cells (dark green) and tooth cells (light green in right panel), induces cell rearrangement in the wing margin. Asterisks indicate presumably prospective tooth cells at 20 h APF. DE-cad (cyan line), which is necessary for forming normal epithelial structure of wing margin cells, becomes more accumulated in both wing margin cells and hair cells through 20 to 30 h APF (upper right panel). Blue line indicates the DV boundary. IRM proteins accumulate at the border between hair cells and the surrounding cells (lower right panel). Different sets of the IRM proteins contribute to the shapes and arrangements of inter-hair cells and tooth cells for zigzag alignment of hair cells. Scale bars: 50 μm in A–H.
In our study, inter-hair cells flanked by hair cells survived in part due to active EGFR signaling (Fig. 4K). In the hid mutant background, the alignment of posterior wing margin hairs was disturbed (data not shown), indicating that apoptosis that occurs in the wing margin is hid-dependent. Thus, EGFR signaling may prevent apoptosis through downregulating hid function in the wing margin as well, consistent with the reports referenced above. However, when we artificially terminated EGFR signaling, a complete loss of the wing margin cells by 40 h APF was not observed (data not shown). This raises the possibility that there are other survival cues for the wing margin cells although hyperactivation of EGFR signaling is sufficient for cell survival (Fig. 4J).

As in eye morphogenesis, IRM proteins are involved in the alignment of the double row of wing margin hairs (Figs. 6, 7). The nephrin homolog Sns is expressed in hair cells, while the NEPH1 homolog Kirre and Rst are expressed in the surrounding cells although another nephrin homolog Hbs is expressed in both of these cells (Fig. 6). Since these IRM proteins are localized at the border between hair cells and the surrounding cells, heterophilic binding between NEPH1 and nephrin subfamily proteins likely contributes to the allocation of wing margin cells. In fact, knockdown of each IRM protein disrupts the normal zigzag alignment of posterior wing margin hairs (Figs. 7A–H). The difference between the expression patterns of Kirre and Rst in wing margin cells (Figs. 6A, B) suggests that they have distinct roles. Specific accumulation of Rst at the border between hair cells and inter-hair cells apparently account for the alternate alignment of these cells in a single row. In contrast, since Kirre is found on tooth cells as well as on inter-hair cells, Kirre may also contribute to the contact between hair cells and tooth cells. These different sets of heterophilic bindings could shape and allocate three different sets of cell types (hair, inter-hair, and tooth) in a well-ordered manner (Fig. 7J). Furthermore, IRM proteins had already accumulated at the interface between the wing margin cells and hair cells at 20 h APF (Fig. S5 in Supplementary material), suggesting that IRM proteins are also involved in the selection of which wing margin cells survive. Another adhesion molecule DE-cad accounts for the adhesion between the dorsal and ventral edges, completing the interlocking structure.

Stronger accumulation of DE-cad in the wing margin region (Fig. 5B) likely strengthens the cell alignment pattern through stabilization of its homophilic binding (Lecuit and Lenné, 2007). Possible preferential adhesion between inter-hair cells and tooth cells across the DV boundary through other heterophilic adhesion molecules may make the interlocking arrangement of wing margin cells more robust.

These mechanisms described above may be a general strategy for geometrically ordered patterning of a two-dimensional epithelium. The zigzag patterning of hairs in the Drosophila wing margin can be compared with the sensory epithelium of the mammalian cochlea, the organ of Corti, where sensory hair cells and surrounding supporting cells align in a highly ordered zigzag manner (Kelly and Chen, 2007). As in the specification of Drosophila bristles, these sensory hair cells are specified by Notch-mediated lateral inhibition (Eddison et al., 2000; Muller and Littlewood-Evans, 2001). A previous study has shown that developing sensory epithelium undergoes convergent extension, during which the cells are rearranged and the final cellular pattern is formed (McKenzie et al., 2004), similar to the rearrangement of the wing margin cells. Apoptosis is also observed in the epithelium of the mouse inner ear during development (Nishikori et al., 1999; Nishizaki et al., 1998) although the relationship between cell rearrangement and apoptosis has not been clarified. Moreover, mKirre, a mouse ortholog of kirre, is expressed in the inner ear (Ueno et al., 2003). Taken together, the cell rearrangement observed in the organ of Corti may have a common mechanism related to the wing margin cell rearrangement shown in this paper.

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