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Dystroglycan is required for polarizing the epithelial cells and the oocyte in *Drosophila*

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SUMMARY

The transmembrane protein Dystroglycan is a central element of the dystrophin-associated glycoprotein complex, which is involved in the pathogenesis of many forms of muscular dystrophy. Dystroglycan is a receptor for multiple extracellular matrix (ECM) molecules such as Laminin, agrin and perlecan, and plays a role in linking the ECM to the actin cytoskeleton; however, how these interactions are regulated and their basic cellular functions are poorly understood. Using mosaic analysis and RNAi in the model organism *Drosophila melanogaster*, we show that Dystroglycan is required cell-autonomously for cellular polarity in two different cell types, the epithelial cells (apicobasal polarity) and the oocyte (anteroposterior polarity). Loss of *Dystroglycan* function in follicle and disc epithelia results in expansion of apical markers to the basal side of cells and overexpression results in a reduced apical localization of these same markers. In *Dystroglycan* germline clones early oocyte polarity markers fail to be localized to the posterior, and oocyte cortical F-actin organization is abnormal. Dystroglycan is also required non-cell-autonomously to organize the planar polarity of basal actin in follicle cells, possibly by organizing the Laminin ECM. These data suggest that the primary function of Dystroglycan in oogenesis is to organize cellular polarity; and this study sets the stage for analyzing the Dystroglycan complex by using the power of *Drosophila* molecular genetics.

Key words: Polarity, Axis, Asymmetry, Oogenesis, Epithelia, Dystroglycan, Actin, Microtubule, ECM, Planar polarity, Signaling, *Drosophila*

INTRODUCTION

Muscular dystrophies are genetic disorders that are characterized by progressive muscle degeneration. These diseases are caused by mutations in different members of the Dystrophin-associated glycoprotein complex (DGC), which is composed of multiple cytocortical, transmembrane and extracellular proteins (Burton and Davies, 2002; Henry and Campbell, 1999; Winder, 2001). How these mutations cause the observed muscle defects is not fully understood and no cures for the diseases exist. In addition to muscle cells, the DGC is required in other cell types such as epithelial cells and neural cells (Durbeej et al., 1995; Durbeej and Campbell, 1999; Williamson et al., 1997; Michele et al., 2002; Moore et al., 2002). Reduced expression of the DGC components is observed in breast and prostate cancers (Henry et al., 2001a). Dystroglycan (DG), a transmembrane protein, is a central player of the DGC. It acts as a receptor for the extracellular matrix (ECM) component Laminin (Ibraghimov-Beskrovnaya et al., 1992), and connects to the actin cytoskeleton through an actin-binding protein, Dystrophin. The cellular function and regulation of these interactions remain elusive, however. *Drosophila melanogaster* is an excellent model organism with which to study basic cellular functions of evolutionarily conserved genes, particularly human disease genes (Bernards and Hariharan, 2001). We have analyzed the DG homolog in *Drosophila* and shown that it is required for the establishment of cellular polarity.

How polarity is established at a cellular level is one of the most fundamental questions in biology. Many cell types undergo certain degrees of polarization to fulfill their specific functions. For example, neurons polarize to form axons and dendrites in order to convey signals; polarization of T cells is needed for their migration. Epithelial cells, however, have a pronounced apicobasal polarity, which is needed for them to cope with different extracellular environments. Studies using genetic model systems such as *Drosophila* and mammalian culture cells have revealed three groups of protein complexes that are involved in the specification and regionalization of the plasma membrane and cortex of the polarized epithelium: Crumbs, Par and Lgl complexes (Tepass et al., 2001). The transmembrane protein, Crumbs (Crb) and its cytoplasmic-

binding partners, the PDZ domain proteins Discs Lost (Dlt) and Stardust as well as the Par-complex [Bazooka(Par3)/DmPar6(Par6)/atypical protein kinase C (aPKC)] are located on the apical membrane and are required for the establishment of this domain (Bachmann et al., 2001; Bhat et al., 1999; Hong et al., 2001; Petronczki and Knoblich, 2001; Tepass et al., 1990; Wodarz et al., 2000). The Lgl-complex [Lethal Giant Larvae (Lgl)/Discs Large (Dlg)/Scribble (Scrib)] is located at the lateral region of the epithelium and is required to restrict Crb to the apical side (Bilder and Perrimon, 2000; Bilder et al., 2000; Woods and Bryant, 1991).

Cell polarity can also be the basis for a body axis. In *Drosophila*, the body polarity is built upon the polarity of the oocyte, and in *C. elegans*, polarization of the single-cell embryo determines the anteroposterior (AP) body axis (reviewed by Wodarz, 2002). In fact, the process of polarity formation in the developing *Drosophila* oocyte provides an excellent model with which to study how the polarity of the cytoskeleton is dynamically regulated. The AP asymmetry of the oocyte cytoskeleton, which is the basis for morphogen localization within different compartments of the egg, is established by a series of dynamic steps (reviewed by Riechmann and Ephrussi, 2001). First, centrioles and the microtubule organizing center (MTOC) are located at the anterior end of the oocyte at stage 1. By stage 3 the MTOC has moved to the posterior of the oocyte. This posterior movement of the MTOC requires function of the Par proteins (Par-complex and Par1) and the action of *maelstrom* gene product (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b; Clegg et al., 2001). At stage 6, posterior follicle cells send an unidentified signal back to the oocyte to re-orient the oocyte microtubule (MT) polarity, which requires the function of an ECM protein, Laminin (Deng and Ruohola-Baker, 2000). At each step, the proper MT polarity is required for localization of key molecules in the oocyte. In addition to MTs, the oocyte has an enriched cortical array of actin cytoskeleton that plays an important role in localizing posterior morphogens in the oocyte (Baum et al., 2000; Erdelyi et al., 1995).

Although the oocyte and the epithelial cells differ profoundly in their morphology and function, polarization of these two cell types uses some of the same genes. For example, the Par genes are required to establish the polarity of both the oocyte and epithelial cells (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b; Petronczki and Knoblich, 2001; Wodarz et al., 2000). This similarly raises the possibility that some common strategies may exist for cellular polarization. We show that DG, a receptor for multiple ECM proteins, is required cell-autonomously to polarize both the epithelial cells and the oocyte in *Drosophila*. We also show a separate, non-cell-autonomous function for DG: disruption of DG affects the organization of the basal actin cytoskeleton in neighboring cells, which suggests the involvement of DG in cell-cell communication.

MATERIALS AND METHODS

Identifying the *Drosophila Dystroglycan* gene

Drosophila Dystroglycan (*Dg*) gene was identified by EP (Rørth, 1996) screen and independently by homology to mouse DG using the Protein Blast program. LD04782 that maps to the genomic region identified by this search contains the 3′ half of the gene. We therefore isolated several cDNAs from the 5′ region by PCR using an embryonic cDNA library. The 5′ end of *Dg* was mapped near the insertion site of EP(2)2241 and ca.4 kb downstream of *Rho1* (Fig. 1A). Several EST 5′ sequences overlap the 5′ UTR of *Dg*. We analyzed the following ESTs: LD11619, GH09323 and SD06707. We fully sequenced LD11619 and partially sequenced three of the cDNAs isolated and found the intron-exon structure to be different from the Gadfly prediction in two incidents (Gadfly annotation has been corrected). We further noticed that at least two exons [exon 8 (265 amino acids) and 9 (83 amino acids)] are subjected to alternative splicing (Fig. 1A). We tested five different cDNAs for presence of exon 8 and 9. Two lacked exon 9, among them LD11619 (1179 amino acids). Two lacked both exon 8 and exon 9, among them SD06707 (914 amino acids). One lacked exon 8 (GH09323, 997 amino acids). None of the cDNAs tested contained both exon 8 and exon 9 (Fig. 1A shows the exon/intron structure and the alternatively spliced exons).

Isolation of deletion mutants at the *Dg* locus

Drosophila melanogaster stocks were raised on standard cornmeal-yeast-agar medium at 25°C. *Dg* alleles Dg^{248}, Dg^{62} and Dg^{323} were obtained by imprecise excision of EP(2)2241 (Fig. 1A). EP(2)2241 was mobilized by crossing the line to a Δ2-3 transposase containing line. 330 White-eyed flies of the F1 generation were established as balanced stocks, and the homozygous lethal (12%) or semi-lethal lines (5%) were tested for complementation with a *CG8414*-allele (EP(2)0525), a deficiency line *Df(2)*JP6 and a *Rho1* allele ($Rho1^{E3.10}$) (Halsell et al., 2000). All three alleles contain deletions that remove the putative transcription start site and the 5′UTR of the *Dg* gene (Fig. 1A). The breakpoints of the deletion mutants were mapped by PCR using combinations of the following genomic primers: forward, GATCAGGGCCAAGGTGTGTCCAGC and AAGCCGCTTTGG-CGTTGC; reverse, GCTCACTCCCACACAAGCGC and GAGCC-CAATGATCCGTGGAAAGCG.

PCR fragments including the breakpoints of Dg^{248} and Dg^{323} were sequenced. Dg^{248} contains a 785 bp deletion between bp 32,514 and bp 33,299 of DS03910. Fifteen basepairs of the inverted repeat of the P element are still present (lowercase in Fig. 1). An A to T mutation and a 2 bp deletion is found at the distal breakpoint: GGA-GCATTCCTTGCT--ATGTTatgttatttcatcatgGGCAGGAGAGTCCCG-AAT.

Dg^{323} contains a 3155 bp deletion between bp 32,345 and bp 35,669 of DS03910. A C to T change was found near the proximal breakpoint: AAAATGGCAGCGTACTTTCG|TTTTGCTTTGCGC-TTCTCTG.

Construction of the transgenic animals with DG-hairpin

The cDNA corresponding to the cytoplasmic domain and 670 bp of the 3′ UTR of *Drosophila* Dystroglycan (CTGTTGCCTGCA to TTGCTTGCATGTTTTTTTTTTTT) was directionally cloned into the *Kpn*I/*Hin*dIII sites in pBluescript II (Stratagene) to form an intermediate vector pKS-dg. The 1 kb *Kpn*I/*Bam*HI fragment of pKS-dg was excised and subcloned into pEGFP-N1 (Clontech) and then digested with *Nhe*I and *Bam*HI. The *Nhe*I/*Bam*HI fragment was inserted together with the 148 bp Sau3A fragment of pEGFP-N1 back into the *Bam*HI/*Spe*I-digested pKS-dg (triple ligation). The *Kpn*I 2.2 kb fragment was subcloned into pUAST. Thus, we constructed a *Dg* hairpin-loop plasmid (pUAST-dg-L-gd). The construct was verified by sequencing and then injected to embryos to obtain stable transformant lines, *UAS-dsDG-RNAi* (*dsDG*). To drive expression of *dsDG* in animals, we crossed the transgenic flies with the *tubP-Gal4* line (Lee and Luo, 1999), which shows ubiquitous expression in follicle cells. *tubP-Gal4/dsDG* causes reduction of DG expression, leading to semi-lethality, consistent with the fact that dg deletion alleles are homozygous lethal. However, some *tubP-Gal4/dsDG* escapers were observed and analyzed for their oogenesis phenotypes.

Loss-of-function mosaic analysis

In order to generate mutant cell clones, *Dg* alleles were recombined to the FRT chromosome (Xu and Rubin, 1993). To obtain follicle cell clones, 1- to 5-day-old flies were heat-shocked as adults for 60 minutes at 37°C and put in freshly yeasted vials with males for 2 or 3 days. To obtain germline clones, 2- to 3-day-old larvae were heat-shocked at 37°C for 2 hours each time during 2 consecutive days. Ovaries from adult female flies at 3-5 days of age were harvested. To generate mutant clones in imaginal discs, the flies were allowed to lay eggs for 24 hours at 25°C and the eggs were allowed to develop 48 hours at 25°C. Thereafter, the larvae were heat shocked for 30-40 minutes at 38°C and returned to 25°C. After 2 days, wandering third instar larvae were collected and dissected for antibody staining.

Dystroglycan antibody production

Three antibodies against DG protein were raised in rabbits: one against the extracellular domain corresponding to exon 8 (amino acids 243-507) and two against the intracellular regions (one against 18 C-terminal amino acids and one against 102 C-terminal amino acids). Most of the staining shown in this paper use the antibody raised against 102 C-terminal amino acids. DG cytoplasmic domain (102 amino acids) was synthesized by PCR using the primer pair: CGGGATCCAAAGGAGCGGCAAAATGGAG and GCTCTAGA-AAGCGGCCGCCGTACGTCCCAGTAAGT (Gibco BRL) and the template GH09323. The PCR product was digested using *Bam*HI and *Not*I and the fragment was cloned in vector pGEX5X. The DGcyto-GST fusion protein was produced in JM109 after induction with IPTG. Then bacteria were harvested and lyzed by French Press. To purify the fusion protein, GST resin was used to bind the protein, and 10 mM gluthione 50 mM Tris (pH 7.5) was used to elude the protein. Polyclonal antisera were produced by R & R rabbitury, and affinity purified by the fusion protein.

Overexpression of Dystroglycan

UAS-DG was constructed by cloning the *Kpn*I/*Not*I insert of an EST clone LD11619 into the pUAST transformation vector. UAS-DGcyto contains a tandem Flag tag sequence inserted at amino acid 37 of DG (amino acids 1-27 constitute the putative signal peptide) fused to the transmembrane (starting WPIVI…) and cytoplasmic domains. The constructs were injected to embryos to obtain stable transformant lines. The UAS-DG and UAS-DGcyto fly-lines were then crossed to different Gal4 driver lines.

To generate DG overexpression follicle cell clones, *hsFLP*, *UAS-Dg* males were crossed to virgin female *act<FRT-CD2-FRT<Gal4; UASGFP* flies (Pignoni and Zipursky, 1997). The F_1 progeny were heat shocked at 37°C for 1 hour and raised at 25°C for 3 days, dissected and analyzed.

Histocytochemistry

Ovarian antibody staining and confocal microscopy was performed as described previously (Deng et al., 2001). Basal actin staining was performed according to the protocol provided by Frydman (Frydman and Spradling, 2001). Imaginal disc staining was as described previously (Woods et al., 1997). A two-photon laser scanning microscope (Leica TCS SP/MP) was used to detect DAPI staining.

The following antibodies were used: rabbit anti-Dlg (1:500) (Woods and Bryant, 1991); mouse anti-Orb (1:20) (Lantz et al., 1994); rabbit anti Baz (1:500) (Wodarz et al., 2000); mouse anti-Crb (CQ4,1:20) (Tepass et al., 1990); rabbit anti DG (1:3000; this study); mouse or rabbit anti-βGal (1:5000, Sigma); mouse anti-Neurotactin (BP102; 1:20) (Hortsch et al., 1990); rabbit anti-Laminin (1:3000) (Fessler et al., 1987); ant-Dlt (1:1000) (Bhat et al., 1999); anti-β-HSpectrin (1:1000) (Thomas and Kiehart, 1994); Alexa 488, 568 or 633 goat anti-mouse (Molecular Probes); Alexa 568 or 633 Goat anti-Rabbit or Rat (Molecular Probes); and Alexa 568 or 633 Phalloidin (Molecular Probes). DAB staining was done with the Vectastain Kit (Vector Laboratories, CA).

RESULTS

Dystroglycan gene structure and protein

To identify genes that affect the polarity of the *Drosophila* oocyte, we used the EP/Gal4 system (Rørth, 1996) to screen for genes that, when overexpressed in follicle cells, cause a polarity defect in the underlying oocyte. From the over 2000 EP insertions screened, two components of the mammalian DGC were identified: Laminin A (Deng and Ruohola-Baker, 2001) and *Drosophila* DG (*EP(2)2241*; Fig. 1A). An independent homology search with mouse DG protein verified the identification of CG18250 as the *Drosophila* homolog of the mammalian *Dystroglycan* gene.

Conceptual translation of the longest cDNA (LD11619) reveals an open reading frame of 1179 amino acids (Fig. 1A). This deduced *Drosophila* DG protein contains all the hallmarks of vertebrate DG: a mucin-like domain, a transmembrane domain and a C-terminal region with WW-, SH2- and SH3-binding domains (Fig. 1B,D). The best conserved region between human and *Drosophila* is the C-terminal half of the protein showing 31% identity (Fig. 1B). The last 12 amino acids of the C terminus include the WW domain-binding motif (PPxY), which is the Dystrophin binding site. Of 12 amino acids within the C terminus, 10 are perfectly conserved in *Drosophila* (Fig. 1C). Vertebrate DG contains a second PPxY motif in its cytoplasmic domain, which is also conserved in *Drosophila*. In addition, two of the six putative SH3 binding sites and all three SH2-binding sites in the cytoplasmic domain of vertebrate DG can be found in *Drosophila* (Fig. 1D). The putative *C. elegans* homolog DGN-1 (T21B6.1) shows 20% identity to *Drosophila* in the C-terminal half. However, T21B6.1 contains no mucin-like domain, Dystrophin-binding site or second PPxY motif (Fig. 1D).

To analyze the expression pattern of DG protein, we raised antibodies against the cytoplasmic domain. Five major bands can be detected on a western blot of wild-type embryonic extracts: 75 kDa, 105 kDa, 120 kDa, 180 kDa and 200 kDa (Fig. 1E). None of these major bands could be seen in the extracts from the deficiency [*Df(2R)JP4*, *Df(2R)JP6*) embryos that completely delete the *Dg* locus, suggesting that all five forms are specific for DG (Fig. 1E)]. Strong *Dg* mutants were isolated by imprecise excisions of *EP(2)2241* element and by generating a transgenic line expressing a double-stranded DG-RNA construct that destroys DG RNA by RNAi-mechanism (Kennerdell and Carthew, 2000) (Fig. 1A). In Dg^{248} or Dg^{323} mutant embryos, of the five major bands derived from the *Dg* locus only the 105 kDa band can be detected weakly (Fig. 1E), indicating that the level of DG expression is highly reduced in these mutants. Furthermore, to test the specificity of the antibodies in tissue samples we analyzed the expression in the follicle cell epithelium. A high level of DG is observed on the basal side of the epithelium, while a lower level is detected on the apical side. This signal is absent in follicle cell clones homozygous for Dg^{248} or Dg^{323}, suggesting that the signal observed with the antibody in the tissue is specific for DG (arrow in Fig. 1F). Similarly, Dystroglycan protein level was highly reduced or patchy because of the expression of DG-RNAi construct (*tubP-Gal4/dsDG*) in follicle cells (Fig. 1G and data not shown).

Dystroglycan is required for apical-basal polarity in epithelial cells

Since DG is highly expressed in the follicle cells, we first asked whether *Drosophila* DG plays a role in establishing or maintaining epithelial morphology in this tissue. The follicle cell epithelium (FE) has a typical apical-basal polarity, with its apical side facing the germline cells (Fig. 2A). As all follicle cells are derived from two to three somatic stem cells, mosaic analysis provides an excellent tool with which to study gene functions in epithelial development (Margolis and Spradling, 1995).

We employed the FLP/FRT system (Xu and Rubin, 1993) to generate follicle cell clones of all three *Dg* alleles and applied the Gal4/UAS-mediated RNAi technique (Kennerdell and Carthew, 2000) to silence DG expression in all follicle cells (*tubP-Gal4/dsDG*). Similar phenotypes are observed in these different *Dg* mutant backgrounds. Some mutant cells lost their epithelial appearance and formed multiple layers (Fig. 2B,E), a typical terminal phenotype for polarity defects in epithelial cells. Within the multi-layer groups, the mutant cells from the mosaic egg chambers were frequently excluded from the layer that contacts the germline cells. Discontinuity of the epithelium

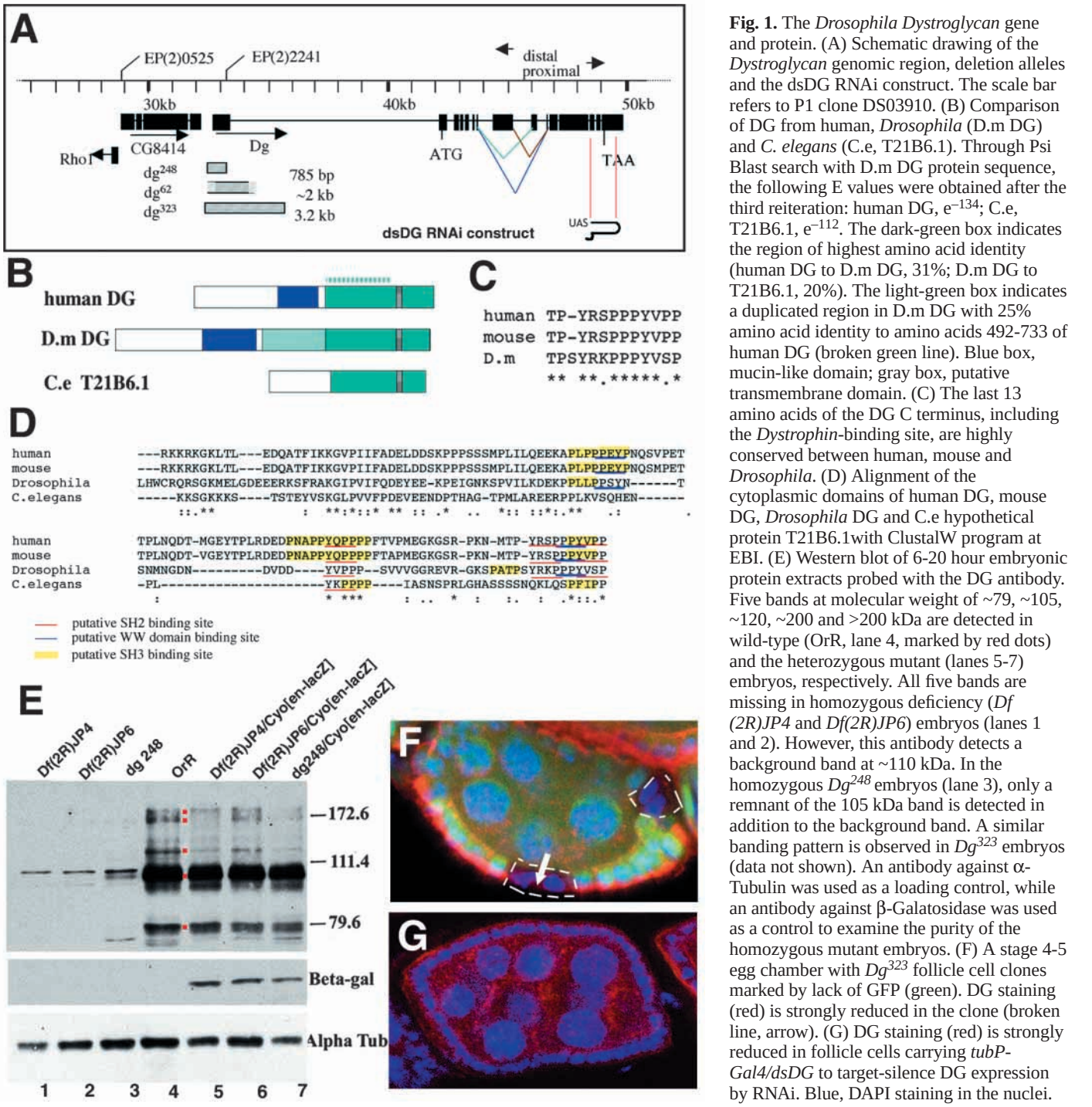

Fig. 1. The *Drosophila Dystroglycan* gene and protein. (A) Schematic drawing of the *Dystroglycan* genomic region, deletion alleles and the dsDG RNAi construct. The scale bar refers to P1 clone DS03910. (B) Comparison of DG from human, *Drosophila* (D.m DG) and *C. elegans* (C.e, T21B6.1). Through Psi Blast search with D.m DG protein sequence, the following E values were obtained after the third reiteration: human DG, e^{-134}; C.e, T21B6.1, e^{-112}. The dark-green box indicates the region of highest amino acid identity (human DG to D.m DG, 31%; D.m DG to T21B6.1, 20%). The light-green box indicates a duplicated region in D.m DG with 25% amino acid identity to amino acids 492-733 of human DG (broken green line). Blue box, mucin-like domain; gray box, putative transmembrane domain. (C) The last 13 amino acids of the DG C terminus, including the *Dystrophin*-binding site, are highly conserved between human, mouse and *Drosophila*. (D) Alignment of the cytoplasmic domains of human DG, mouse DG, *Drosophila* DG and C.e hypothetical protein T21B6.1with ClustalW program at EBI. (E) Western blot of 6-20 hour embryonic protein extracts probed with the DG antibody. Five bands at molecular weight of ~79, ~105, ~120, ~200 and >200 kDa are detected in wild-type (OrR, lane 4, marked by red dots) and the heterozygous mutant (lanes 5-7) embryos, respectively. All five bands are missing in homozygous deficiency (*Df(2R)JP4* and *Df(2R)JP6*) embryos (lanes 1 and 2). However, this antibody detects a background band at ~110 kDa. In the homozygous Dg^{248} embryos (lane 3), only a remnant of the 105 kDa band is detected in addition to the background band. A similar banding pattern is observed in Dg^{323} embryos (data not shown). An antibody against α-Tubulin was used as a loading control, while an antibody against β-Galatosidase was used as a control to examine the purity of the homozygous mutant embryos. (F) A stage 4-5 egg chamber with Dg^{323} follicle cell clones marked by lack of GFP (green). DG staining (red) is strongly reduced in the clone (broken line, arrow). (G) DG staining (red) is strongly reduced in follicle cells carrying *tubP-Gal4/dsDG* to target-silence DG expression by RNAi. Blue, DAPI staining in the nuclei.

was also visible in egg chambers containing *Dg* follicle cell clones but not in control egg chambers (Fig. 2B) (yellow arrow). These phenotypes are similar to loss-of-function phenotypes of *crb*, *dlt*, *dlg* or *lgl* in follicle cells (Tanentzapf et al., 2000; Bilder et al., 2000) and suggest that DG is required for proper epithelial polarity. The mutant follicle cells eventually died off, as we rarely saw mutant clones 9-10 days after heat shock, while sister clones (twin spots) were readily observed.

To characterize the apicobasal polarity defect in more detail, we examined the expression and distribution of molecular markers in mutant cells that still maintained their columnar shape (Table 1). In *Dg* follicle cell clones and *tubP-Gal4/dsDG* follicle cells, mislocalization of apical markers, Dlt and β-Heavy-Spectrin (βH-Spec) (Table 1) was observed. Instead of a strict apical localization, Dlt and βH-Spec were present at both the apical and basal sides of the mutant epithelia (Figs 2C,C′,F′,F″). Dlg, a basolateral marker, exhibited a significant reduction of staining in the basolateral domain in *Dg* RNAi follicle cells (Fig. 2G′). The function of DG in apicobasal polarity formation was not restricted to the FE, as mislocalization of Dlt to the lateral and basal sides was also observed in the mutant epithelial cells in an antennal disc (Fig. 2D, arrows). Taken together, these results suggest that DG is required in different epithelial cells for proper formation or maintenance of apicobasal polarity.

Overexpression of Dystroglycan in epithelial cells disrupts the localization of apical markers

To ask whether DG, when overexpressed, is sufficient to interfere with the epithelial cell polarity we used two UAS constructs, the full-length DG-construct (*UAS-DG*; Fig. 3A) and the short construct with cytoplasmic and transmembrane domains (*UAS-DGcyto*; Fig. 3B) and expressed them in the FE and in the embryonic salivary glands (Fig. 3). Both constructs expressed proteins of the expected sizes (Fig. 3A,B) and were induced by the following Gal4 driver lines: *daughterlessGal4* (*daGal4*), for maternal expression: *elavGal4*, for the salivary gland expression; and the flip-out Gal4 system (Pignoni and Zipursky, 1997) for the FE expression. Similar defects in epithelial polarity were observed with all three drivers.

In wild-type salivary glands, Crb was localized to the apical membrane of the epithelium, facing the lumen of the gland (Fig. 3D,G), while DG expression was undetectable (Fig. 3G). Embryos that overexpress DG showed strong ectopic DG staining on both the apical and basolateral membranes of the salivary gland (Fig. 3H). In about 75% of these salivary glands, the expression of Crb was strongly reduced (n=50; Fig. 3E,H,H′). Whereas Crb localization was disrupted by overexpression of full-length DG (*UAS-DG*), it was unaffected by overexpression of the form of DG lacking the extracellular domain (*UAS-DGcyto*; data not shown). These results suggest

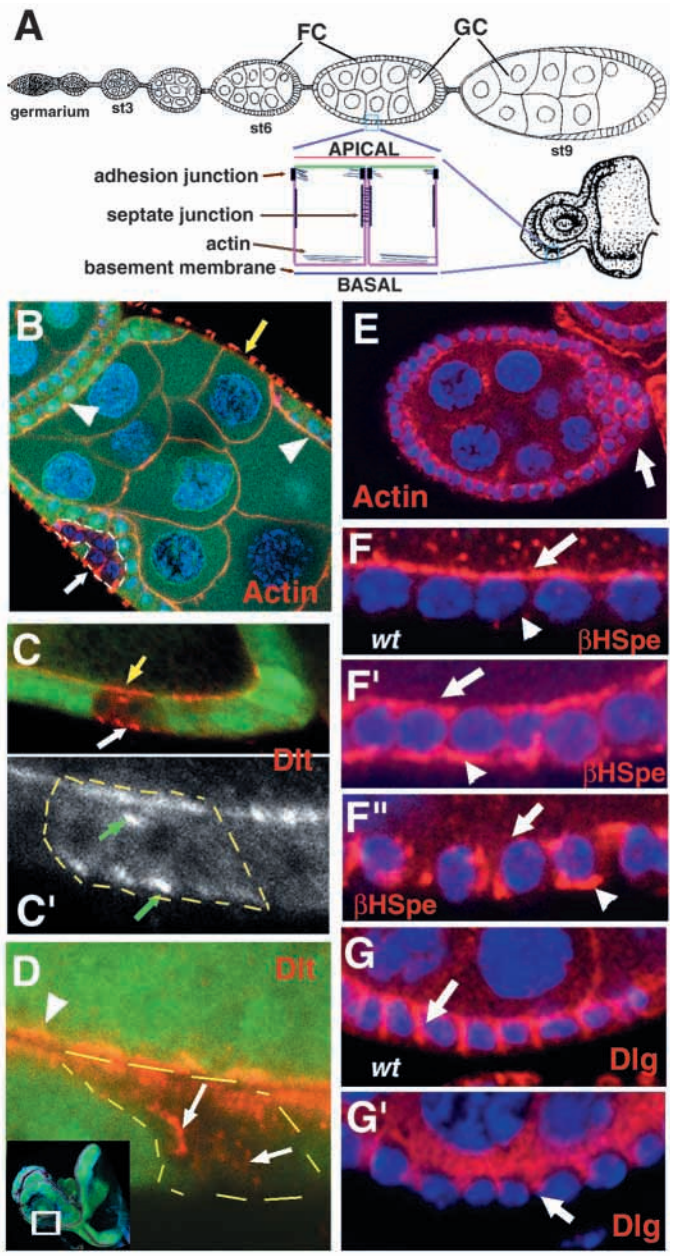

Fig. 2. Dystroglycan function is required for apicobasal polarity in epithelial cells. (A) Schematic drawing of an ovariole and an eye-antennal imaginal disc. The ovariole contains egg chambers at different developmental stages (st). A layer of somatically derived follicle cells (FC), the majority of which have a typical epithelial apicobasal polarity, covers the germline cells (GC). Epithelial cells in the imaginal discs also show apical-basal polarity. (B-D) Mosaic analysis of Dg mutations. GFP in green marks wild-type cells. (E-G′) RNAi analysis. (B) In a *Dg* mutant clone (broken lines in B, Dg^{248}), the apicobasal polarity of the FE is disrupted, as mutant cells form a multi-layer epithelium (white arrow) and also cause discontinuance in the epithelium (yellow arrow) (red, actin; blue, DNA). White arrowheads show the wild-type region. (C,C′) In a Dg^{323} follicle cell clone where follicle cells have not lost their columnar shape yet, an apical marker Dlt (red in C and white in C′) is detected at both the apical and basal (white arrow in C and green arrow in C′) side. C′ is an enlarged view of the mutant clone region (broken yellow line) and vicinity shown in C. (D) In a Dg^{248} clone in an antennal imaginal disc (inset, the white box indicates where the mutant clone is) Dlt (red) is also mislocalized, expanding from the apical (arrowhead) to lateral side (arrow). (E) Multi-layered FE is also detected in *tubPGal4/dsDG* flies. (F,F′,F″) An apical marker βH-Spec (red; F shows the wild-type pattern, arrow) is mislocalized to the basal side (arrowheads in F′ and F″) in *dsDG* follicle cells. F″ shows a more severe loss-of-polarity phenotype than does F′. (G,G′) A basolateral marker, Dlg (red in G shows the wild-type pattern; arrow), is greatly reduced at the basolateral membrane (arrow in G′). DNA is shown in blue at B and E-G′.

Table 1. Nomenclature and putative gene functions for molecular markers used in this study

Protein name used	*Drosophila* gene name	Putative function	Marker used to detect	Reference
Crb (Crb-complex)	*crumbs* (*crb*)	Transmembrane protein; establishment and/or maintenance of cell polarity	Apical staining in epithelial cells	Tepass et al., 1990
Dlt (Crb-complex)	*discs lost* (*dlt*)	PDZ protein; interacts with Crb	Apical staining in epithelial cells	Bhat et al., 1999
β-Heavy-spectrin (βH-Spec)	*karst* (*kst*)	Actin cross-linking; plasma membrane organization	Apical staining in epithelial cells	Thomas and Kiehart, 1994
Baz (Par-complex)	*bazooka* (*baz*)	Par3 homolog, PDZ protein	Apical staining in epithelial cells	Wodarz et al., 2000
Dlg (Lgl-complex)	*discs large* (*dlg*)	MAGUK protein	Basolateral staining in epithelial cells	Woods and Bryant, 1991
Nrt	*neurotactin* (*nrt*)	Plasma membrane protein	Basolateral staining in epithelial cells	Hortsch et al., 1990
Orb	*oo18 RNA-binding protein* (*orb*)	Cytoplasmic polyadenylation element binding protein	MTOC in early oocytes	Lantz et al., 1994
Nod-β-Gal	(an artificial gene; contains head domain of Nod, coiled coil domain of kinesin and a β-galactosidase reporter)		MTOC in early oocytes	Clark et al., 1997

that the mislocalization of Crb was not due to nonspecific interference with the secretory apparatus but due to a defect on cell polarity. The lateral membrane domain was unaffected as assayed by the localization of Neurotactin, a lateral marker (Table 1; data not shown). As seen in the salivary glands, we found that follicle cells that overexpressed DG (Fig. 3I, arrow) lost the apical markers: βH-Spec and Bazooka (Baz), while normal apical localization of these proteins was observed in neighboring wild-type cells (Fig. 3J,J′,K,K′; Table 1). Again, overexpression of the DGcyto-form did not cause any obvious defects in the follicle epithelial polarity.

Dystroglycan is required in the germline for oocyte polarity

As Laminin A is required in the posterior follicle cells for proper oocyte polarity at stages 7-10 (Deng and Ruohola-Baker, 2000), we attempted to ask whether DG functions in the germline cells to receive the polarity signal from the Laminin ECM by clonal analysis. Unfortunately, egg chambers bearing germline clones of all deletion alleles are arrested at pre-vitellogenic stages (Fig. 4A, arrow), prior to the stage we could detect signaling between the posterior follicle cells and the oocyte. Therefore, we concentrated on analyzing the establishment of oocyte polarity in earlier stages, a process that is marked by a posterior movement of the MTOC (Fig. 4B,C). During these stages, a low-level expression of DG is detected at the oocyte membrane (data not shown).

To detect whether the early oocyte polarity is properly established in *Dg* germline clones, we examined the localization of two MTOC markers, Nod-β-Galactosidase (Nod-β-Gal) and ORB (Table1), which (in the wild type) are localized at the anterior of the oocyte at stage 1 (Fig. 4B) and move to the posterior in later stages (Fig. 4C,F). Mislocalization of both markers was observed in the mutant germline clones [Nod-β-Gal mislocalization: 60%, n=32; ORB mislocalization: 76% in Dg^{323} (n=25); 60% Dg^{248} (n=38)]. In half of the mislocalization cases, the markers either remained in the anterior of the oocyte or surrounded the nuclei after stage 3 (Fig. 4D,E,G). The remaining egg chambers exhibited diffuse staining (data not shown). Compared with wild type, the staining was significantly reduced. Furthermore, no accumulation of α-tubulin was observed in the mutant oocytes, while normal posterior accumulation was detected in the control oocytes between stages 2 and 6 (data not shown) (Clegg et al., 2001; Cox et al., 2001a). In conclusion, these data suggest that DG is required in the early oocyte for the maintenance or translocation of the MTOC from the anterior to the posterior of the oocyte (Fig. 4J-K). This step is crucial in establishing AP polarity in the oocyte and the future embryo (Riechmann and Ephrussi, 2001).

Enrichment of the actin cytoskeleton in the oocyte is disrupted in *Dystroglycan* germline clones

Although links between DG and MT cytoskeleton have been suggested (Lumeng et al., 1999), the linkage between DG and the actin cytoskeleton via dystrophin/utrophin is far more evident. We therefore examined the actin distribution in the developing oocyte in the wild-type and *Dg* germline clones. Previous studies demonstrated that actin is enriched at the cortex of early wild-type oocytes (Fig. 4H) (González-Reyes and St Johnston, 1998). Interestingly, this actin enrichment is disrupted in the *Dg* germline clones (Fig. 4I). In addition, ‘spreading’ of the ring canals normally observed in stage 1-2 oocyte is not detected in egg chambers that lack germline DG (Fig. 4I, arrowhead).

Basal actin array is disrupted non-cell-autonomously in *Dystroglycan* follicle cell clones

At the basal side of the FE, actin filaments have a planar polarity that is perpendicular to the long axis, the AP axis, of the egg chamber (Fig. 5A,C). Integrins and receptor tyrosine phosphatase Lar are involved non-cell-autonomously in organizing this basal actin orientation (Bateman et al., 2001). In our analysis of the βH-Spec staining in follicle cells that express *dsDG*, we noticed that βH-Spec is mislocalized to the basal side of the FE to bind the basal actin fibers. Noticeably, the fibers decorated with βH-Spec in different follicle cells appeared to be oriented in a random fashion. To test whether

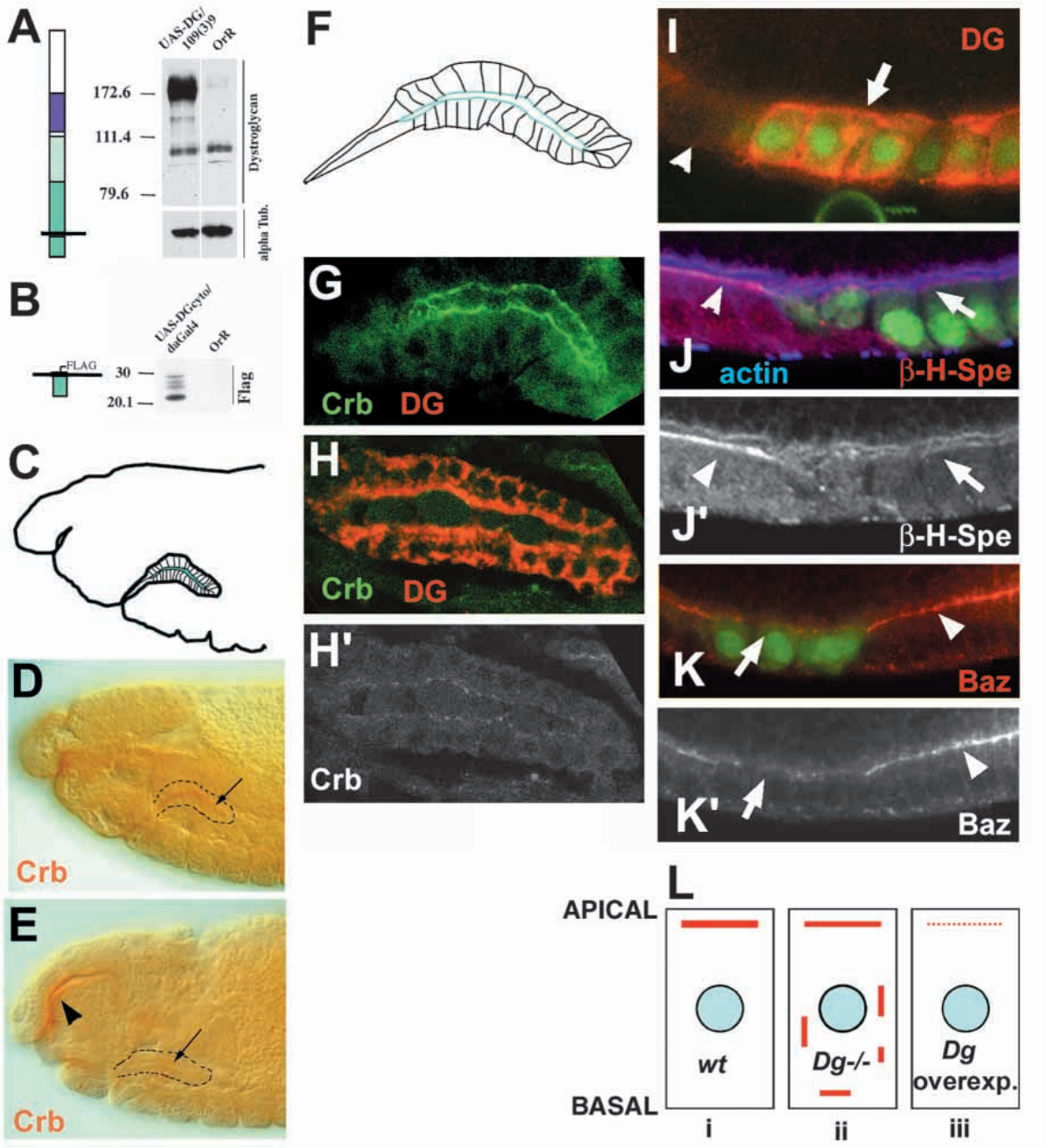

Fig. 3. Ectopic expression of Dystroglycan interferes with epithelial cell polarity. (A) A schematic drawing of the *UAS-DG* construct and a Western blot to detect overexpression of *UAS-DG* driven by a Gal4 driver. (B) *UAS-DGcyto* contains the cytoplasmic and the transmembrane domain of DG linked to a FLAG-tag. Western blot analysis of *UAS-DGcyto* (20kDa) driven by *daGal4* using anti-Flag antibody. (C) Drawing of an embryo at stage 13 to show the location of the salivary gland. The apical surface of the salivary epithelium is indicated by green line (in the center of the salivary gland). (D) A Nomarski image of a wild-type embryo at stage 12-13 stained with the Crb antibody, which shows apical staining (arrow). (E) Stage 13 embryo derived from da-*GAL4* × *UAS-DG* cross. No Crb staining in the salivary gland can be detected (arrow). Notice that Crb staining in the pharynx is normal (arrowhead). (F) An enlarged drawing of an embryonic salivary gland at stage 13. The apical surface of the epithelial cells is shown in green. (G) A confocal image of the wild-type salivary gland around stage 13 stained with Crb (green) and DG (red) antibodies. Wild-type DG expression is below detection level. (H,H′) Salivary gland of stage 13 embryo expressing UAS-DG driven by *elav-GAL4*. Notice the strong staining of DG (red, H) and the strong reduction of Crb (green, H; white, H′) at the apical membrane. (I) Stage 10 follicle cells that overexpress UAS-DG (marked with GFP, green) accumulate high levels of DG protein (red) both in the apical (arrow) and basal surfaces. By contrast, stage 10 wild-type follicle cells express very low levels of DG (arrowhead). (J,J′) Apical localization of βH-Spec (red in J, white in J′) is reduced in DG-overexpressing follicle cells (green; arrow). (K,K′) Overexpression of DG in follicle cells also causes reduction (arrow) of the apical localization (arrowhead) of Baz (red in K, white in K’). (L) Summary of the localization of apical markers (red line) in the wild-type (i), *Dg* mutant epithelial cell clones (ii) and cells that overexpress DG (iii). Apical markers are expanded to the basolateral surface of the epithelium in *Dg* mutant clones, and their apical localization is substantially reduced because of DG overexpression.

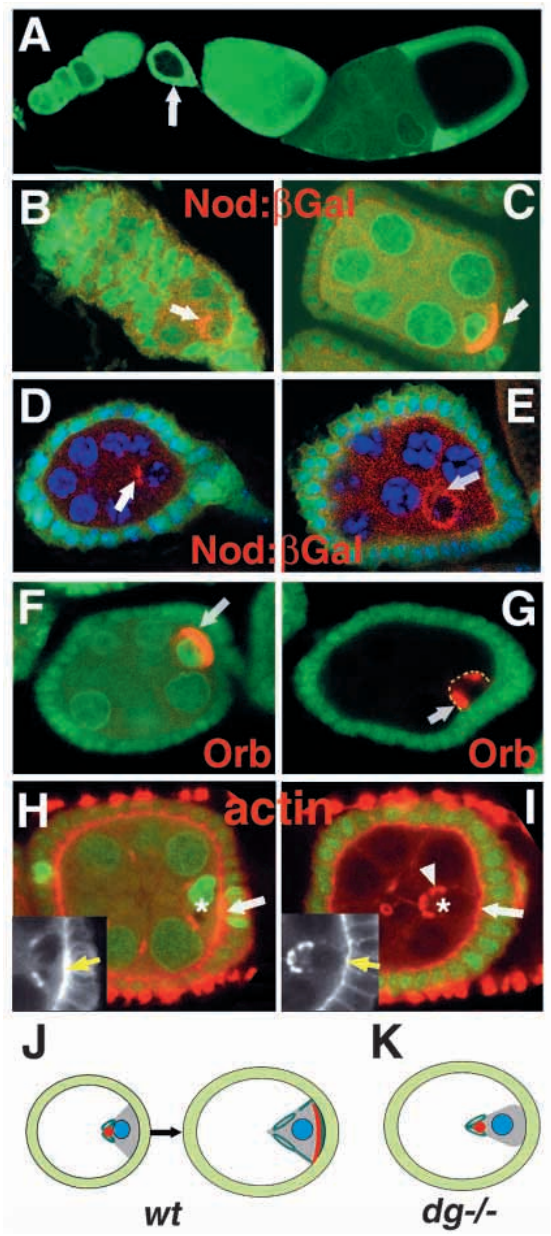

Fig. 4. Dystroglycan is required for the establishment of oocyte polarity. (A) *Dg* germline clones are usually arrested at around stage 6 of oogenesis (arrow, a Dg^{248} germline clone is smaller than the two neighboring egg chambers). (B,C) Nod-β-Gal (red), a marker that co-localizes with the MTOC in early oocytes, moves from the anterior of the oocyte (red in B) to the posterior and stays there until stage 6 (arrow in C). (D,E) In Dg^{248} germline clones, Nod-β-Gal (red) is frequently mislocalized (arrows). (F) ORB (red), is also localized to the posterior of the oocyte at stages 2-6 (arrow). (G) In a Dg^{323} germline clone, ORB (red) is mislocalized (surrounds the oocyte nucleus, arrow) or undetectable (not shown). (H,I) DG is required for the enrichment of the actin cytoskeleton in the oocyte (marked by an asterisk). In the wild-type oocyte (H), actin (red; white in inset) is enriched at the posterior of the oocyte (arrows). In a Dg^{323} germline clone (I), actin (red; white in inset) failed to be enriched at the posterior of the oocyte (arrow), and ring canals accumulate tightly in the anterior of the oocyte (arrowhead). (J) Schematic drawing depicting the anterior-to-posterior migration of MTOC in an early wild-type oocyte. This movement is defective and correlates with defect in posterior enrichment of actin in *Dg* germline clones (K).

this defect reflects problems in basal actin orientation, we analyzed planar polarity of the actin arrays in control egg chambers and in the mutant *Dg* follicle cell clones. Instead of normal perpendicular orientation to the AP axis, random misorientation was observed in the *Dg* mutant egg chambers (Fig. 5B,D). Moreover, the basal actin fibers in follicle cells adjacent to the mutant clones were also misoriented, revealing a non-cell autonomous requirement for DG function (Fig. 5E,F). Although the actin filaments were not organized perpendicular to the AP axis in the mutant cells, they aligned with the neighboring cells, suggesting that some communication of the orientation from one cell to the other still existed. These results suggest that DG has a non-cell-autonomous role in organizing the actin cytoskeleton in the follicle cells, similar to other receptors such as Integrin and Lar. Losing any one of these receptors still allows some orientation transfer but the global direction is defective (Frydman and Spradling, 2001; Bateman et al., 2001) (this study) suggesting that multiple receptor-ECM interactions are required for precise orientation.

Previous data have shown that Laminin stripes in the basement membrane of the FE are organized in the same orientation as the basal actin fibers (Gutzeit et al., 1991; Bateman et al., 2001) (Fig. 5C,G), suggesting an instructive interaction between the actin cytoskeleton and the ECM through a receptor(s). One explanation for the non-cell-autonomous role of DG in basal actin organization is that DG functions through organizing the Laminin ECM to affect the basal actin in the neighboring cell. To test this idea further, we analyzed the orientation of Laminin stripes in the wild-type and the *Dg* mutant follicle cells. Instead of the orientation perpendicular to the AP axis in the wild type (Fig. 5G), overall reduction and misorganization of Laminin ECM occurred in the mutant clone and neighboring regions (Fig. 5H).

To test whether DG is sufficient to organize the Laminin ECM, we asked whether overexpression of DG had any effect on Laminin localization. In stage 10 follicle cells, the majority of the Laminin staining is observed at the basal side (Fig. 5I, arrowhead). Noticeably, Laminin is accumulated at the lateral and apical sides of the follicle cells that overexpressed DG (Fig. 5I, arrow), which is consistent with the fact that high-level DG expression is visible at the apical and basal surfaces of these cells (Fig. 3I). This result suggests that DG can effectively organize the Laminin ECM in *Drosophila*. The dotted instead of stripe/line appearance of ectopic Laminin because of DG overexpression is consistent with a previous report that DG is required for Laminin binding, while Integrin is required for further formation of the Laminin stripe/line-like structures (Henry et al., 2001b).

DISCUSSION

Disruption of DGC function is tightly linked with the pathogenesis of various forms of muscular dystrophy. Previous work has revealed a central role for the transmembrane protein DG in this protein complex (reviewed by Winder, 2001). The cellular function and regulation of DG interactions remain elusive, however. In this paper, we describe the isolation and analysis of *Drosophila* mutants of *Dg*. Using genetic tools in this model organism, we show that

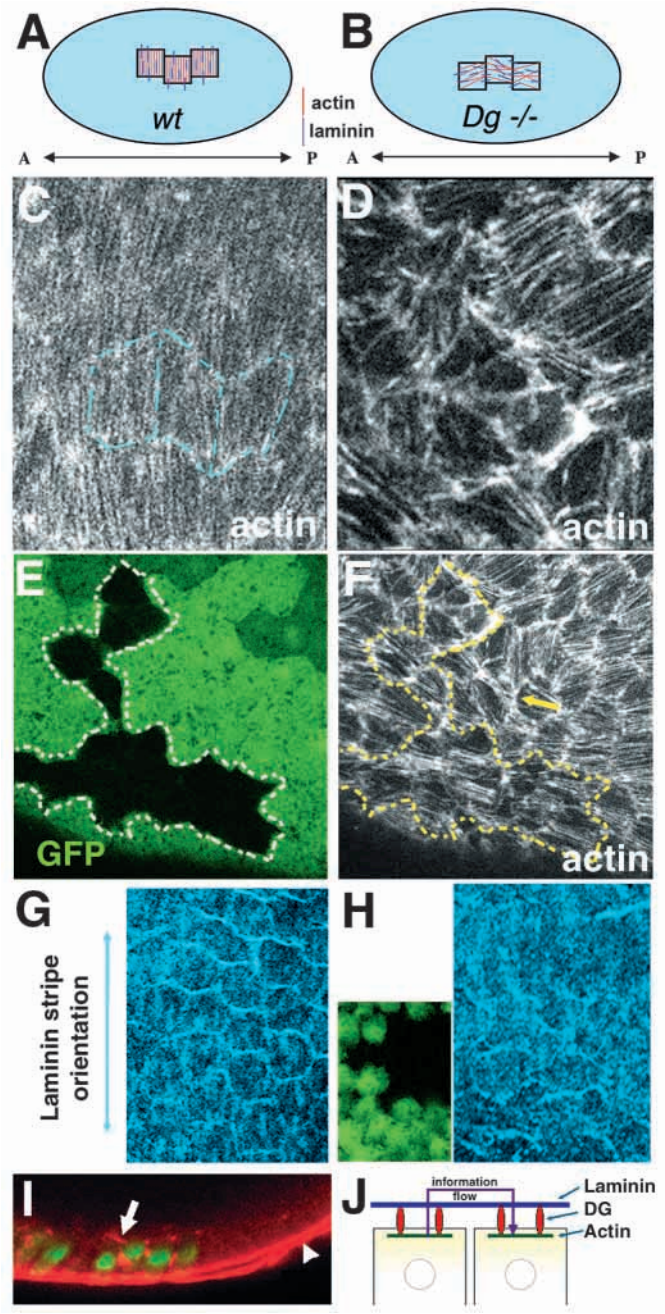

Fig. 5. Laminin and basal actin organization in the follicle cell epithelium is disrupted in a *Dystroglycan* mutant clone. (A) Drawing of a wild-type egg chamber with a basal surface view of three follicle cells. The basal actin fibers (red lines) in these cells are arrayed at a direction perpendicular to the long (AP) axis of the egg chamber from stage 8 to 12. Laminin stripes in the ECM (blue lines) shows the same orientation. (B) Drawing of a *Dg* follicle cell clone with misoriented basal actin fibers and Laminin stripes. (C) Phalloidin staining shows the basal actin array in the wild-type FE. Three cells are outlined. (D) In a Dg^{323} mutant egg chamber, the basal actin organization is disrupted. (E,F) The role of DG in basal actin organization is non-cell-autonomous, as cells adjacent to the follicle cell clone (broken outline) also frequently show a disrupted basal actin distribution (arrow in F). In wild-type egg chambers, Laminin is oriented in stripes (surface stripes in G), similar to the basal actin. This Laminin orientation is disrupted in *Dg* mutant clones (H). The left-hand image in H shows where the mutant follicle cell clone (black area) is located. (I) At stage 10, Laminin is mainly detected at the basal surface (arrowhead) of the wild-type FE (area lacking green). However, overexpression of DG (marked by co-expression of GFP, green) causes accumulation of Laminin ECM to the apical and lateral surfaces (arrow). (J) A model for Dystroglycan function in planar polarity (basal actin organization). In this study, we have shown that the DG function is involved in cell-cell communication, which is essential for basal actin planar polarity. This communication probably involves the cyto-architecture of the Laminin ECM. DG directs the orientation of the Laminin stripes. This information is transmitted into a neighboring cell to coordinate the orientation of the actin fibers. GFP (green) marks the wild-type cells in E and H; Actin is white in C,D,F; Laminin is turquoise in G,H.

DG is required cell-autonomously for polarizing two different cell types: epithelial cells and the oocyte. In *Dg* mutant epithelial cells, apicobasal polarity is disrupted, while in oocytes, anteroposterior polarity is abnormal. Loss of DG function in follicle and disc epithelia results in expansion of apical markers to the basal side of the cells and overexpression results in a seemingly opposite phenotype, reduced localization of apical markers. *Dg* mutations in the germline, however, disturb the enrichment of the oocyte cortical actin and the movement of the MTOC to the posterior oocyte: a process that is the prerequisite for the establishment of all polarity within the egg chamber and embryo. In addition, DG has a non-cell-autonomous effect on the planar polarity of basal F-actin in follicle cells. The non-cell-autonomous phenotype probably results from a lack of instructive interaction between the actin cytoskeleton and the ECM, as DG is required for and sufficient in organizing Laminin in follicle cells (Fig. 5H-J).

Comparison of *Drosophila* Dystroglycan to its mammalian orthologs

Drosophila DG contains most of the hallmarks of vertebrate DG, but is significantly longer than its vertebrate orthologs, due to a ~250 amino acid duplication in the extracellular domain. The N-terminal half of fly DG harbors a mucin-like domain, similar to vertebrate DG, but is otherwise only weakly conserved. As the mucin-like sugars have been implicated in Laminin binding it is interesting to note that splicing variants of DG that lack exon 8, also lack most of this domain. In addition, altered glycosylation of DG is related to two forms of congenital muscular dystrophy (Brockington et al., 2001; Hayashi et al., 2001; Michele et al., 2002; Moore et al., 2002), and reduced expression of DG is observed in a mouse model of Duchene's muscular dystrophy (Ervasti and Campbell, 1993).

The C-terminal half of *Drosophila* DG is conserved with 31% identity (46% similarity, Fig. 1A,B). Especially well conserved are the protein-protein interaction sites in the cytoplasmic domain of DG, including the binding site for Dystrophin. Seven of the eight amino residues, which are crucial for Dystrophin binding (Huang et al., 2000) are conserved in *Drosophila*. Recent studies demonstrate that phosphorylation of the tyrosine residue within the dystrophin/utrophin binding motif can interfere with binding to utrophin, leading to recruitment of SH2 domain proteins (Sotgia et al., 2001; Ilsley et al., 2002). The putative SH2-binding motif involved in this interaction is conserved in

Drosophila. The third protein-protein interaction described for vertebrate DG is the binding of the SH2-SH3 adaptor GRB2. GRB2 helps initiate the Ras-MAP kinase signal transduction cascade and is involved in controlling cytoskeletal organization (Yang et al., 1995). However, the SH3-binding motif, thought to mediate GRB2 binding, is not fully conserved in *Drosophila*.

The role of Dystroglycan in epithelial polarity formation

Reduced expression of DG is often associated with tumor formation, suggesting that DG can act as a tumor suppressor (Henry et al., 2001a). It is likely that loss of DG function in some cancers leads to abnormal cell-ECM interactions and thus contributes to progression to a metastatic state. Defects in epithelial interactions normally result in cell death; when associated with abnormal cell growth and division; however, such defects could induce metastasis. Our analysis supports this hypothesis: lack of DG function in *Drosophila* results in tumor-like structures (Fig. 2B) and abnormal cell movement because of the lack of epithelial integrity and cellular polarity.

Reduction of DG function expands the apical domain and overexpression of DG reduces this domain in epithelial cells. In *Dg* loss-of-function follicle cell clones, a component of the Lgl-complex, Dlg, is mislocalized. This mislocalization could explain the expansion of apical markers in the clones, as Dlg and Scrib are each required for the lateral localization of each other and their function is essential to restrict the apical markers Crb and Dlt to the apical surface (Bilder et al., 2000; Bilder and Perrimon, 2000). Further experiments are directed to distinguish whether mislocalization of Dlg is caused directly by lack of physical interaction with DG or indirectly by lack of proper cytoskeletal arrangements.

The role of Dystroglycan in oocyte polarity formation

Drosophila oocyte polarity is essential for morphogen localization and therefore for the formation of the major body axes. The establishment of oocyte polarity is a gradual process that involves multiple steps (reviewed by Riechmann and Ephrussi, 2001). Key events in the process are cytoskeletal rearrangements. First, the MTOC is present in the anterior region of an early oocyte. By stage 3, the first rearrangement has occurred and the MTOC is positioned in the posterior portion of the developing oocyte. By the end of stage 6, a signal from the posterior follicle cells has initiated a new MT rearrangement, the posterior MTOC disappears and a new anterior MTOC forms. Although this signaling pathway remains a mystery, several molecules including Laminin A have been shown to be involved (Riechmann and Ephrussi, 2001; Deng and Ruohola-Baker, 2000). As for the first rearrangement, genes encoding the *Drosophila* Par3/Par6/aPKC-complex, Par-1, and Maelstrom are required, (Cox et al., 2001a; Cox et al., 2001b; Huynh et al., 2001a; Huynh et al., 2001b; Clegg et al., 2001; Vaccari and Ephrussi, 2002). However, the mechanism for the MTOC movement or anchoring is not clear. We show that DG, similar to the Par proteins, is required in the germline for this first rearrangement step. As *Dg* germline clones also exhibited a defect in cortical actin enrichment in the oocyte, it is possible that the cortical actin plays an important role in MTOC movement and/or anchoring. Alternatively, as DGC contains proteins that can interact with either actin or microtubular cytoskeletons, it could play a role in coordinating actin and microtubule functions in this process.

Molecular similarities in the establishment of epithelial and oocyte polarity

The fact that DG is required for both epithelial and oocyte polarity re-iterates the idea that common strategies may exist for polarizing these two very different cell types. In addition to DG, Par proteins also act in polarity formation in both cell types, suggesting that the Par proteins and DG complex have functional similarities. Interestingly, DG can affect localization of the Par complex as one of the members, Baz (Par3), is mislocalized when DG is overexpressed. In addition, both Par-proteins and the DG-complex interact with molecules that can associate with either actin or microtubular cytoskeletons. Par-1 associates with Myosin II heavy chain and also phosphorylates a MT-associated protein (Drewes et al., 1997; Guo and Kemphues, 1996). DG can interact with actin through Dystrophin-like proteins. Furthermore, the Dystrophin-associated protein, Syntrophin, interacts with MT-associated proteins via a two-hybrid assay (Lumeng et al., 1999). It is possible that both Par proteins and the DG complex facilitate interactions between actin and microtubules and that these interactions between the two cytoskeletal systems are key regulators for establishment of polarity in both cell types.

Cell non-autonomous phenotype and the function of DG in signaling to neighboring cells

To our surprise, *Dg* mutant follicle cell generated actin defects in neighboring cells; the basal actin was misoriented in adjacent follicle cells (Fig. 5F). How would a defective DG in one cell alter the dynamics of actin organization in the neighboring cell? We propose that the interaction between ECM and DG is bi-directional (Fig. 5J): on one hand, DG organizes the Laminin ECM architecture (Henry et al., 2001b; this study), suggesting that a defect in DG will be transmitted to a defect in ECM organization; on the other hand, a defective Laminin lattice will extend to the surface of the neighboring cell and there this architectural information could be transmitted to the cellular actin cytoskeleton by DG in the neighboring cell (Colognato et al., 1999). Three pieces of evidence support this hypothesis. First, *Drosophila* DG is capable of organizing the Laminin lattice (Fig. 5H,I). Second, the Laminin lattice in the basal side of follicle cells is oriented in the same orientation as the underlying basal actin lattice (Fig. 5C,G) (Gutzeit et al., 1991; Bateman et al., 2001). Third, Laminin, similar to DG, could also be involved in basal actin organization (Bateman et al., 2001; Frydman and Spradling, 2001). Interestingly, two other Laminin receptors, Integrin and Lar, are also required for basal actin planar polarity in follicle cells (Bateman et al., 2001; Frydman and Spradling, 2001). It is possible that one connector alone would not give enough rigidity or allow enough flexibility in relaying information between the ECM and the basal actin.

In summary, we have shown that DG has two separate functions in cell polarity: cell autonomous in apical-basal and anteroposterior polarity, and non-cell-autonomous in planar polarity. Future research aims to take advantage of *Drosophila* as a model organism to genetically dissect the partners of DG in these two functions.

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