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The cadherin Fat2 is required for planar cell polarity in the *Drosophila* ovary

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Planar cell polarity is an important characteristic of many epithelia. In the *Drosophila* wing, eye and abdomen, establishment of planar cell polarity requires the core planar cell polarity genes and two cadherins, Fat and Dachsous. *Drosophila* Fat2 is a cadherin related to Fat; however, its role during planar cell polarity has not been studied. Here, we have generated mutations in *fat2* and show that Fat2 is required for the planar polarity of actin filament orientation at the basal side of ovarian follicle cells. Defects in actin filament orientation correlate with a failure of egg chambers to elongate during oogenesis. Using a functional fosmid-based *fat2-GFP* transgene, we show that the distribution of Fat2 protein in follicle cells is planar polarized and that Fat2 localizes where basal actin filaments terminate. Mosaic analysis demonstrates that Fat2 acts non-autonomously in follicle cells, indicating that Fat2 is required for the transmission of polarity information. Our results suggest a principal role for Fat-like cadherins during the establishment of planar cell polarity.

KEY WORDS: *Drosophila*, Ovary, Follicle cell, Cadherin, Fat2, Planar cell polarity

INTRODUCTION

The polarization of cells within the plane of the tissue is an important characteristic of many epithelia (reviewed by Adler, 2002; Saburi and McNeill, 2005; Seifert and Mlodzik, 2007; Strutt and Strutt, 2005; Wang and Nathans, 2007; Zallen, 2007). Examples include the orientation of stereocilia in the inner ear, oriented outgrowth such as hair, and oriented cell divisions and tissue movements. A molecular pathway controlling planar cell polarity was first delineated in *Drosophila melanogaster*. Establishment of planar cell polarity in the wing, eye and abdomen of the fly requires an evolutionarily conserved set of 'core' planar-cell-polarity genes and their effectors. More recently, Fat and Dachsous, two members of the cadherin superfamily of Ca^{2+}-dependent cell-adhesion molecules that provide molecular links between neighboring cells, were shown to be important for establishing planar cell polarity in these epithelia (Casal et al., 2006; Ma et al., 2003; Yang et al., 2002). Four Fat homologs (Fat1-4) have been identified in vertebrates (Tanoue and Takeichi, 2005), and a requirement for Fat4 during the establishment of planar cell polarity has recently been shown (Saburi et al., 2008).

A second excellent system in which to study planar cell polarity is the *Drosophila* ovarian follicle epithelium. Follicle cells display actin filaments at their basal side that are oriented perpendicular to the anteroposterior (long) axis of the developing egg chamber (Gutzeit, 1990). These actin filaments resemble stress fibers, which are bundles of actin filaments observed at the basal side of some cultured epithelial and fibroblast cells (Pellegrin and Mellor, 2007). The formation of stress fibers is influenced by integrins, transmembrane proteins composed of heterodimers of α and β subunits, that connect the actin cytoskeleton to the extracellular matrix at focal adhesions. Like stress fibers, the ends of actin filaments within follicle cells are associated with integrins (PSβ-integrin), and integrins are required for the proper polarized orientation of these actin filaments (Bateman et al., 2001). In addition, proper actin filament orientation requires the receptor tyrosine phosphatase Lar, which is involved in signaling between the extracellular matrix and the actin cytoskeleton (Bateman et al., 2001; Frydman and Spradling, 2001), a receptor for extracellular matrix proteins called Dystroglycan (Deng et al., 2003; Mirouse et al., 2009), Dystrophin, a cytoplasmic protein binding to Dystroglycan (Mirouse et al., 2009), and the Pak family serine/threonine kinase (Conder et al., 2007). The functions of these proteins in signaling between the extracelluar matrix and the actin cytoskeleton suggest an important role for cell-to-matrix interactions in the establishment of planar cell polarity in the follicle epithelium.

The first mutations shown to disrupt the polarized actin filament orientation in follicle cells were in the gene *kugelei* (also known as *kugel*) (Gutzeit et al., 1991). The analysis of *kugelei* mutants also first showed a link between the planar polarity of actin filaments in follicle cells and overall egg shape (Gutzeit et al., 1991). Whereas normal eggs are elongated along their anteroposterior axis, *kugelei* mutants produce eggs that are spherical in shape. Based on these observations, it was proposed that the planar-polarized actin filaments provide a 'molecular corset' that restrains the increase in size of the growing egg chamber perpendicular to the anteroposterior axis and, thereby, contributes to the elongation of the egg chamber (Gutzeit et al., 1991). Even though *kugelei* mutants were isolated several decades ago, the product of the *kugelei* gene has not been identified.

In *Drosophila*, Fat2 (also known as Fat-like) is highly related to *Drosophila* Fat as well as to the vertebrate cadherins Fat1, Fat2 and Fat3 (Castillejo-Lopez et al., 2004). A recent study, which used RNA interference to knock down *fat2* function, has revealed a role for *Drosophila* Fat2 during tubulogenesis in the embryo (Castillejo-Lopez et al., 2004). However, whether Fat2 has a role during planar cell polarity has not been reported.

Here, we show that *Drosophila fat2* is essential for the planar polarity of basal actin filaments in follicle cells and the elongation of egg chambers. We demonstrate that *fat2* is allelic to *kugelei*.

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Moreover, we show that the distribution of a Fat2-GFP fusion protein is polarized within the plane of the follicle epithelium and that it accumulates on cell membranes where the planar oriented actin filaments terminate. Finally, we demonstrate that Fat2 acts non-cell-autonomously to establish planar cell polarity and proper egg chamber shape. Our results suggest that cell-to-cell interactions, mediated by Fat2, play an important role in the establishment of planar cell polarity in the follicle epithelium.

MATERIALS AND METHODS

Drosophila stocks

Mutant alleles of *fat2* were generated by imprecise excision of the EP-element *GE20158* (GenExel). Out of 1181 excision lines analyzed by PCR, two contained large deletions in the *fat2* gene. The allele $fat2^{58D}$ contains a genomic deletion of 4162 bp spanning from 2100 bp 5′ to 2059 bp 3′ of the translational start codon (Castillejo-Lopez et al., 2004) deleting the coding sequence for amino acids 1-687. The allele $fat2^{103C}$ contains a genomic deletion of 2688 bp spanning from 2366 bp 5′ to 319 bp 3′ of the translational start codon deleting the coding sequence for amino acids 1-107. Additional mutant alleles used were kug^{003}, kug^{040}, kug^{054}, kug^{285}, kug^{602}, kug^{622}, kug^{649}, $Lar^{5.5}$, $Lar^{13.2}$, ds^{1}, ds^{UAO71}, ft^{1}, $ft^{G\text{-}rv}$, dsh^{1}, dgo^{380}, pk^{1} and $stbm^{6}$. Alleles are described in FlyBase (www.flybase.org). *Df(3L)BSC2* and *Df(3L)XS533* are deficiencies uncovering *fat2*.

Generation of a *fat2-GFP* transgenic construct

A fosmid containing the genomic region spanning nucleotides 19998559-20035492 on chromosome 3L (see FlyBase www.flybase.org) including *fat2* was kindly provided by R. K. Ejsmont and P. Tomancak (Ejsmont et al., 2009). GFP was inserted at the 3′ end of *fat2* replacing its stop codon by homologous recombineering in bacteria (Sarov et al., 2006). Transgenic flies were generated using *phiC31*-mediated integration into the *attP16* docking site (Markstein et al., 2008). Unless otherwise stated, flies homozygous for the *fat2-GFP* transgene were analyzed. The genotype of $Lar^{5.5}/Lar^{13.2}$ mutant flies expressing Fat2-GFP was: *w*; $Lar^{5.5}$, *fat2-GFP*/$Lar^{13.2}$, *fat2-GFP*.

Clonal analysis

Marked clones of cells were generated using the FRT-Flp system (Xu and Rubin, 1993). Clones lacking *fat2-GFP* were generated by subjecting 1- to 2-day-old adult flies two times per day to a 38°C heat shock for 1 hour on three successive days. Ovaries were dissected 5-7 days after the last heat shock. The genotypes of these flies were: *y w hsp-flp*; *FRT42D fat2-GFP*/*FRT42D hsp-CD2* and *y w hsp-flp*; *FRT42D fat2-GFP*/*FRT42D hsp-CD2*; $fat2^{58D}/fat2^{103C}$. Marked clones of $fat2^{58D}$ mutant cells were specifically generated in the follicular epithelium through Flp-mediated recombination using *e22c-Gal4, UAS-flp* (Duffy et al., 1998). Germline clones were generated using the dominant female-sterile technique (Chou et al., 1993).

Sequencing of *kugelei* mutants

Genomic DNA from heterozygous *kugelei* mutant adult flies was amplified by PCR and sequenced using standard methods. Mutations were detected in the sequence chromatogram by ‘double peaks’. Mutations were verified by sequencing both DNA strands. Mutations were verified to reside on the *kugelei* mutant chromosomes by sequencing genomic DNA from *kugelei*/*Df(3L)BSC2* flies.

Immunohistochemistry

Ovaries dissected from adult flies were fixed and stained using standard protocols. We raised three antibodies directed against different parts of Fat2. All three antibodies, however, failed to give specific immunostainings in ovaries (data not shown). To generate antibodies against Fat2, GST-fusion proteins containing amino acids 1-269 or 270-1076 of Fat2 were used to immunize rats and rabbits. To raise antibodies against the C-terminal part of Fat2, a synthetic peptide corresponding to amino acids 4057-4075 (CEIEDSELEEFLPQQQTNN) was used for immunization of rabbits. Sera were affinity purified. The following additional primary antibodies were used: rabbit anti-Oskar (1:3000) (Vanzo and Ephrussi, 2002), mouse anti-Gurken 1D12 [Developmental Studies Hybridoma Bank (DSHB); 1:100], mouse anti-Fasciclin III (DSHB; 1:50), mouse anti-Lar 9D82B3-c (DSHB; 1:100), mouse anti-PSβ-integrin CF.6G11 (DSHB; 1:100), rabbit anti-GFP (Clontech; 1:2000) and mouse anti-CD2 (Serotec; 1:2000). Secondary antibodies, all diluted 1:200, were goat anti-mouse Alexa fluor 488 (Molecular Probes), goat anti-rabbit Alexa fluor 488 (Molecular Probes), and donkey anti-mouse CY5 (Jackson ImmunoResearch Laboratories). Rhodamine-phalloidin and DAPI (both Molecular Probes) were used at a dilution of 1:200 and 1:500, respectively. Images were recorded on a LSM510 Zeiss confocal microscope. Basal views of stage 5-8 egg chambers show projections of three to four images recorded at a z-distance of 0.3 μm.

Image analysis and statistics

Pixel intensities of Fat2-GFP were measured using ImageJ. Statistical analysis was performed using a two-sample, unpaired Student’s *t*-test.

RESULTS

Fat2 is required for proper egg chamber shape

To analyze the function of Fat2 in planar cell polarity, we generated two mutations in the *fat2* gene, $fat2^{58D}$ and $fat2^{103C}$, by imprecise excision of the EP-element *GE20158* (Fig. 1A). Both mutations removed parts of the 5′ end of the coding sequence of *fat2*, indicating that they are molecular null alleles of *fat2* (see Materials and methods). $fat2^{58D}$ and $fat2^{103C}$ mutant flies were viable; however, *fat2* mutant females were sterile and displayed a highly reduced rate of oviposition (data not shown), indicating a role of Fat2 during oogenesis. The *Drosophila* ovary is composed of chains of egg chambers proceeding through 14 stages from the germarium to the oviduct (Spradling, 1993) (Fig. 1B). Each egg chamber consists of 16 germline cells, one oocyte and 15 nurse cells, encapsulated by a monolayer of somatic, epithelial follicle cells. Egg chambers budding off from the germarium are spherical in shape; however, they elongate along their anteroposterior axis as they proceed through oogenesis, giving rise to highly elongated egg chambers at stage 14.

In contrast to control egg chambers, egg chambers of $fat2^{58D}$ and $fat2^{103C}$ mutant flies failed to elongate and remained almost spherical until stage 14 (Fig. 1C,D, and data not shown). In addition, mutant egg chambers displayed abnormally short dorsal appendages (Fig. 1C,D). The spherical shape of mutant stage 14 egg chambers correlated with the failure of follicle cells to elongate along their anteroposterior axis (Fig. 1E,F). These results demonstrate a role for Fat2 in the elongation of the egg chamber during oogenesis.

kugelei mutations are alleles of *fat2*

Similar to *fat2* mutants, egg chambers fail to elongate and remain spherical in the previously identified *kugelei* mutants (Gutzeit et al., 1991). *kugelei* and *fat2* have been mapped to a similar genomic interval (FlyBase: www.flybase.org), raising the possibility that *kugelei* is allelic with *fat2*. To assess this notion, we tested complementation between $fat2^{58D}$, $fat2^{103C}$ and seven *kugelei* mutant alleles. None of these *kugelei* mutants complemented the *fat2* mutants, indicating that *kugelei* and *fat2* are allelic (Fig. 2A-C, and data not shown). Moreover, genomic sequencing identified premature stop codons in the *fat2* gene of all seven *kugelei* mutants (Table 1). Finally, we tested whether the expression of a functional *fat2* gene could revert the spherical egg chamber phenotype of *kugelei* mutants to wild type. To this end, we employed homologous recombineering to tag a *fat2* gene located on a fosmid by GFP and used this modified fosmid to generate *fat2-GFP* transgenic flies (see Materials and methods). In this genomic construct, *fat2-GFP* was expressed under its own regulatory elements. Female $fat2^{103C}/fat2^{58D}$ flies carrying *fat2-GFP* were fertile and produced normal-shaped egg chambers (see Fig. S1 in the supplementary material), demonstrating that *fat2-GFP* is a functional transgene that

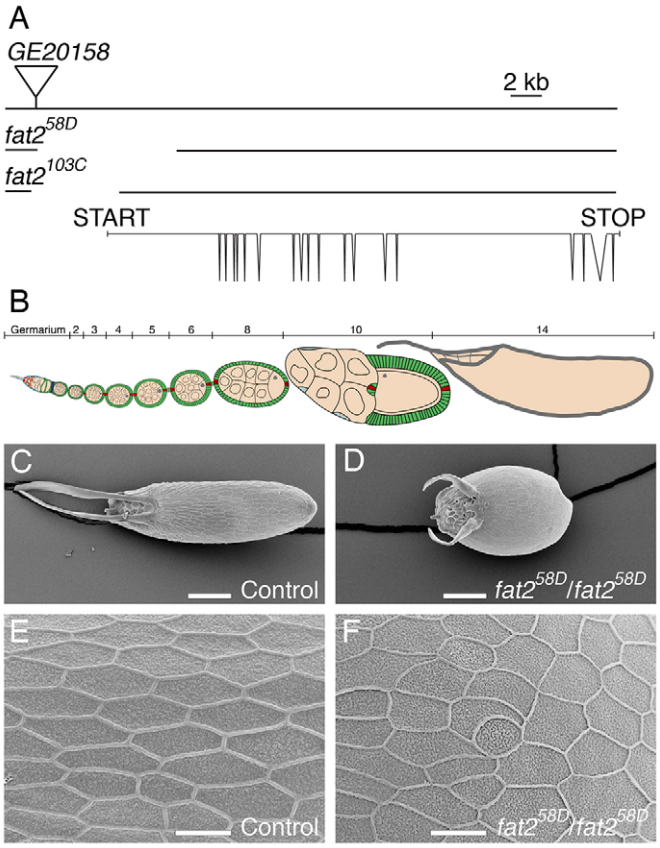

Fig. 1. Fat2 is required for proper egg chamber shape. (**A**) Extent of the deletions in *fat2* mutant alleles. Position of the EP element *GE20158*, and intron-exon structure of *fat2* are indicated. (**B**) Scheme of ovariole development. Follicle cells (green) surround the germline cells (yellow). The basal side of follicle cells faces the outside of the egg chamber. Polar cells are labeled in red. Developmental stages are indicated at the top. (**C-F**) Scanning electron micrographs of control (C,E) and $fat2^{58D}$ mutant (D,F) stage 14 egg chambers. (E,F) Magnified views of C,D to show the follicle cell imprints in the eggshell. In this and the following figures anterior is to the left. Scale bars: 100 μm in C,D; 5 μm in E,F.

contains all the regulatory elements of *fat2* essential for ovarian development. Notably, kug^{003}/kug^{602} flies carrying *fat2-GFP* were fertile and deposited normal-shaped eggs (Fig. 2D). These data demonstrate that *kugelei* mutations are alleles of *fat2*.

Fat2 is not required for oocyte polarity

Similar to *fat2/kugelei* mutants (henceforth referred to as *fat2*), mutations in *Lar* result in a spherical egg chamber shape (Bateman et al., 2001; Frydman and Spradling, 2001). Moreover, *Lar* mutants are also defective in oocyte polarity and fail to properly localize Oskar, a protein essential for posterior patterning and germ cell development (reviewed by Riechmann and Ephrussi, 2001), to the posterior pole of the oocyte (Frydman and Spradling, 2001). To test whether the spherical egg chamber shape of *fat2* mutants resulted from the failure to properly establish oocyte polarity, we analyzed the localization of Oskar and Gurken, a dorsal marker (reviewed by Riechmann and Ephrussi, 2001), in *fat2* mutant egg chambers. Oskar localized to the posterior pole (n=18 egg chambers) and Gurken to the dorsal anterior corner of the oocyte (n=50 egg chambers), indistinguishable from control egg chambers (Fig. 3A-D). Moreover, in *fat2* mutants the oocyte nucleus migrated to the dorsal anterior corner of the oocyte (n=50 egg chambers), as in wild-type ovaries (Fig. 3C,D). Finally, the number of pairs of polar cells, specialized follicle cells implicated in directing planar actin filament polarity (Frydman and Spradling, 2001), was normal in *fat2* mutants (Fig. 3E,F; n=100 egg chambers). These results indicate that the failure of *fat2* mutants to produce normal shaped egg chambers is not a result of defects in oocyte polarity or an altered number of polar cell pairs.

Table 1. *kugelei* alleles display mutations in *fat2*

Allele	Mutation	Allele	Mutation
kug^{003}	$Lys^{75}\rightarrow Stop$	kug^{602}	$Gln^{184}\rightarrow Stop$
kug^{040}	$Tyr^{243}\rightarrow Stop$	kug^{622}	$Gln^{1038}\rightarrow Stop$
kug^{054}	$Lys^{4}\rightarrow Stop$	kug^{649}	$Trp^{1157}\rightarrow Stop$
kug^{285}	$Tyr^{3665}\rightarrow Stop$		

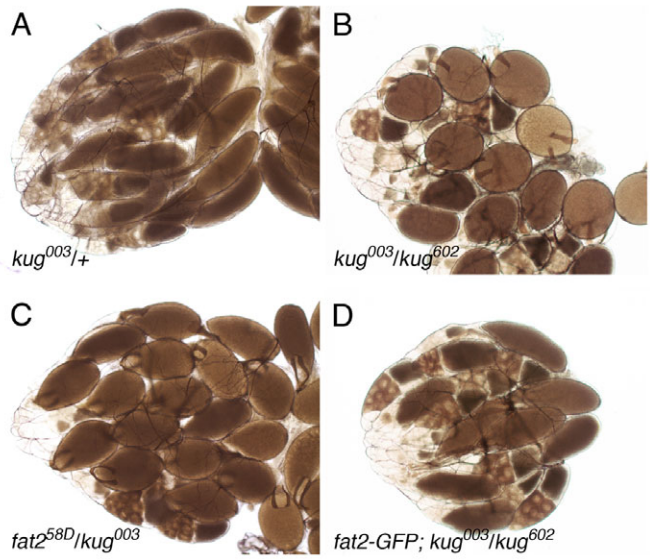

Fig. 2. *kugelei* mutations are alleles of *fat2*. (**A-D**) Ovaries of control (A), kug^{003}/kug^{602} (B), $fat2^{58D}/kug^{003}$ (C) and *fat2-GFP*; kug^{003}/kug^{602} (D) mutant flies. *fat2-GFP* reverts the spherical egg chamber shape of kug^{003}/kug^{602} mutants to normal.

Fat2 is required for the planar-polarized orientation of actin filaments

From stages 6-7 onwards, wild-type follicle cells display bundles of parallel actin filaments at their basal side. The orientation of these actin filaments is planar polarized perpendicular to the anteroposterior (long) axis of the egg chamber (Gutzeit, 1990). The appearance of basal actin filaments changes during follicle development. During early stages, actin filaments form long and thin bundles, whereas at later stages actin bundles are more densely packed. In kug^{003}/kug^{003} mutant egg chambers, the appearance of basal actin filaments is similar to wild-type and actin filaments form parallel bundles within cells (Gutzeit et al., 1991). Actin filaments are, however, no longer strictly oriented perpendicular to the anteroposterior axis in kug^{003}/kug^{003} mutant egg chambers (Gutzeit et al., 1991). Consistent with our finding that *kugelei* is allelic to *fat2*, we also found that in *fat2* mutant flies actin filaments were still parallel within cells; however, in contrast to the controls, actin filaments were no longer strictly oriented perpendicular to the anteroposterior axis of stage 8 or stage

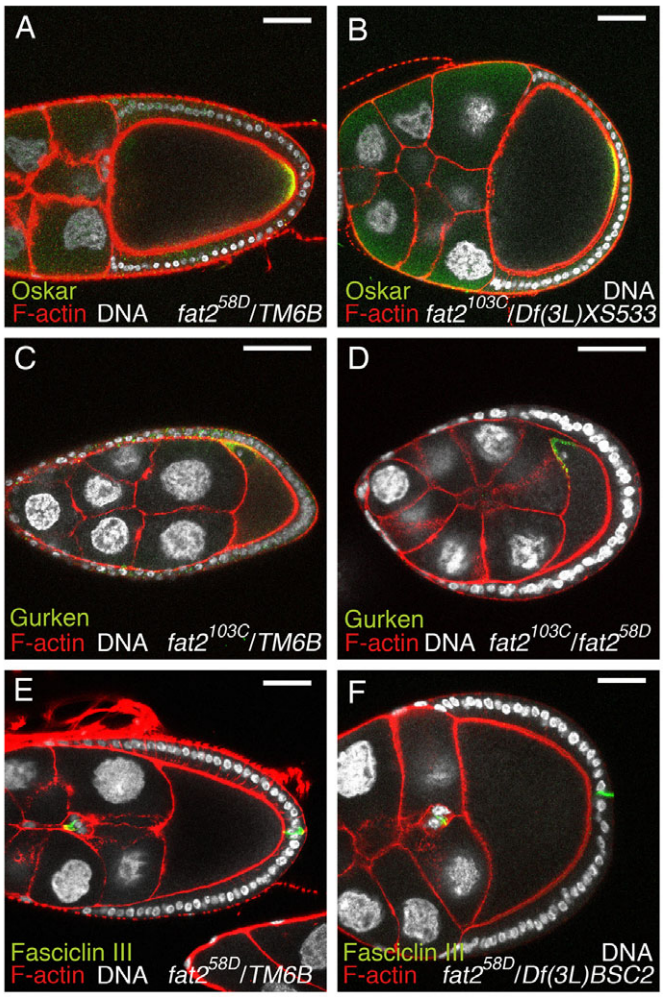

Fig. 3. Fat2 is not required for oocyte polarity. (**A**,**B**) Control (A) and *fat2* mutant (B) stage 10 egg chambers stained for Oskar (green), rhodamine-phalloidin to reveal F-actin (red) and DNA (white). (**C**,**D**) Control (C) and *fat2* mutant (D) stage 9 egg chambers stained for Gurken (green), F-actin (red) and DNA (white). (**E**,**F**) Control (E) and *fat2* mutant (F) stage 10 egg chambers stained for Fasciclin III (a marker for polar cells, green), F-actin (red) and DNA (white). As in the control, two clusters of Fasciclin III-positive cells are present in a single *fat2* mutant egg chamber. Scale bars: 50 μm.

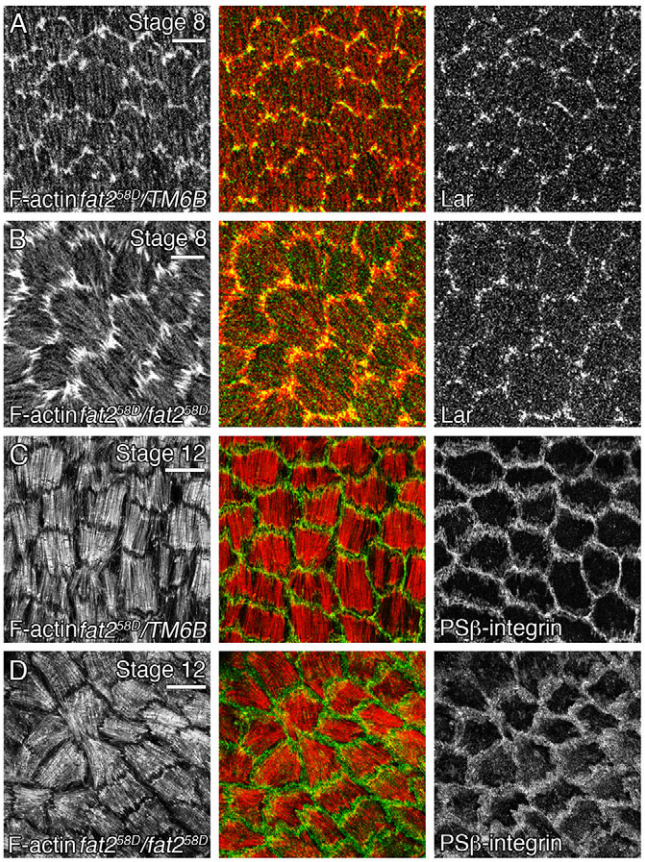

Fig. 4. Fat2 is required for the planar-polarized orientation of actin filaments. (**A**,**B**) Follicle cells of control (A) and *fat2*^{58D} mutant (B) stage 8 egg chambers stained for F-actin (red in the merge) and Lar (green in the merge). (**C**,**D**) Follicle cells of control (C) and *fat2*^{58D} mutant (D) stage 12 egg chambers stained for F-actin (red) and PSβ-integrin (green). Basal views of egg chambers are shown. Scale bars: 5 μm in A,B; 20 μm in C,D.

12 egg chambers (Fig. 4A-D). As reported for *kug*^{003}/*kug*^{003} mutant egg chambers (Gutzeit et al., 1991), actin filaments did not appear to be randomly oriented in *fat2* mutant follicle epithelia, but frequently were oriented in parallel in neighboring cells (Fig. 4B,D). These data confirm that *kugelei*/*fat2* is required for the normal planar-polarized orientation of basal actin filaments perpendicular to the anteroposterior axis of egg chambers.

Fat2 is required for the planar-polarized localization of Lar and PSβ-integrin

In wild-type egg chambers, Lar protein is enriched at cell membranes oriented nearly parallel to the anteroposterior axis during stage 8, and it localizes to the ends of basal actin filaments (Bateman et al., 2001). To test whether the planar-polarized localization of Lar is dependent on Fat2, we stained *fat2* mutant egg chambers using an antibody specific for Lar. In contrast to control egg chambers, Lar protein was no longer enriched at cell membranes oriented nearly parallel to the anteroposterior axis in *fat2* mutant stage 8 egg chambers (Fig. 4A,B). Lar protein, however, was still enriched on the cell membranes where the basal actin filaments terminated, regardless of their orientation (Fig. 4B). Moreover, PSβ-integrin shows a prominent enrichment at the ends of basal actin filaments only during the later stages of oogenesis (Bateman et al., 2001). In *fat2* mutant stage 12 egg chambers, PSβ-integrin was still associated with actin filament ends, regardless of their orientation (Fig. 4C,D). Taken together, these results show that Fat2 is not required for the localization of PSβ-integrin and Lar to actin filament ends. Instead, Fat2 is required for the planar-polarized distribution of PSβ-integrin and Lar.

Dynamic distribution of Fat2-GFP during oogenesis

We next used our functional *fat2-GFP* transgene to analyze the localization of Fat2 protein in ovaries. Fat2-GFP was detected during most of ovarian development. In egg chambers budding from the germarium, Fat2-GFP was present in punctate structures within follicle cells (Fig. 5A). During stages 3-8, Fat2-GFP was detected in the oocyte and at both the apical and lateral sides of follicle cells (Fig. 5B-E). Fat2-GFP levels were increased at the apical side in posterior follicle cells compared to other follicle

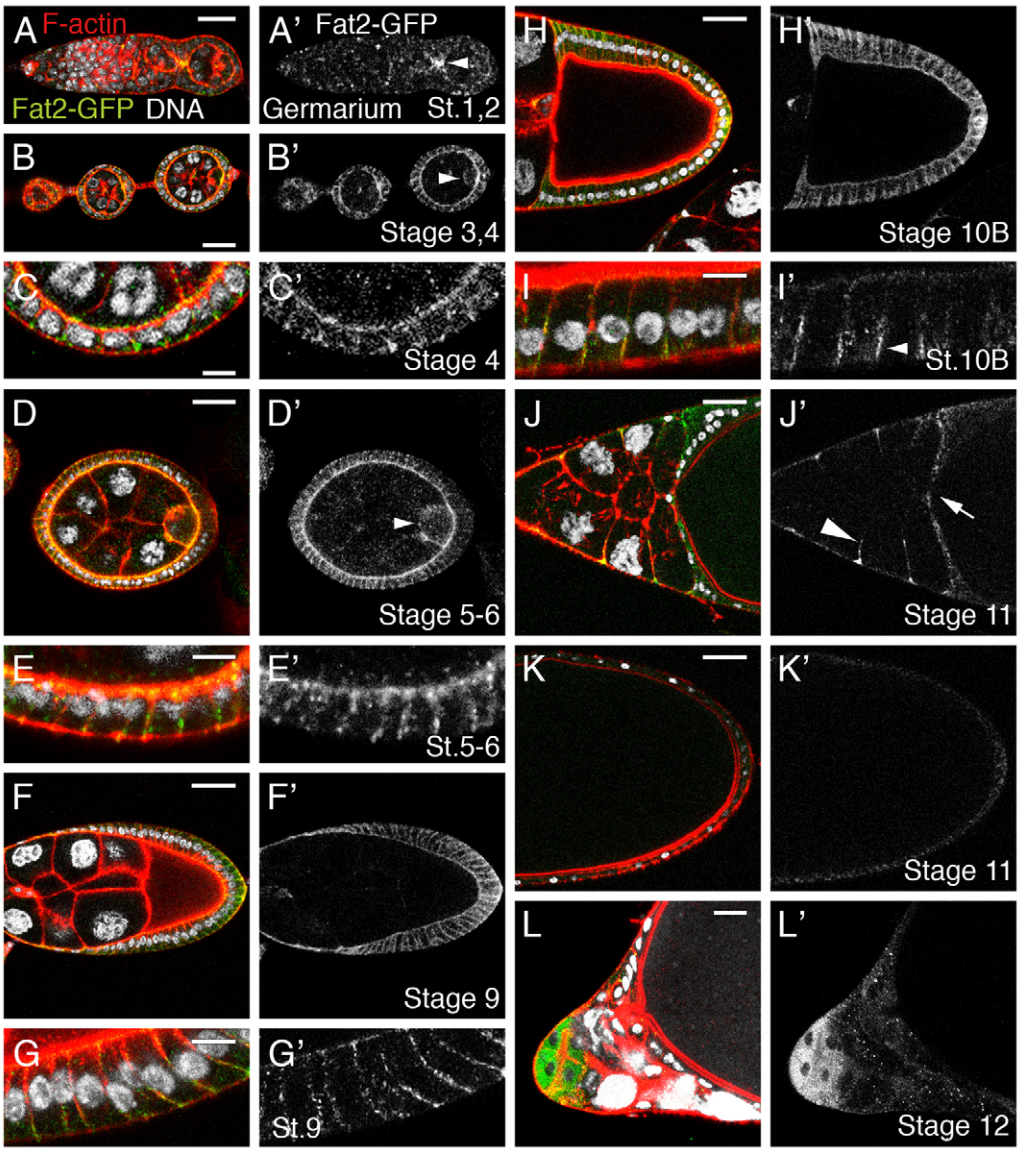

Fig. 5. Distribution of Fat2-GFP in egg chambers. (**A-L'**) Egg chambers of the indicated stages of *fat2-GFP* flies stained for GFP (green), F-actin (red) and DNA (white). Fat2-GFP is detected at the anterior side of stage 2 egg chambers (arrowhead in A'). During stages 3-8, Fat2-GFP is detected in the oocyte (arrowheads in B' and D') and at both the apical and lateral sides of follicle cells. In stage 10B, Fat2-GFP is detected in follicle cells mainly at the basal side of the lateral membrane (arrowhead in I'). In stage 11, Fat2-GFP is detected in centrepetal follicle cells (arrow in J') and in nurse cells (arrowhead in J'). C,E,G, and I show higher magnification views (apical is to the top). Scale bars: 20 μm in A,B,D,L; 5 μm in C,E; 40 μm in F,H,J,K; 10 μm in G,I.

cells surrounding the oocyte (Fig. 5B,D). During stages 9-10A, Fat2-GFP was gradually lost from the apical side of follicle cells and, in stage 10B, Fat2-GFP was mainly detected at the basal side of the lateral membrane of follicle cells (Fig. 5F-I). Fat2-GFP was also expressed in migrating border cells (see Fig. S2A in the supplementary material) and polar cells (see Fig. S2B,C in the supplementary material). In stage 11, Fat2-GFP was undetectable in follicle cells surrounding the oocyte except for the centripetral follicle cells present between the oocyte and the nurse cells (Fig. 5J,K). Fat2-GFP was detectable in the remnants of the stretched follicle cells during stage 12 (Fig. 5L). These data reveal a dynamic distribution of Fat2-GFP during oogenesis.

Fat2-GFP distribution is planar polarized at the basal side of follicle cells

The distribution of Lar protein is planar polarized at the basal side of follicle cells in stage 7-8 egg chambers (Bateman et al., 2001). Therefore we tested whether Fat2-GFP distribution is also polarized in the plane of the follicle epithelium. Fat2-GFP was detectable in the same focal plane as the basal actin filaments (Fig. 6A-C). In stage 5, before these actin filaments were strictly oriented perpendicular to the anteroposterior axis, Fat2-GFP was enriched at tricellular junctions (Fig. 6A, see Fig. S3A in the supplementary material). Lar protein was barely detectable at this stage (Fig. 6A). During stages 6 and 7, the time when actin filament orientation becomes polarized, Fat2-GFP was enriched on the plasma membranes that were oriented nearly parallel to the anteroposterior axis of egg chambers, similar to Lar protein (Fig. 6B,C,G; see Table S1 and Fig. S3B in the supplementary material). Fat2-GFP and Lar localized to cell membranes where the parallel actin filaments terminated. These data show that Fat2-GFP protein is planar polarized at the basal sides of follicle cells during early oogenesis. The presence of Fat2-GFP at the sites where actin filaments terminate is consistent with a mechanism whereby Fat2 aligns the orientation of actin filaments perpendicular to the anteroposterior axis by interacting, directly or indirectly, with actin filaments.

Fat2-GFP is restricted to one of the two sides of follicle cells where actin filaments terminate

In the wing, the distribution of many core planar cell polarity proteins is planar polarized and often restricted to one or the other side of the cell (Zallen, 2007). To test whether Fat2 is present at both

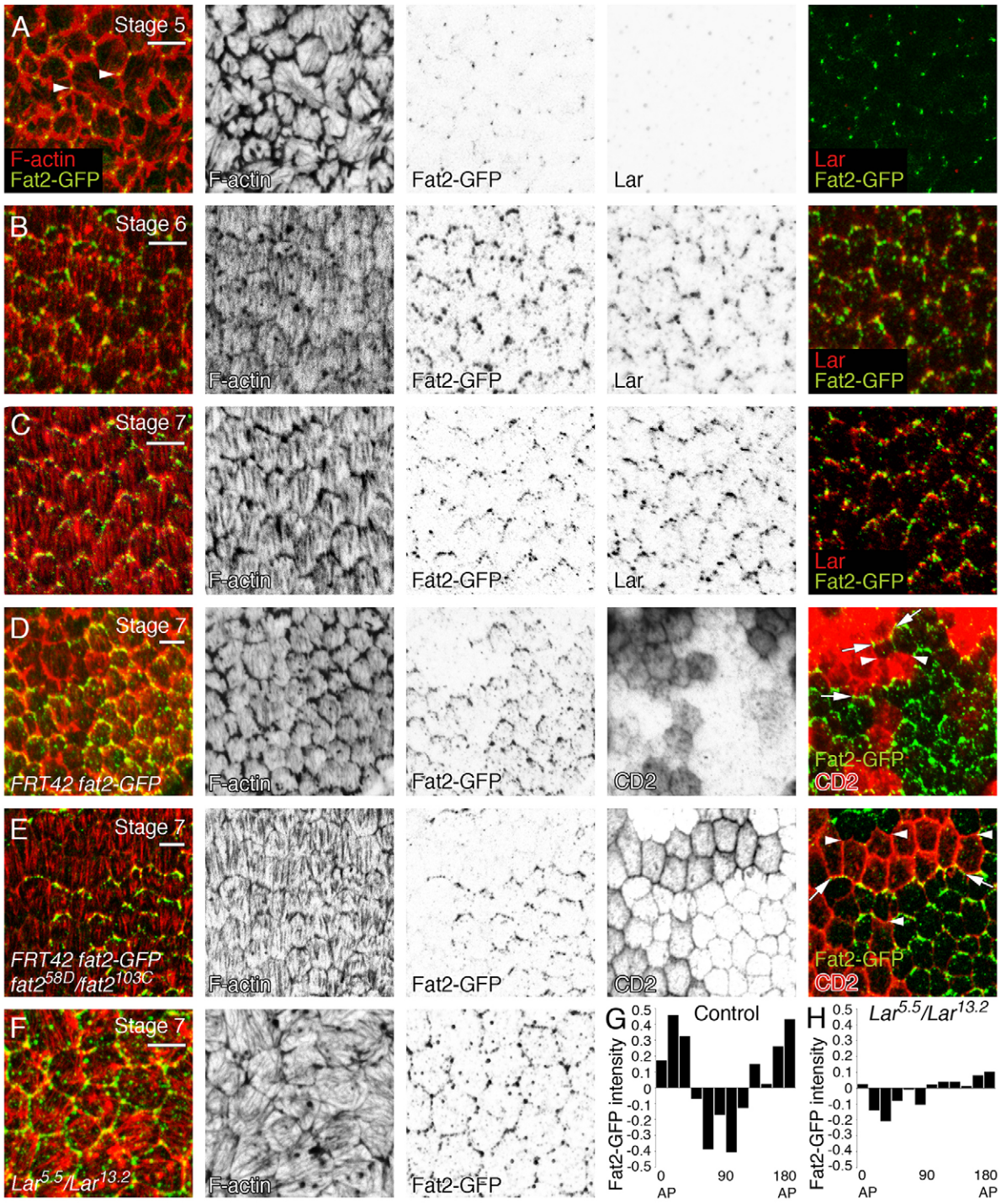

Fig. 6. The distribution of Fat2-GFP is planar polarized. (**A-C**) Egg chambers of the indicated stages of *fat2-GFP* flies stained for GFP, Lar and F-actin. Fat2-GFP is enriched at tricellular junctions during stage 5 (arrowheads in A) and on the plasma membrane domains where planar-oriented actin filaments terminate during stages 6 and 7. (**D**,**E**) Stage 7 egg chambers lacking *fat2-GFP* in clones of cells marked by the elevated levels of CD2 in flies wild type for *fat2* (D) or in $fat2^{58D}/fat2^{103C}$ mutant flies (E). In these images, Fat2-GFP is present at the clonal borders facing to the bottom (arrows), but is absent from the clonal borders facing to the top (arrowheads). Assigning basal as the top of the cell and viewed from anterior to posterior, Fat2-GFP localizes to the left side of the follicle cells in the egg chambers shown. (**F**) Stage 7 egg chamber of a $Lar^{5.5}/Lar^{13.2}$ mutant fly expressing Fat2-GFP and stained for F-actin. Fat2-GFP is distributed evenly on the plasma membrane. (**G**,**H**) The average relative Fat2-GFP pixel intensities for cell membranes oriented in 15° intervals in respect to the anteroposterior axis of stage 7 control egg chambers expressing *fat2-GFP* (G) or $Lar^{5.5}/Lar^{13.2}$ mutant egg chambers expressing *fat2-GFP* (H) are shown. See Table S1 in the supplementary material for a statistical analysis of the data. AP denotes cell membranes nearly parallel to the anteroposterior axis of egg chambers. Control: *n*=367 cell membranes of four egg chambers. Mutant: *n*=417 cell membranes of four egg chambers. In A-F, views of the basal actin filaments are shown. The panels in the left column show merged Fat2-GFP (green)–F-actin (red) channels. The panels in the right column show merged Fat2-GFP (green)–Lar (red) (A-C) or merged Fat2-GFP (green)–CD2 (red) channels (D-E). The middle panels show inverted signals from the individual channels. Scale bars: 5 μm.

sides of a cell where the parallel actin filaments terminate, or whether it is only present at one of these two sides, we used the FRT-Flp system to generate positively marked clones of cells lacking the *fat2-GFP* construct within an follicle epithelium otherwise expressing *fat2-GFP*. As expected, these positively marked clones of cells, which lacked the *fat2-GFP* construct, did not show Fat2-GFP expression (Fig. 6D). The distribution of Fat2-GFP on cell membranes was analyzed in Fat2-GFP-expressing cells facing cells not expressing Fat2-GFP. As shown in Fig. 6D, Fat2-GFP was detectable only on one of the two sides of the cell, left or right, where actin filaments terminated. In a given egg chamber, Fat2-GFP invariably localized to the same side of the Fat2-GFP-expressing cells at the borders of all clones analyzed. A similar result was obtained when the distribution of Fat2-GFP was analyzed in Fat2-GFP-expressing cells facing non-Fat2-GFP expressing cells in $fat2^{58D}/fat2^{103C}$ mutant egg chambers (Fig. 6E), suggesting that the endogeneous Fat2 protein did not interfere with the distribution of Fat2-GFP. These results demonstrate that Fat2-GFP is restricted to one of the two sides of follicle cells where the basal actin filaments terminate and that Fat2-GFP localizes to the same side of each cell throughout the follicle epithelium of a given egg chamber.

Planar-polarized distribution of Fat2-GFP depends on Lar

We next tested whether the planar-polarized distribution of Fat2-GFP at the basal side of follicle cells was dependent on Lar. Flies mutant for *lar* and expressing Fat2-GFP at the same time, were generated and the distribution of Fat2-GFP was analyzed. In contrast to the control flies, Fat2-GFP was no longer enriched on the plasma membranes that were oriented nearly parallel to the anteroposterior axis of $Lar^{5.5}/Lar^{13.2}$ mutant egg chambers (Fig. 6F-H, see Table S1 in the supplementary material). Moreover, Fat2-GFP appeared to be more uniformly distributed on cell membranes at the basal side of follicle cells compared to the controls and was no longer enriched on cell membranes where actin filaments terminated (Fig. 6F). These results demonstrate that the planar-polarized distribution of Fat2-GFP, and the enrichment of Fat2-GFP at sites where actin filaments terminate, depends on Lar.

Fat2 acts non-autonomously in follicle cells to determine normal egg chamber shape and planar-polarized actin filament orientation

We next tested in which cells Fat2 is required: germline cells or somatic follicle cells. $fat2^{58D}$ mutant germline clones, eliminating Fat2 function in the germline, resulted in 14.4% (n=174) spherical stage 14 egg chambers. We attribute this low frequency of spherical egg chambers to the occasional formation of large $fat2^{58D}$ mutant clones in the follicle epithelium that are inevitably generated in parallel with the germline clones. By contrast, generation of large $fat2^{58D}$ mutant cell clones specifically in the follicular epithelium frequently resulted in spherical egg chambers (Fig. 7A,B), demonstrating that Fat2 acts in follicle cells to specify egg chamber shape.

To test whether *fat2* acts cell-autonomously, we generated $fat2^{58D}$ mutant follicle cell clones. Egg chambers fell into two phenotypic classes dependent on the total size of $fat2^{58D}$ mutant cell clones within the follicle epithelium. Egg chambers in which fewer than approximately 60% of all follicle cells were mutant had a shape indistinguishable from the control egg chambers (Fig. 7C,I; see Table S2 in the supplementary material). This was the case irrespective of the position these clones occupied within the follicle epithelium (Fig. 7D). Furthermore, actin filaments were normally oriented in these $fat2^{58D}$ mutant follicle cell clones (Fig. 7E). Some of the phenotypically normal $fat2^{58D}$ mutant clones were as large as ~150 cells; it is therefore unlikely that cells in these clones still contained amounts of Fat2 protein that were sufficient to direct normal actin filament orientation. We conclude that Fat2 is not required cell-autonomously for the planar-polarized orientation of actin filaments in any particular position within the follicular epithelium.

The second phenotypic class was comprised of egg chambers in which more than approximately 60% of all follicle cells were mutant. These egg chambers had a spherical shape indistinguishable from the shape of egg chambers of $fat2^{58D}$ mutant flies (Fig. 7F,I, see Table S2 in the supplementary material). This was again the case irrespective of the position these clones occupied within the follicle epithelium (Fig. 7G). In these mutant cells, basal actin filaments were no longer properly oriented perpendicular to the anteroposterior axis of the egg chamber (Fig. 7H). Strikingly, actin filaments were also no longer properly oriented in the remaining control cells, irrespective of whether the control cells were located immediately adjacent to $fat2^{58D}$ mutant cells or not (Fig. 7H). These data show that, in large clones, *fat2* mutant cells act non-autonomously on the planar polarized orientation of basal actin filaments in wild-type cells. Moreover, these findings suggest that the fraction of wild-type follicle cells to *fat2* mutant follicle cells is important for the global actin filament orientation and egg chamber shape, indicating that the planar-polarized orientation of basal actin filaments involves the orchestrated action of a large number of follicle cells.

DISCUSSION

The planar polarization of cellular structures is an important feature displayed by many epithelia. In this study, we have addressed the role of the cadherin Fat2 in the establishment of planar cell polarity. We show that Fat2 is required for the planar-polarized orientation of actin filaments in follicle cells and the proper shape of egg chambers. Moreover, we found that Fat2-GFP distribution in follicle cells is planar polarized and that Fat2 is required non-cell-autonomously in the follicle epithelium. Our results suggest an important role for Fat2-mediated cell-to-cell interactions in the establishment of planar cell polarity in the follicle epithelium.

Fat2 is not required for apical-basal polarity or oocyte polarity

fat2 mutants share defects with *Lar* and integrin mutants (*mys*, *mew*, *if*) in establishing planar cell polarity in the follicle epithelium. However, *Lar* and integrin mutants display additional phenotypes in follicle cells not observed in *fat2* mutants, indicating that Fat2 acts independently of Lar and integrins in various processes that might not be related to planar cell polarity. *Lar* mutants, for example, are associated with oocyte polarity defects and defects in the number and localization of polar cells (Frydman and Spradling, 2001). Although we detect Fat2-GFP in the oocyte and polar cells, both oocyte polarity, polar cell number and localization appear normal in *fat2* mutants. Integrin mutants, in addition to failing to properly organize basal actin filaments, display apical-basal defects and multi-layering of the follicle epithelium (Fernandez-Minan et al., 2007), defects not observed in *fat2* mutants (Fig. 3, and data not shown). *fat2*, *Dystroglycan* and *Dystrophin* mutants, however, all display defects in the formation of the posterior cross vein in wings (Christoforou et al., 2008) (see Fig. S4A,B in the supplementary material), indicating that Fat2, Dystroglycan and Dystrophin also might play a common role during wing development.

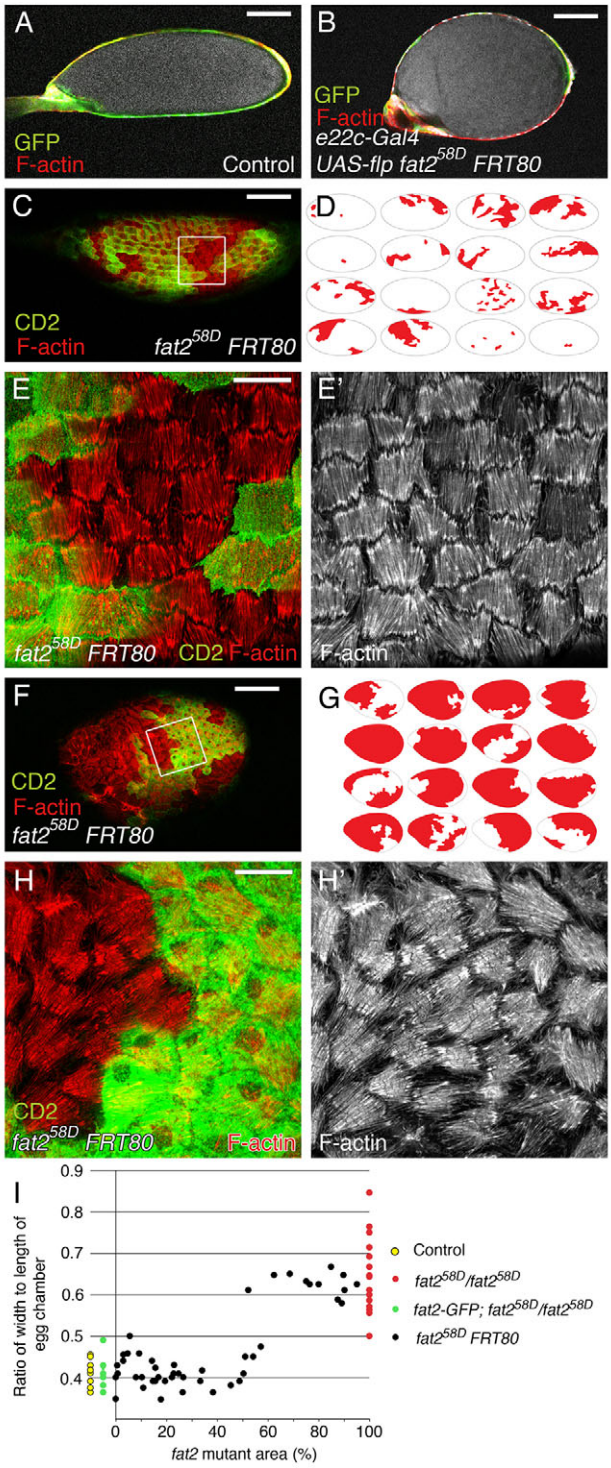

Fig. 7. *fat2* is required non-cell-autonomously in follicle cells. (**A**,**B**) A control stage 14 egg chamber (A) and an egg chamber displaying large clones of *fat2*^{58D} mutant cells (B), marked by the absence of GFP (green), specifically generated in the follicular epithelium (*e22c-GAL4 UAS-flp fat2*^{58D} *FRT80*). (**C**) Small clones of *fat2*^{58D} homozygous mutant cells of a stage 12 egg chamber stained for F-actin (red) and marked by the absence of CD2 (green). (**D**) Schemes of single confocal planes of normal shaped egg chambers displaying small *fat2* mutant clones (red). Note that due to the ellipsoid shape of the egg chamber each confocal plane visualizes approximately 30-40% of the total follicle epithelium. (**E**,**E′**) Magnified view of the basal actin filaments of the area indicated by a square in C. (**F**) A large clone of *fat2*^{58D} homozygous mutant follicle cells of a stage 12 egg chamber stained for F-actin (red) and marked by the absence of CD2 (green). (**G**) Schemes of single confocal planes of abnormal shaped egg chambers displaying large *fat2* mutant clones (red). (**H**,**H′**) Magnified view of the basal actin filaments of the area indicated by a square in F. (**I**) The ratio of width to length of stage 12 egg chambers with different percentages of total *fat2*^{58D} mutant clone area observed in a single confocal plane (black dots). The width-to-length ratios of stage 12 control (yellow), *fat2*^{58D} mutant (red) and *fat2*^{58D} mutant egg chambers expressing *fat2-GFP* (green) are shown for comparison. Scale bars: 100 μm in A-C,F; 20 μm in E,H.

Fat2 distribution is planar polarized

Planar-polarized orientation of basal actin filaments arises gradually during stages 5-6 of egg chamber development and is fully established by stage 7 (Frydman and Spradling, 2001). Establishment of planar-polarized actin filament orientation parallels a redistribution of Fat2, as visualized by Fat2-GFP. Fat2-GFP, at stage 5, is initially enriched at the tricellular junctions between follicle cells. By stages 6 and 7, however, Fat2-GFP is preferentially distributed along the cellular junctions at which the oriented actin filaments terminate. This result, taken together with our observation that actin filament orientation fails to be established in *fat2* mutant egg chambers, indicates that Fat2 plays a role in the initial establishment of planar-polarized actin filament orientation. The localization of Fat2 to sites where actin filaments terminate is consistent with a mechanism whereby Fat2 directs actin filament orientation by interacting, directly or indirectly, with actin filaments. Of note, mammalian Fat1, which is required for renal slit junction formation and normal development of the eye and forebrain (Ciani et al., 2003), has previously been shown to control actin polymerization by binding to Ena/vasodilator-stimulated phosphoprotein (VASP) (Moeller et al., 2004; Tanoue and Takeichi, 2004). The binding sites for the Ena/VASP homology 1 (EVH1) domain of Ena/VASP proteins, present in mammalian Fat1, are, however, not conserved in *Drosophila* Fat2 (Moeller et al., 2004; Tanoue and Takeichi, 2004) (data not shown).

Members of the cadherin superfamily can form homophilic or heterophilic interactions through their extracellular cadherin repeats with cadherin molecules on neighboring cell membranes at cellular junctions (Pokutta and Weis, 2007). By using mosaic expression of Fat2-GFP in follicle cells, we found that Fat2-GFP was detectable at the lateral plasma membrane only on one of the two sides of follicle cells where the basal actin filaments terminate. This result, therefore, is consistent with the view that Fat2 does not form homophilic interactions between neighboring follicle cells at the basal side of the lateral membrane. As Fat2-GFP appears to localize to the same side of each cell throughout the tissue, this data furthermore suggest that a unique direction perpendicular to the anteroposterior axis is specified in the follicle epithelium early during oogenesis.

A non-cell-autonomous function for Fat2

Mutations affecting the planar-polarized orientation of basal actin filaments in follicle cells fall in two classes: *Dystroglycan* or *Dystrophin* mosaic mutants show strictly cell-autonomous defects in the orientation of basal actin filaments (Mirouse et al., 2009). By contrast, in *Lar* and *myospheroid* (*mys*, encoding PSβ-integrin) mosaic mutants, both mutant and neighboring wild-type cells display abnormal actin filament orientation (Bateman et al., 2001;

Frydman and Spradling, 2001). This non-cell-autonomous behaviour is also observed in mosaic *fat2* mutants, indicating that Fat2, like Lar and PSβ-integrin, is required for the transmission of polarity information.

Two observations indicate that Fat2 is not required for the local transmission of polarity information. First, the polarized orientation of actin filaments remains normal within small *fat2* mutant follicle cell clones. Secondly, in *fat2* mutants, orientation of actin filaments is not randomized, but neighboring cells frequently display a parallel organization of actin filaments. Our observation that the fraction of wild-type follicle cells to *fat2* mutant follicle cells is important for actin filament orientation, indicates that the planar-polarized orientation of basal actin filaments involves the orchestrated action of a large number of follicle cells, and that Fat2 is required in this process.

Non-autonomy and local coordination of planar cell polarity are also two features of mutant clones of planar cell polarity genes such as *fat* or *frizzled* in the *Drosophila* wing (Strutt and Strutt, 2002; Vinson and Adler, 1987). These observations indicate that wing cells and follicle cells might use a conserved molecular logic to communicate planar polarity information.

Fat-like cadherins mediate planar cell polarity in wing and follicle cells

In wings, pathways including Fat and Dachsous, and the core planar cell polarity proteins Frizzled, Dishevelled, Diego, Prickle and Strabismus/Van Gogh are required for the planar-polarized orientation of hairs (Adler et al., 1998; Feiguin et al., 2001; Gubb and Garcia-Bellido, 1982; Gubb et al., 1999; Ma et al., 2003; Taylor et al., 1998; Vinson and Adler, 1987; Wolff and Rubin, 1998). Fat2 (see Fig. S4C,D in the supplementary material) and Lar (Frydman and Spradling, 2001) are dispensable for this process. By contrast, Fat2 and Lar are required for the planar-polarized orientation of actin filaments in follicle cells. By using mutant analysis, we did not find evidence for a role of Fat, Dachsous, and the core planar cell polarity proteins Dishevelled, Diego, Prickle and Strabismus/Van Gogh, in establishing the planar-polarized orientation of actin filaments at the basal side of follicle cells or in the elongation of the egg chamber (see Fig. S5 in the supplementary material). Thus, it appears that there are at least two largely distinct pathways required for the establishment of planar cell polarity in wings and follicle cells. One pathway, dependent on Frizzled, Dishevelled and other core planar cell polarity proteins, is required to establish planar cell polarity in the wing. A second pathway, involving Lar, integrins and Dystroglycan, establishes planar cell polarity in the follicle cells. The only proteins known to act in the establishment of planar cell polarity in both wings and follicle cells are Fat-like cadherins. These findings suggest that Fat-like cadherins in general play an important role in the establishment of planar cell polarity.

Integrins, Lar, and the Dystroglycan complexes are known to interact both with extracellular matrix proteins and the actin cytoskeleton (Barresi and Campbell, 2006; Bokel and Brown, 2002; Frydman and Spradling, 2001), suggesting an important role for interactions between the extracellular matrix and the actin cytoskeleton for the planar polarization of follicle cells. Furthermore, similar to basal actin filaments, Laminin A, a component of the extracellular matrix, is polarized perpendicular to the long axis of the egg chamber (Gutzeit et al., 1991), and mutations in *LanA*, the gene encoding Laminin A, result in spherical eggs (Frydman and Spradling, 2001). Our finding that Fat2, a member of the cadherin superfamily of proteins, is required for planar-polarized orientation of actin filaments in follicle cells suggests that also cell-to-cell interactions are important for establishing planar cell polarity in the follicle epithelium.

In addition to its role in planar-polarizing wing hairs, Fat is also required for the proper shape of the wing (Garoia et al., 2000; Ma et al., 2003; Mohr, 1923). Likewise, we report here that *fat2* is also required for normal planar cell polarity and tissue shape in the ovary. It is intriguing to speculate that Fat-like cadherins provide a common mechanistic link between tissue shape and planar cell polarity in both tissues.

In summary, we found that Fat2, like Fat, is required to establish a planar polarity of cells, indicating that Fat-like cadherins may play a principle role in this process. It will, therefore, be interesting to test whether vertebrate Fat1, Fat2 and Fat3, which are related to *Drosophila* Fat2, are also involved in establishing planar cell polarity.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/24/4123/DC1

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