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Lgl2 and E-cadherin act antagonistically to regulate hemidesmosome formation during epidermal development in zebrafish

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The integrity and homeostasis of the vertebrate epidermis depend on various cellular junctions. How these junctions are assembled during development and how their number is regulated remain largely unclear. Here, we address these issues by analysing the function of Lgl2, E-cadherin and atypical Protein kinase C (aPKC) in the formation of hemidesmosomes in the developing basal epidermis of zebrafish larvae. Previously, we have shown that a mutation in *lgl2* (*penner*) prevents the formation of hemidesmosomes. Here we show that Lgl2 function is essential for mediating the targeting of Integrin alpha 6 (Itga6), a hemidesmosomal component, to the plasma membrane of basal epidermal cells. In addition, we show that whereas aPKCλ seems dispensable for the localisation of Itga6 during hemidesmosome formation, knockdown of E-cadherin function leads to an Lgl2-dependent increase in the localisation of Itga6. Thus, Lgl2 and E-cadherin act antagonistically to control the localisation of Itga6 during the formation of hemidesmosomes in the developing epidermis.

KEY WORDS: Lgl2 (Llgl2), E-cadherin (Cadherin 1), Hemidesmosome formation, Epidermis, Zebrafish

INTRODUCTION

Epithelial cells contain a variety of junctions that are essential for the maintenance of tissue integrity, barrier function and regulation of tissue growth. Some of these cellular junctions exhibit a strict polarised distribution in epithelial cells. Whereas intercellular junctions, such as adherens junctions and tight junctions, exhibit apicolateral localisation, focal adhesions and hemidesmosomes are localised basally to mediate cell-matrix adhesion. The formation and positioning of adherens and tight junctions (or septate junctions in invertebrates) are dependent on the activity of genes involved in the establishment and maintenance of cell polarity (reviewed by Bryant, 1997; Knust and Bossinger, 2002; Humbert et al., 2003; Shin et al., 2006; Suzuki and Ohno, 2006).

The balanced activities of the Crumbs, Par3 (Bazooka, Pard3)-atypical Protein kinase C (aPKC) and Lgl-Scrib-Dlg pathways are essential for the formation of adherens junctions or tight junctions in epithelia. Whereas the Par3-aPKC pathway is required for the maintenance of the apical domain, the Lgl pathway provides the basolateral cues. In the absence of basolateral cues, the apical domain expands at the expense of the basolateral domain, and vice versa, thus affecting the formation of adherens junctions or tight junctions (Bilder et al., 2003; Tanentzapf and Tepass, 2003; Yamanaka et al., 2003; Chalmers et al., 2005). The localisation of Lgl to the basolateral membrane domain depends on its phosphorylation status, which is regulated by the apical Par3-aPKC pathway (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003; Hutterer et al., 2004; Betschinger et al., 2005). *Drosophila* Lgl and its vertebrate orthologues, Lgl1 and Lgl2, possess conserved serine residues that can be phosphorylated by aPKC (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003; Sonawane et al., 2005). It has been proposed that aPKC phosphorylates Lgl at the apical domain, leading to its release from the cortex. Because the Par3-aPKC complex is absent from the basolateral domain, Lgl cannot be phosphorylated in this domain and thus remains localised to the cortex (Betschinger et al., 2003).

In *Drosophila* neuroblasts, basolaterally localised Lgl [L(2)gl – FlyBase] is essential for the targeting of proteins such as Miranda, a determinant of ganglion mother cell fate, to the basolateral cortex (Ohshiro et al., 2000; Peng et al., 2000; Betschinger et al., 2003). It has been proposed that this targeting is actomyosin-dependent (Ohshiro et al., 2000; Peng et al., 2000). In mammalian epithelia, Lgl1 interacts with syntaxin 4, a t-SNARE involved in the fusion of post-Golgi vesicles to the target membranes (Musch et al., 2002). In yeast, Lgl homologues interact with the exocyst complex and are involved in polarised exocytosis (Zhang et al., 2005). Thus far, it is not clear whether Lgl is essential for targeting any component to the basolateral domain in epithelia, be it by polarised exocytosis or by an actomyosin-dependent mechanism.

The interaction between the Lgl pathway and the formation of adherens junctions is reciprocal. The function of *Drosophila* Lgl is essential for localising adherens junctions (Bilder et al., 2003; Tanentzapf and Tepass, 2003), and these junctions are also necessary for the segregation of Dlg to the basolateral domain to establish epithelial cell polarity (Harris and Peifer, 2004). In addition, the formation of adherens junctions is essential for the maintenance of apical-basal cell polarity and for the formation of other cellular junctions. E-cadherin has been shown to be essential for the formation of tight junctions in MDCK cells and in the mouse epidermis (Gumbiner et al., 1988; Tunggal et al., 2005; Capaldo and Macara, 2007). Another adherens junction component, α-catenin, has a function in the maintenance of cell polarity in the mouse epidermis (Vasioukhin et al., 2001). The formation of focal adhesions is linked to the loss of adherens junctions and the endocytosis of E-cadherin (Balzac et al., 2005). Moreover, β1 integrin, a transmembrane component of focal adhesions, influences cadherin-dependent intercellular junction formation (Gimond et al., 1999). These latter observations

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indicate an interaction between cadherins and integrins (reviewed by Chen and Gumbiner, 2006). However, it is not clear whether the formation of hemidesmosomes is regulated by E-cadherin in any way.

We are investigating the role of polarity genes in the formation of cellular junctions in the larval epidermis of the zebrafish. In zebrafish larvae, the epidermis is bi-layered, consisting of the outer periderm and the underlying basal epidermis (see Fig. 1A). The basal epidermal cells exhibit three distinct plasma membrane domains. The basal domain connects the cells to the extracellular basal lamina, the lateral domains are in contact with the neighbouring basal epidermal cells, and the apical domain attaches to the outer peridermal cells (Fig. 1A). The basal domains of epidermal cells that cover the larval head and flanks are equipped with hemidesmosomes, whereas the lateral and apical domains of all epidermal cells contain adherens junctions and desmosomes (Fig. 1A,B). A number of mutants exhibiting defects in the larval epidermis have been isolated (van Eeden et al., 1996). In the *penner* (*pen*) mutant, the larval epidermis detaches from the basal lamina. We have shown that *pen* encodes Lgl2. Strikingly, in *pen* mutant larvae, hemidesmosomes are absent from the basal epidermal cells (Sonawane et al., 2005). However, the precise function of Lgl2 in hemidesmosome formation remained to be elucidated.

We have analysed the functions of Lgl2, aPKC and E-cadherin (Llgl2, Prkci and Cadherin 1, respectively – ZFIN) in the formation of cellular junctions in basal epidermal cells in zebrafish larvae. We show that Lgl2 and E-cadherin localise to the lateral domain in basal epidermal cells. At the lateral domain, Lgl2 promotes the formation of hemidesmosomes by mediating the targeting of a hemidesmosomal component, Integrin alpha 6 (Itga6), to the plasma membrane. By contrast, E-cadherin negatively regulates hemidesmosome formation, presumably by regulating the Lgl2-mediated Itga6 targeting. Thus, Lgl2 and E-cadherin localised at the lateral domain act antagonistically in controlling the formation of hemidesmosomes at the basal membrane domain.

MATERIALS AND METHODS

Fish strains

For immunostainings, *pen* or *albino* mutant or wild-type larvae were used. For transplantations, embryos from β-actin::GFP transgenic fish (Maderspacher and Nüsslein-Volhard, 2003) were used as donors and embryos from *albino* fish as recipients.

Generating antibodies against Lgl2 and Itga6

The cDNA corresponding to amino acids 994-1094 of Lgl2 was cloned into the pQE-30 UA vector in M15(pREP4) cells. The expressed His-tagged protein was isolated and affinity purified under native conditions (The Qiaexpressionist, Qiagen) and used for immunisation. A peptide polyclonal antibody against zebrafish Itga6 (accessions: XP_683444, XP_707029, XP_707030), corresponding to the sequence KRKHKILSCSGDAR, was generated in guinea pigs (Genosphere Biotechnologies). The sera were used in immunohistology without any further purification.

To test its specificity, the anti-Itga6 serum was diluted (1:500), preincubated with different concentrations of the peptide epitope in PBS for 2 hours at room temperature, centrifuged at 16,100 ***g*** for 10 minutes and used for immunostaining. The specific signal was completely eliminated at a peptide concentration of 1000 μg/ml.

Morpholino injections and cell transplantations

Antisense morpholino oligos (Gene Tools, Corvallis) against *lgl2* (Sonawane et al., 2005), *e-cadherin* (Knaut et al., 2005) and *aPKC* (Horne-Badovinac et al., 2001), along with their corresponding five-base mismatch control morpholinos, were used at the following concentrations: *lgl2*, 5′-GCCCATGACGCCTGAACCTCTTCAT-3′ (200 μM); *lgl2* control, 5′-GCACATAACGCCTCAACCTGTTAAT-3′ (200 μM); *e-cadherin*, 5′-ATCCCACAGTTGTTACACAAGCCAT-3′ (100 μM); *e-cadherin* control, 5′-ATCCGACACTTCTTACAGAACCCAT-3′ (100 μM); *aPKC*, 5′-TGTCCCGCAGCGTGGGCATTATGGA-3′ (750 μM yielded consistent knockdown in clones at 5 dpf).

For transplantations, morpholino oligos were injected into β-actin::GFP donor embryos (1- to 2-cell stage). For transplantation from *has* (*prkci*) mutants, embryos were injected with Alexa 488-dextran (Molecular Probes/Invitrogen). At the blastula stage, cells were transplanted to recipient *albino* embryos that were at the same stage to obtain epidermal clones. For electron microscopy analysis of clones, GFP was detected using rabbit anti-GFP antibody and biotinylated anti-rabbit antibody with the Elite ABC System (Vectastain) and DAB.

Brefeldin A (BFA) treatment

Three-day-old *albino* larvae were incubated in 1.8, 2.7 and 3.6 mM BFA in the fish medium until 5.5 dpf and analysed for morphological phenotype. The larvae raised in 3.6 mM BFA showed a distinct blistering phenotype over the head and were fixed in 4% paraformaldehyde (PFA).

Immunohistochemistry and lectin GS-II staining

Larvae were fixed overnight in 4% PFA in PBS for anti-Lgl2, monoclonal anti-E-cadherin, anti-GFP (Torrey Pines, Roche), anti-aPKC (Santa Cruz Biotechnology), anti-Itga6 and anti-Alexa 488 antibodies. For anti-Cytokeratin II (Ks-pan1-8, Progen Biotechnik) staining, larvae were fixed in Dent's fixative overnight at –20°C. Larvae were incubated overnight at 4-6°C in the following antibody dilutions: anti-Lgl2 (1:400), anti-E-cadherin (1:100), anti-aPKC (1:1000), anti-Cytokeratin (1:10), anti-Itga6 (1:500), anti-GFP (1:200), anti-Alexa 488 (1:250). Afterwards, larvae were washed in PBT and incubated with appropriate secondary antibodies conjugated with Cy3, Cy5, Alexa 488 or Alexa 546. Larvae were washed, developed using the ABC Elite Kit and DAB as required, post-fixed in 4% PFA and either mounted in glycerol or embedded for sectioning.

For immunoelectron microscopy, larvae were prestained with anti-Itga6 antibody and anti-guinea-pig antibody conjugated with HRP (1:500). For detection, DAB (0.2 mg/ml in PBS containing 0.1% Tween 20) was supplemented with nickel ammonium sulphate (10 mg/ml) and Imidazole (1 mM). After staining, larvae were post-fixed and processed for electron microscopy.

For Golgi staining, anti-Itga6-stained larvae were embedded in 2% agarose, sectioned (100 μm) on a Vibratome and incubated for 4 hours in lectin GS-II (Molecular Probes/Invitrogen) diluted (1:250) in PBS containing 1 mM $CaCl_2$. After the incubation, sections were washed with PBS with $CaCl_2$ and mounted in 70% glycerol for confocal microscopy.

Histology: fluorescence and electron microscopy

Immunostained larvae were embedded in Technovit 7100 for sectioning, and in Epon for electron microscopy as previously described (Sonawane et al., 2005).

Image acquisition

Almost all fluorescence microscopy images were acquired on a Zeiss LSM 510 Meta confocal microscope at 40×/1.3 with a 2× digital zoom. Images of DAPI-counterstained sections were acquired on a Zeiss Axioplan 2 microscope at 40×/0.75 using a digital Axiocam camera without digital zoom.

Quantification of fluorescent signal intensities

The *z*-stacks were obtained by scanning the epidermis every 0.4 μm by confocal microscope with the pinhole adjusted to 83 μm. The fluorescent intensity scans were performed on a single section from a *z*-stack using LSM 510 software (Zeiss). To quantify Itga6 staining intensity, the cell area was demarcated and intensity histograms obtained. The software displays the data in tabular form as the mean pixel intensity and standard deviation for the selected cell area. The mean intensities for three cells from the mutant clone and three host cells were obtained and represented in a histogram. We further compared the mean of each of these clonal cells with each host cell in a 3×3 matrix using Student's *t*-test.

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RESULTS

Lgl2 and aPKC localise to the lateral and basal domain, respectively, whereas Itga6 localises to both lateral and basal domains in the developing basal epidermis

Previously, we have shown that *lgl2* function is essential for the formation of hemidesmosomes during epidermal development (Sonawane et al., 2005). However, its cellular localisation and precise role in hemidesmosome formation have yet to be resolved. To analyse the localisation of Lgl2 during epidermal development, we generated an antibody against the C-terminal epitope of Lgl2. This antibody stains basal epidermis and periderm in developing wild-type larvae (Fig. 1C,H). The *lgl2* (*pen*) mutant larvae do not exhibit staining in the basal epidermis or periderm (Fig. 1D), indicating that the antibody specifically recognises Lgl2. In histological sections, we never observed Lgl2 localisation at the basal cortex of the basal epidermal cells, where hemidesmosomes form (Fig. 1H). Instead, Lgl2 was predominantly localised to the lateral domain in the basal epidermal cells (Fig. 1H). The apparent apical staining in the basal epidermal cells might in fact represent the basolateral localisation of Lgl2 in juxtaposed peridermal cells.

The localisation of Lgl proteins is dependent on their phosphorylation status. According to a current model, apically localised aPKC phosphorylates Lgl proteins, which leads to their release from the apical cortex but leaves the basolateral localisation unperturbed (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003; Hutterer et al., 2004; Betschinger et al., 2005). It was not clear whether aPKC is expressed in basal epidermal cells in zebrafish and whether it regulates the localisation of Lgl2 in a similar manner. We used an anti-aPKC antibody (Horne-Badovinac et al., 2001) to analyse aPKC localisation in the epidermis. In the basal epidermis, aPKC expression begins at 3 days post-fertilisation (dpf), peaks at ~4-5 dpf and then declines at 6 dpf (Fig. 1E and data not shown). Interestingly, histological sections revealed that aPKC localises to the basal domain (Fig. 1I). To test whether the antibody is specifically recognising the aPKCλ isoform, we injected a morpholino against aPKCλ (Horne-Badovinac et al., 2001). These morpholino-injected larvae did not develop after 2 dpf. Therefore, we transplanted cells (marked with GFP) from morpholino-injected embryos into wild-type recipient embryos at the blastula stage to obtain clones in the basal epidermis (Fig. 1F). The aPKC expression was strongly attenuated in these clones, indicating that the aPKCλ isoform is expressed in the epidermis (Fig. 1G).

We then asked whether aPKC localisation coincides with the hemidesmosomal components at the basal domain. We found that Integrin alpha 6 (Itga6) localises to the basal domain and that aPKC localisation overlaps with that of Itga6 (Fig. 1J,K). Surprisingly, a minor fraction of Itga6 also localised to the lateral domain along with Lgl2 (Fig. 1L-N) and E-cadherin (Fig. 1O-Q) in 4-day-old larvae. We further analysed the localisation of Lgl2, E-cadherin and Itga6 in histological sections to check whether any of these localise to a sub-domain of the lateral membrane domain. However, all three proteins localised to the entire lateral domain and also exhibited perfect overlap with each other (Fig. 1R-T′). In histological sections, Itga6 localisation was apparent in the apical side of the basal cell, just like Lgl2 and E-cadherin. It is not clear whether this apical staining represents the basal localisation in the peridermal cells. The Itga6 staining at the basal, as well as lateral, membrane domain proved specific in a competition assay using the peptide antigen (data not shown).

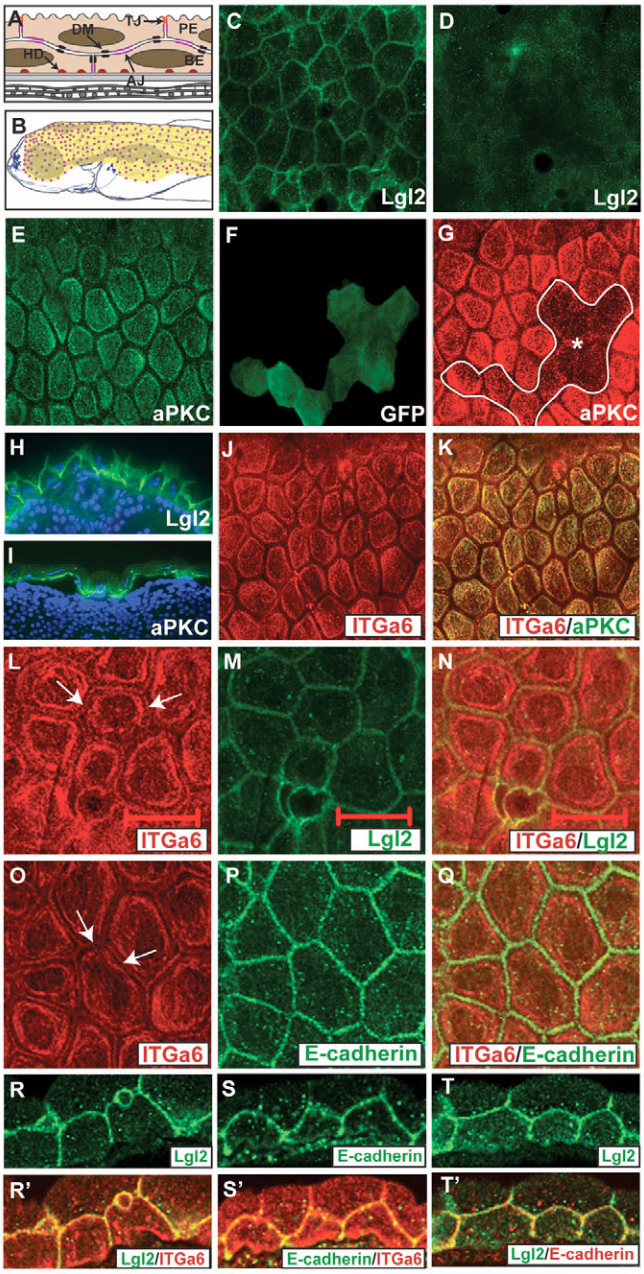

Fig. 1. The localisation of Lgl2, aPKC, Itga6 and E-cadherin in 4- to 5-day-old zebrafish basal epidermis. (**A**,**B**) Schematic representation of the zebrafish bi-layered epidermis (A) and the epidermal region of 5-day-old larvae (B), which exhibits hemidesmosomes (red dots). (**C-T′**) Immunostaining using anti-Lgl2 (C,D,H,M,R,T), anti-aPKC (E,G,I,K), anti-GFP (F), anti-Itga6 (J,K,L,O,R′,S′) and anti-E-cadherin (P,S) antibodies. In the basal epidermis, Lgl2 localises to the lateral domain (C,H). Control *lgl2* mutant epidermis does not show any specific Lgl2 staining (D). By contrast, aPKC is localised to the basal domain (E,I). Epidermal clones injected with a morpholino against *aPKCλ* (F) exhibit a reduction in aPKC expression (G). At the basal domain, Itga6 (J,K) and aPKC (K) co-localise. Co-staining of Itga6 and Lgl2 in whole-mounts (L-N) and sections (R,R′), and of Itga6 and E-cadherin in whole-mounts (O-Q) and sections (S,S′), reveals that a minor Itga6 fraction is localised at the lateral domain (arrows in L,O). Co-staining of Lgl2 and E-cadherin in sections reveals an overlap in their localisation (T,T′). Asterisk, basal epidermal clone. PE, periderm; BE, basal epidermis; AJ, adherens junctions; DM, desmosomes; HD, hemidesmosomes; TJ, tight junctions. Scale bar: 40 μm in C-G,J,K; 22.5 μm in H,I; 20 μm in L-T′.

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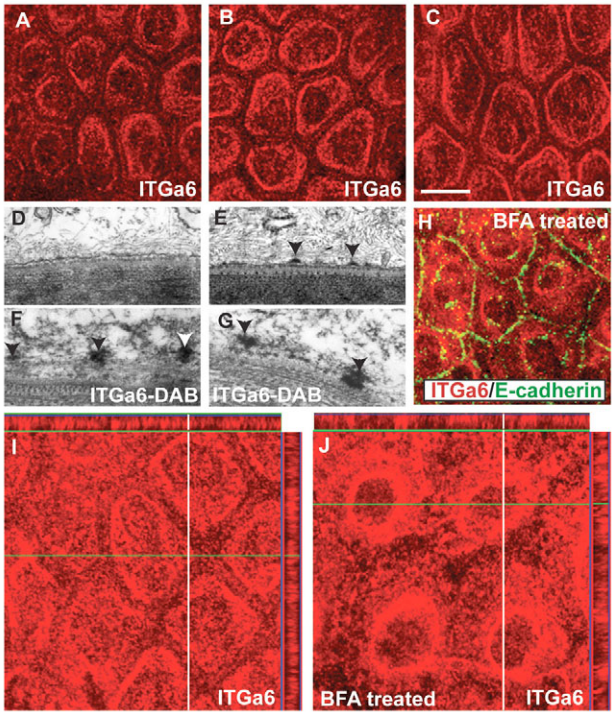

Fig. 2. Dynamics of hemidesmosome formation in the zebrafish epidermis. (**A-C**) Immunostaining using anti-Itga6 antibody in wild-type larvae analysed at 3, 4 and 5 dpf in whole-mount. The localisation of Itga6 to the basal domain increases, while at the lateral domain it progressively decreases during 3-5 dpf. (**D-G**) Electron microscopy (D,E) and immunoelectron microscopy using anti-Itga6 antibody and nickel-enhanced DAB (F,G) in wild type. Electron-dense hemidesmosomes (arrowheads) are absent from the epidermis at 3 dpf (D) but present at 4 dpf (E). Itga6 localises to intermediate filaments (arrowheads) at 3.5-dpf (F). At 4 dpf, Itga6 becomes incorporated in hemidesmosomes (G). (**H**) Co-immunostaining using anti-Itga6 (red) and E-cadherin (green) antibodies. (**I**,**J**) Immunostaining using anti-Itga6 antibody followed by analysis in *x-y* and *x-z* planes. The basally localised Itga6 (I) accumulates around the nucleus after BFA treatment (H,J). Scale bar: 13.5 μm in A-C,H; 200 nm in D-G.

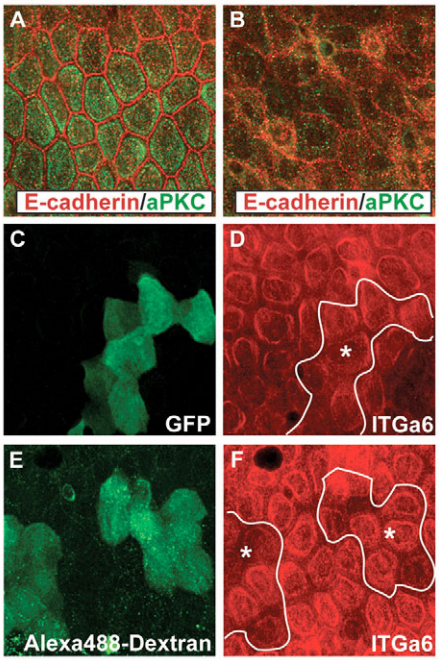

Fig. 3. The function of aPKC is dispensable for Itga6 localisation. Co-immunostaining in zebrafish larvae at 5-6 dpf using (**A**,**B**) anti-aPKC (green) and anti-E-cadherin (red) antibodies, (**C**,**D**) anti-GFP (green) and anti-Itga6 (red) antibodies, and (**E**,**F**) anti-Alexa 488 (green) and Itga6 (red) antibodies. The basal aPKC localisation in wild-type larvae (A) is lost in 5-day-old *lgl2* mutant larvae (B). The aPKCMO clone marked with GFP (C), and *has* (*prkci*) clones marked with Alexa 488-dextran (E), show normal Itga6 localisation (D,F) at 6 dpf. Asterisk, basal epidermal clone.

We analysed the temporal changes in Itga6 localisation in the basal epidermis during larval development. Whereas electron-dense hemidesmosomes become apparent after 4 dpf in the developing larval epidermis (Sonawane et al., 2005), Itga6 localised to the basal domain as early as 2.5 dpf (data not shown). The signal intensity for Itga6 at the basal domain increased considerably between 3 and 4 dpf and reached a plateau thereafter (Fig. 2A-C). By contrast, the intensity of Itga6 staining at the lateral domain diminished after 4 dpf and was difficult to detect in larval epidermis at 5 dpf (Fig. 2A-C). We performed immunoelectron microscopy on larvae prestained with anti-Itga6 antibody. At 3.5 dpf, although hemidesmosomes were not apparent (Fig. 2D), DAB precipitate was clearly seen clustered at the intermediate filaments at the basal cortex (Fig. 2F). By 4 dpf, electron-dense hemidesmosomes became apparent in the epidermis (Fig. 2E) and the DAB precipitate was associated with the hemidesmosomes that were being assembled (Fig. 2G). Based on these observations, we propose that the increase in the amount of Itga6 at the basal domain between 3 and 5 dpf indicates additional targeting of Itga6 to the basal domain, and that this increase is essential for driving Itga6, which is clustered at intermediate filaments at 3.5 dpf, into hemidesmosomal plaques by 4-5 dpf. To test whether Itga6 is indeed targeted to the membrane between 3 and 5 dpf, we treated 3-day-old larvae with Brefeldin A (BFA), which inhibits Golgi function (reviewed by Nebenführ et al., 2002). In the BFA-treated larvae, Itga6 exhibited a broad accumulation around the nucleus at 5 dpf, and Itga6 localisation at the basal domain was lost (Fig. 2H-J). These observations (Fig. 2A-C,H-J) clearly indicate that substantial amounts of Itga6 are newly synthesised and targeted to the basal domain between 3 and 5 dpf, and that this later targeting is essential for stabilising the early Itga6 fraction, which is targeted before 3 dpf, at the basal domain.

aPKCλ function is dispensable for the localisation of Itga6 to the basal membrane domain

To explain the hemidesmosomal phenotype in *lgl2* mutant larvae, we proposed that the function of Lgl2 is essential for the maintenance of the basal localisation of aPKCλ, which might be serving as the primary regulator of hemidesmosome formation because it co-localises with Itga6 during hemidesmosome formation. To test this hypothesis, we asked whether aPKCλ localisation is altered in the absence of *lgl2* function in the basal epidermal cells. Indeed, in the *lgl2* mutant larvae, the basal localisation of aPKCλ was disrupted (Fig. 3A,B), raising the possibility that the loss of hemidesmosomes is a consequence of the loss of aPKC localisation. To test this, we analysed hemidesmosome formation in aPKCλMO clones (Fig. 3C). Interestingly, Itga6 exhibited normal basal localisation in aPKCλMO clones, indicating that hemidesmosome formation does not require aPKCλ (Fig. 3D). Similarly, we did not observe any significant difference in the

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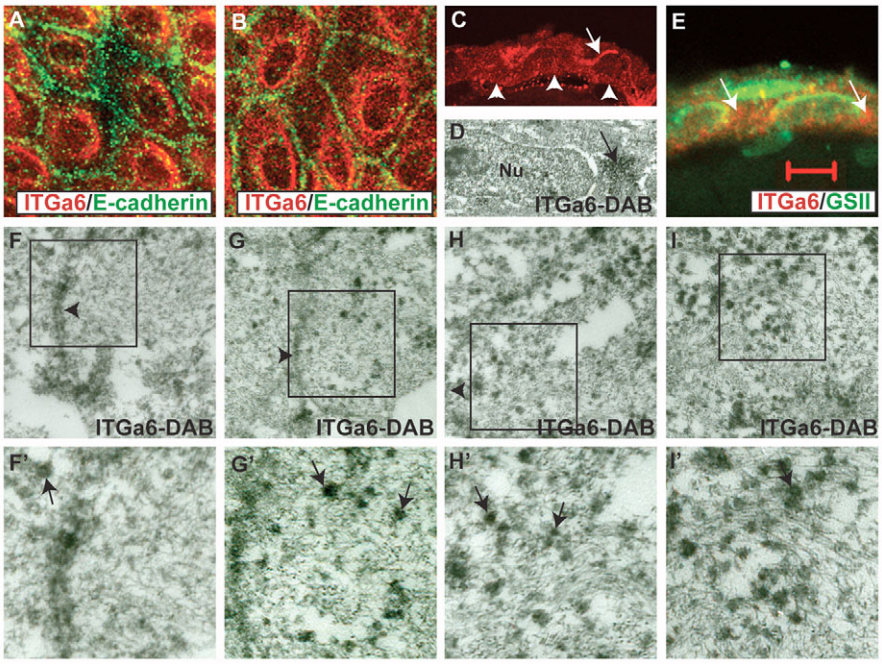

Fig. 4. Lgl2 mediates targeting of Itga6 to the plasma membrane during hemidesmosome formation in basal epidermal cells. (**A-C**,**E**) Immunostaining of *lgl2* mutant epidermis with anti-Itga6 (red) and anti-E-cadherin (green) antibodies (A,B), anti-Itga6 antibody (C), or anti-Itga6 antibody (red) and lectin GS II (green) in section (E). In mutant larvae at 4-5 dpf, Itga6 exhibits diffuse cytoplasmic staining along with strong perinuclear accumulation (A,B). This perinuclear Itga6 staining is not due to Itga6 accumulation in the Golgi apparatus (E). Basal and lateral Itga6 localisation is lost in *lgl2* larvae (C); arrowheads mark the basal domain and the arrow marks the apical domain juxtaposed to a peridermal cell where the Itga6 staining occasionally persists. (**D**,**F-I′**) Immunoelectron microscopy using anti-Itga6 antibody and nickel-enhanced DAB in wild-type (F,F′) and *lgl2* mutant (D,G-I′) larvae. F′-I′ are 2× enlargements of the boxed regions in F-I. The perinuclear accumulation of Itga6 is evident in immunoelectron micrographs (arrow in D). In wild-type larval epidermis at 4 dpf, electron-dense Itga6 vesicles are relatively rare (F) around the lateral domain. By contrast, in *lgl2* mutant larvae at 4 dpf, Itga6 vesicles are frequently present in the lateral cortex (G,H) and in the cytoplasm in general (I). Arrowheads in F-I indicate the lateral domain. In F′-I′, some of the Itga6 vesicles are indicated by arrows. Scale bar: 13.5 μm in A,B; 27 μm in C; 5 μm in E; 200 nm in F-I.

localisation of Itga6 in *aPKCλ* (*heart and soul*) mutant clones (Fig. 3E,F). These results indicate that aPKCλ function is dispensable for Itga6 localisation during hemidesmosome formation, and that Lgl2 does not act in hemidesmosome formation via aPKCλ.

Lgl2 regulates hemidesmosome formation by mediating the targeting of Itga6 to the membrane beyond 3.5 dpf

The co-localisation of Itga6 and Lgl2 at the lateral domain led us to test the alternative hypothesis that Lgl2 has a primary function in hemidesmosome formation by mediating the targeting of Itga6. We investigated whether Itga6 localisation is altered in *lgl2* mutant larvae at 3-5 dpf. As the *lgl2* epidermal phenotype is subtle at 4 dpf and not apparent at 3 dpf, mutant larvae were isolated based on the absence of Lgl2 staining in the epidermis. At 3 dpf, *lgl2* mutant larvae exhibited lateral as well as basal localisation of Itga6, as in wild-type epidermis (see Fig. S1A in the supplementary material). This might be caused by low levels of maternal Lgl2 persisting at this stage that are sufficient to execute the proper localisation of Itga6. To test this, we injected antisense ATG morpholino to knock down maternal as well as zygotic Lgl2. As these morpholino-injected larvae exhibit early phenotypes and remain retarded, we transplanted cells (marked with GFP) from morphant embryos into wild-type recipient embryos at the blastula stage to obtain morphant *lgl2* (lgl2MO) clones in the epidermis. The morpholino selectively knocked down the Lgl2 in these epidermal clones at 3.5 dpf. Out of 33 clones analysed from 3.5-day-old recipient larvae (n=26), 27 showed complete loss of Lgl2 staining (see Fig. S1B,C in the supplementary material). In separate experiments, we analysed Itga6 localisation in lgl2MO clones and found that most of the clones (28 out of 34) in 3.5-day-old recipient larvae (n=25) exhibited normal basal, as well as lateral, localisation of Itga6 in spite of the absence of Lgl2 (see Fig. S1D-F in the supplementary material). Our data clearly indicate that prior to 3.5 dpf, Lgl2 function is not essential for Itga6 localisation.

At ~3.75 dpf, several mutant larvae exhibited selective loss of the lateral membrane staining of Itga6, whereas the basal localisation appeared largely unperturbed (see Fig. S1G-L in the supplementary material). Interestingly, however, at 4-5 dpf, *lgl2* mutant larvae exhibited faint cytoplasmic Itga6 staining with some enrichment around the nucleus (Fig. 4A,B,D; compare with Fig. 2A-C). Furthermore, analysis of lgl2MO clones at 6 dpf indicated that, as in *lgl2* mutant larvae, Itga6 accumulates in the cytoplasm (data not shown). GSII lectin staining revealed that Itga6 does not accumulate in the Golgi apparatus (Fig. 4E). Rather, it appeared to accumulate in post-Golgi compartments such as recycling endosomes or exocytotic vesicles, which play important roles in protein targeting. At these time points, we never observed Itga6 staining at the lateral or basal domain in *lgl2* mutant larvae (Fig. 4C), indicating that Lgl2 is involved in mediating the targeting of Itga6 to the plasma membrane as well as in its maintenance at the plasma membrane.

Next, we analysed the distribution of Itga6 vesicles in 4-day-old wild-type and *lgl2* mutant larvae by immunoelectron microscopy. The Itga6 vesicles were difficult to locate in the cytoplasm of basal

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epidermal cells of wild-type larvae, possibly owing to the rapid targeting of exocytotic vesicles to the membrane (Fig. 4F). Nevertheless, we occasionally observed Itga6 vesicles in the vicinity of the lateral membrane domain (Fig. 4F′). By contrast, Itga6 vesicles were abundant in the basal epidermal cells of *lgl2* mutant larvae (Fig. 4G-I′). Moreover, in *lgl2* mutant larvae, these Itga6 vesicles were distributed in the lateral and apical cortical region of the basal epidermal cells (Fig. 4G-I′). In the *lgl2* mutant larvae, which exhibit a slightly stronger phenotype, Itga6 vesicles were seen to accumulate in the cytoplasm (Fig. 4I,I′).

To conclude, in *lgl2* mutant larvae, the basal and lateral Itga6 localisation is lost after 4 dpf and Itga6 vesicles accumulate in the lateral cortical region as well as in the cytoplasm in general, further indicating that Lgl2 function is essential for mediating the targeting of Itga6 to the plasma membrane beyond 3.5-4 dpf. Since the basal and lateral localisation of Itga6 is not altered prior to 3.5 dpf in the absence of *lgl2* function, we conclude that Lgl2 is dispensable for the initial Itga6 targeting.

E-cadherin negatively regulates the formation of hemidesmosomes during epidermal development

In addition to its role in adherens junction formation, E-cadherin is involved in the maintenance of epithelial cell polarity and in the formation of other cellular junctions such as desmosomes and tight junctions (Gumbiner et al., 1988; Tunggal et al., 2005; Capaldo and Macara, 2007). We tested whether E-cadherin plays a role in hemidesmosome formation and in the localisation of Lgl2 in the developing basal epidermis. E-cadherin has essential functions in many early developmental processes, and mutation or morpholino knockdown of *e-cadherin* (*half-baked*, *cadherin 1*) leads to an early phenotype that is lethal (Kane et al., 2005). Therefore, we analysed late *e-cadherin* knockdown phenotypes in clones of epidermal cells (ecadMO clones). ecadMO clones from 5- to 6-day-old larvae did not exhibit any appreciable E-cadherin expression (Fig. 5A,B). Of 120 clones (n=10), 76 exhibited complete knockdown, whereas 22 exhibited partial knockdown of E-cadherin expression by 6 dpf. Surprisingly, further analysis of ecadMO clones revealed augmented levels of Itga6 localisation to the basal domain (Fig. 5C,D). We analysed 35 clones from 5- to 6-day-old larvae (n=27) of which 28 exhibited this augmented localisation phenotype. Quantification revealed that the mean intensity for Itga6 in ecadhMO clones was consistently higher than that in the surrounding host cells (t-test, $P \leqslant 0.001$; see Materials and methods for details), a phenomenon never exhibited by clones carrying control morpholinos (Fig. 5E,F,I,J). We then investigated whether this increase in Itga6 represents an increase in the number of hemidesmosomes. Electron microscopy analysis revealed that whereas in wild-type cells hemidesmosomes appear as discrete electron-dense punctae, in ecadMO clones the number of hemidesmosomes was so high that these punctae coalesced to form an electron-dense mat at the basal domain (Fig. 5K,L). We observed intermediate filaments projecting out of these electron-dense mats, indicating that these mats are formed from functional hemidesmosomes (Fig. 5K). We further argued that if increased Itga6 levels represent increased numbers of hemidesmosomes, this should lead to an increase in the hemidesmosome-associated keratin cytoskeleton. By contrast, if Itga6 is associated with its other partner, Integrin beta 1, in ecadMO clones then keratin levels should not change. In 75% of ecadMO clones (n=16), more keratin localisation was observed as compared with the surrounding basal epidermal cells (Fig. 5G,H). We conclude that Itga6 localisation to the basal domain increases in ecadMO clones and that this increased fraction is assembled in hemidesmosomes, leading to the increase in the number of hemidesmosomes.

Because aPKCλ colocalises with Itga6 at the basal domain and Lgl2 is involved in targeting Itga6 during hemidesmosome formation, we asked whether aPKC or Lgl2 localisation is altered in ecadMO clones. Whereas Lgl2 localisation was unaltered in ecadMO clones (Fig. 5M,N), we observed a clear increase in the basal localisation of aPKC in ecadMO clones (Fig. 5O,P).

Our data show that E-cadherin negatively regulates Itga6 localisation during the formation of hemidesmosomes. Although the number of hemidesmosomes is increased in the absence of E-cadherin, we did not find elevated levels of Lgl2 at the lateral domain. Thus, we hypothesised that existing Lgl2 levels are sufficient for the increased basal localisation of Itga6 when negative regulation by E-cadherin is lost. To test this hypothesis, we reduced the levels of Lgl2 in ecadMO clones by co-injecting *lgl2* morpholino at a concentration (100 μM) that reduces, but does not completely eliminate, Lgl2 levels (Fig. 6C,D). Analysis of these lgl2-ecadMO clones revealed that the reduced levels of Lgl2 normalised the basal localisation of Itga6 in 82% of clones (29 out of 35) analysed from 6-day-old larvae (n=28) (Fig. 6B,F). Furthermore, punctate hemidesmosomal morphology was restored in lgl2-ecadMO clones (Fig. 6E). This experiment demonstrated a quantitative requirement of Lgl2 in Itga6 localisation during hemidesmosome formation, and proved that the increased Itga6 localisation observed in the absence of E-cadherin is mostly Lgl2-dependent.

Finally, we asked whether E-cadherin function is essential for Itga6 localisation prior to 3 dpf. We did not observe a difference in Itga6 localisation in ecadMO clones (10 out of 12) in 3- to 3.5-day-old larvae (n=6), indicating that the early Itga6 localisation is independent of E-cadherin function (Fig. 6G,H).

DISCUSSION

In the developing epidermis, cellular junctions are essential for maintenance of tissue homeostasis and integrity. How cellular junctions are formed and how their number is regulated in the developing epidermis are two central questions in developmental cell biology. Here, we have analysed the function of Lgl2, E-cadherin and aPKCλ in the formation of hemidesmosomes in the developing basal epidermis. Our data indicate that Lgl2 regulates the formation of hemidesmosomes by mediating the targeting of a hemidesmosomal component, Itga6, to the membrane beyond 3.5 dpf. Lgl has been shown to mediate the basolateral targeting of cell fate determinants in *Drosophila* neuroblasts (Ohshiro et al., 2000; Peng et al., 2000; Betschinger et al., 2003; Betschinger et al., 2006). However, it was not clear whether Lgl2 functions in targeting any protein in epithelia. Here, we have identified Itga6 as the first membrane protein whose targeting is mediated by Lgl2.

Similar to Lgl2 and Itga6, E-cadherin is localised to the lateral domain in basal epidermal cells. Given the role of E-cadherin in the formation of cellular junctions, such as desmosomes and tight junctions (Gumbiner et al., 1988; Tunggal et al., 2005; Capaldo and Macara, 2007), we analysed the role of E-cadherin in hemidesmosome formation. Interestingly, the loss of E-cadherin in the basal epidermis leads to increased Itga6 localisation at 5-6 dpf, in turn leading to an increase in the number of hemidesmosomes. The reduction of Lgl2 levels in E-cadherin knockdown clones rescues this hemidesmosomal phenotype. Our analysis has led to the surprising discovery that the lateral domain of the basal epidermal cells harbours two signals that act antagonistically to regulate the formation of hemidesmosomes in the basal domain. This is the first

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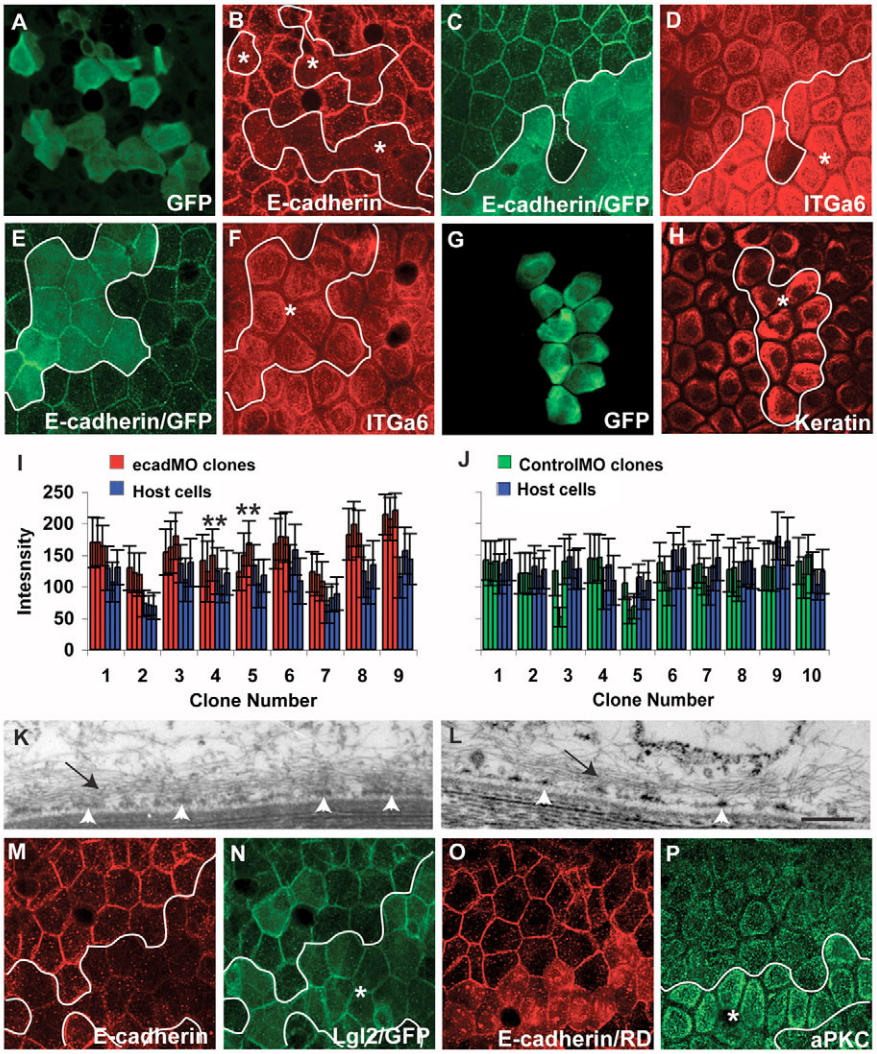

Fig. 5. The loss of E-cadherin function results in augmented localisation of Itga6 and increased hemidesmosome formation in the basal domain. (**A-H**) Immunostaining with anti-GFP, anti-E-cadherin, anti-Itga6 and anti-keratin antibodies as labelled. *e-cadherin* morpholino (ecadMO) clones (A) show consistent knockdown of E-cadherin in 5-day-old larvae (B). The knockdown of E-cadherin in clones (C) results in the enhanced localisation of Itga6 at the basal domain in 6-day-old larvae (D). The control morpholino clones (E), do not show any increase in Itga6 localisation to the basal domain (F). Furthermore, ecadMO clones (G) also exhibit enhanced localisation of hemidesmosomal keratin (H). Asterisks, epidermal clones. (**I**,**J**) Quantification of Itga6 signal intensity in ecadMO and control morpholino (controlMO) clones and surrounding host cells at 6 dpf. The mean intensity per cell is shown with s.d. This quantification reveals that in ecadMO clones, signal intensities for Itga6 are consistently higher than in the host cells (I), whereas in control morpholino clones they are not (J). Three comparisons out of nine (in clone number 4) and one comparison out of nine (in clone number 5) were not statistically significant by Student's *t*-test (**$P \leqslant 0.05$). (**K**,**L**) Electron micrograph of an ecadMO clonal cell (K) and control wild-type cell in the same region (L). In ecadMO clones, hemidesmosomes coalesce to form electron-dense mats (arrowheads in K), which contrast with electron-dense punctae in control cells at 6 dpf (arrowheads in L). Note the macrofilaments projecting out of the hemidesmosomal mats and punctae (arrows). (**M-P**) Co-immunostaining using anti-E-cadherin (M) and anti-Lgl2 (N) antibodies, or anti-E-cadherin (O) and anti-aPKC (P) antibodies. *e-cadherin* knockdown in the basal epidermis (M) does not alter the localisation of Lgl2 at 5 dpf (N). However, ecadMO clones (O) do exhibit increased localisation of aPKC to the basal domain (P). In C,E,H, the cytoplasmic green fluorescence represents GFP, whereas in O the cytoplasmic red fluorescence represents Rhodamine-dextran (RD). Our fixation conditions do not lead to complete quenching of GFP and Rhodamine fluorescence. Scale bar: 27 μm in A-H; 270 nm in K,L.

evidence indicating that in developing epidermal cells, an interaction between proteins localised at the lateral domain is essential to regulate the formation of junctions in the basal domain.

How do Lgl2 and E-cadherin, localised at the lateral domain, regulate the formation of hemidesmosomes formed at the basal domain in epidermal cells? We have been able to show that at the lateral domain, Itga6 localises with Lgl2 as well as with E-cadherin. This observation indicates that after its synthesis, a fraction of Itga6 is first targeted to the lateral domain. This lateral Itga6 fraction diminishes by 5 dpf, indicating that Itga6 localisation at the lateral domain is dynamic. In early *lgl2* mutant larvae (3.75 dpf), there is a selective loss of Itga6 localisation at the lateral membrane domain. Moreover, in *lgl2* mutant larvae, Itga6 vesicles accumulate in the cytoplasm, especially near the lateral and apical domains. Thus, it is plausible that beyond 3.5 dpf, a fraction of the Itga6 synthesised is targeted to the lateral membrane domain first and that Lgl2 mediates this targeting. This fraction at the lateral domain then translocates to the basal domain, where it joins the existing Itga6 fraction (localised

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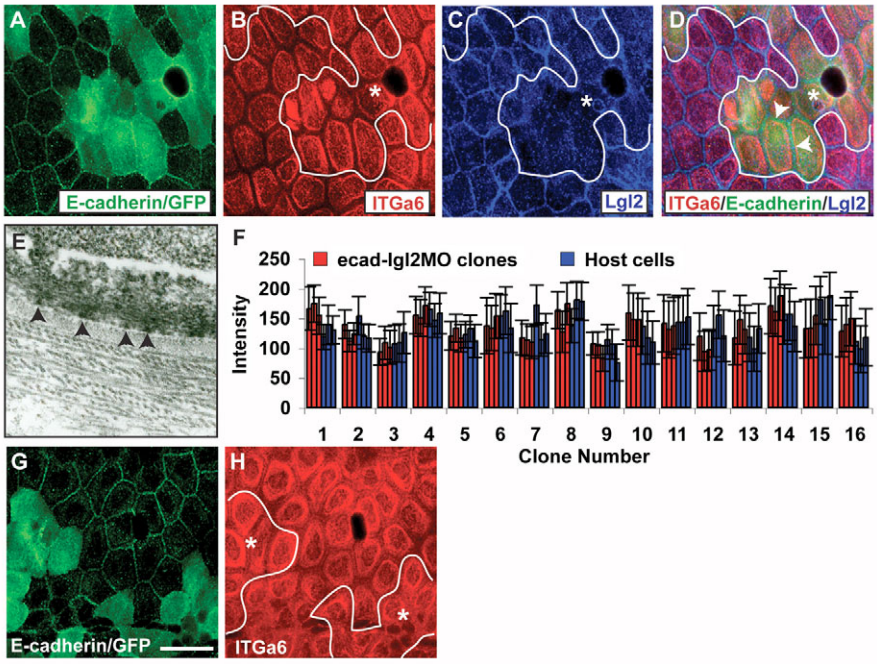

Fig. 6. The reduction of Lgl2 levels in ecadMO clones rescues the hemidesmosomal phenotype. (**A-D**) Co-immunostaining using anti-E-cadherin/GFP (A), anti-Itga6 (B) and anti-Lgl2 (C) antibodies and overlay (D). In ecadMO clones (A), Itga6 does not show enhanced localisation at 6 dpf (B) when Lgl2 levels (C) are reduced by co-injecting *lgl2* morpholino. Note that Lgl2 is not completely absent after injection of suboptimal concentrations (100 μM) of *lgl2* morpholino (arrowheads in D). Quantification of signal intensities reveals that there are no significant differences in the Itga6 intensities in clonal cells and surrounding host cells (D). Asterisks, epidermal clones. In A and E, the cytoplasmic green fluorescence represents GFP. Our fixation conditions do not lead to complete quenching of GFP fluorescence. (**E**) Electron micrograph of lgl2-ecadMO clone. (**F**) The signal intensity for Itga6 was quantified from three host cells and from three cells per clone that received *e-cadherin* as well as *lgl2* (at sub-optimal concentrations) morpholinos. The mean intensity for each cell and the standard error are shown. (**G**,**H**) Co-immunostaining using anti-E-cadherin (G) and anti-Itga6 (H). This clonal analysis in 3.5-day-old larvae reveals that ecadMO clones (G) do not exhibit enhanced localisation of Itga6 at the basal or lateral domain (H). Scale bar: 27 μm in A-D,G,H; 270 nm in E.

prior to 3.5 dpf) clustered at the intermediate filaments, and becomes assembled into functional hemidesmosomes. The translocation of the lateral Itga6 fraction to the basal domain may occur by passive diffusion or by transcytosis. In the latter case, a likely mechanism might be Rab21/Rab5-mediated endocytosis and trafficking of Itga6 from the lateral domain and Rab11-mediated delivery to the basal domain through recycling endosomes (for a review, see Pellinen and Ivaska, 2006). Since, in *lgl2* mutant larvae, the Itga6 fraction targeted beyond 3.5 dpf fails to reach the lateral membrane domain and thus also the basal domain, the existing levels of Itga6 at the basal domain (localised before 3.5 dpf) remain insufficient to form functional hemidesmosomes.

In contrast to the *lgl2* mutant, where Itga6 targeting is perturbed and its localisation lost, knockdown of E-cadherin function leads to enhanced Itga6 localisation at the basal domain. Reduction of Lgl2 levels in ecadMO clones leads to the normalisation of Itga6 levels at the basal domain. This indicates that E-cadherin negatively regulates Lgl2-mediated targeting of Itga6. Alternatively, E-cadherin could be (indirectly) repressing Itga6 synthesis such that in the absence of E-cadherin, more Itga6 is synthesised and hence localised to the basal domain. If the latter hypothesis were correct, then continued Itga6 synthesis in the absence of E-cadherin would lead to Itga6 accumulation in the cytoplasm when the membrane targeting is perturbed by reducing Lgl2 levels. We do not observe any such accumulation of Itga6 in lgl2-ecadMO clones. In all events, further experiments need to be performed to understand the precise function of E-cadherin in this process.

In mouse, E-cadherin deficiency has thus far not been correlated with increased hemidesmosome formation, although beta 4 integrin localisation studies have been performed in E-cadherin knockout mice (Tinkle et al., 2004; Tinkle et al., 2008). In the light of our data, these knockout mice models should be re-evaluated by constructing mutant-wild type chimeras to examine quantitative differences in hemidesmosome formation and to check whether the mechanism we have described in zebrafish is conserved in mammals. Our analyses demonstrate a clear, quantifiable effect of the loss of *e-cadherin* function on hemidesmosomes in a basal vertebrate. Such analyses will be important in the quest to understand how mechanisms that establish polarity and hemidesmosome formation have evolved in vertebrates.

Although hemidesmosomes are not formed, Itga6 is localised to the basal domain as early as 2.5 dpf. At 3.5 dpf, Itga6 exhibits a clustered association with intermediate filaments, and this appears to be an intermediate step prior to hemidesmosome formation. This early basal localisation of Itga6, as well as the lateral localisation, were mostly unperturbed in *lgl2* mutant larvae at 3.5 dpf and in *lgl2* and *e-cadherin* morphant clones. Thus, the initial targeting of Itga6, prior to 3.5 dpf, is Lgl2- and E-cadherin-independent. This early Itga6 fraction that is localised to the basal domain prior to 3.5 dpf is lost in *lgl2* mutant larvae at 4-5 dpf. This indicates that in addition to its primary function in targeting, Lgl2 function is also essential for the maintenance of Itga6 at the basal domain beyond 3.5 dpf. It is not clear what happens to the early Itga6 fraction, which is localised to the basal domain, in *lgl2* mutant larvae. One plausible

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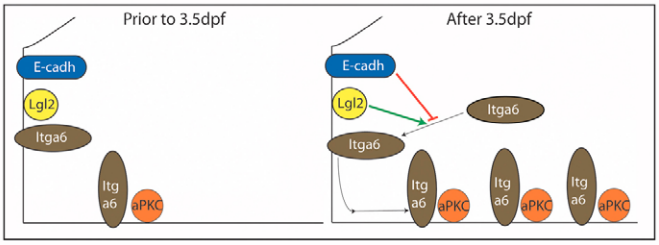

Fig. 7. Steps involved in hemidesmosome formation and the establishment of polarity in developing zebrafish basal epidermis. At early stages, prior to 3.5 dpf, Itga6 is localised to the basal domain. However, it is not clear whether Itga6 is targeted directly to the basal domain or whether it follows an indirect route via the lateral domain. After 3.5 dpf, additional Itga6 is targeted to the basal domain, probably via the lateral domain. This targeting is positively regulated by Lgl2 and negatively regulated by E-cadherin at the lateral domain. aPKC localises to the basal domain along with Itga6, and this localisation of aPKC is dependent on hemidesmosome formation.

explanation is that the turnover of Itga6 that is not yet assembled in hemidesmosomes is high, leading to its endocytosis and degradation at subsequent stages.

In many epithelia, a balance in the activities of the Par3-aPKC and Lgl pathways is essential for the establishment and maintenance of apical-basal cell polarity (Bilder et al., 2003; Tanentzapf and Tepass, 2003; Yamanaka et al., 2003; Hutterer et al., 2004; Chalmers et al., 2005). In *Xenopus* blastula epithelium, beta 1 integrin, a basolateral marker, has been shown to mislocalise to the apical domain when the function of aPKC is inhibited or Lgl2 is overexpressed (Chalmers et al., 2005). Interestingly, however, Itga6 localisation is not altered in the *aPKCλ* mutant or when *aPKCλ* is knocked down. Thus, Itga6 does not respond to polarity cues like beta 1 integrin and adherens as well as tight junction components do. However, there is a strong correlation between Itga6 localisation, the formation of hemidesmosomes and the localisation of aPKCλ during development. Moreover, the loss of hemidesmosomes in *lgl2* mutants and the increase in the number of hemidesmosomes when E-cadherin function is knocked down also correlate with the absence and increase, respectively, in aPKCλ localisation. Therefore, we propose that aPKC localisation is dependent on hemidesmosome formation.

To summarise, our analysis has revealed hitherto unidentified steps in the formation of hemidesmosomes and in the establishment of polarity in the developing basal epidermis of vertebrates (Fig. 7). Prior to 3.5 dpf, neither Lgl2 nor E-cadherin function is essential for the targeting of Itga6 to the basal domain. During 3.5-5 dpf, the assembly of hemidesmosomes requires progressive targeting of Itga6 to the membrane. This latter Itga6 targeting depends on Lgl2, which localises to the lateral domain. We propose that after its synthesis, Itga6 is targeted to the lateral domain first and from there it is translocated to the basal domain, where it participates in hemidesmosome formation. Whereas Lgl2 positively regulates hemidesmosome formation by mediating Itga6 targeting and maintaining its localisation, E-cadherin negatively regulates the Lgl2-mediated Itga6 targeting. These antagonistic signals control the precise levels of Itga6 at the basal domain during hemidesmosome formation in the developing zebrafish epidermis. The localisation of aPKCλ in the basal epidermis is tightly correlated with, and might be dependent on, Itga6 localisation and hemidesmosome formation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/8/1231/DC1

References

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J. V. and Retta, S. F. (2005). E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. *J. Cell Sci.* **118**, 4765-4783.

Betschinger, J., Mechtler, K. and Knoblich, J. A. (2003). The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* **422**, 326-330.

Betschinger, J., Eisenhaber, F. and Knoblich, J. A. (2005). Phosphorylation-induced autoinhibition regulates the cytoskeletal protein Lethal (2) giant larvae. *Curr. Biol.* **15**, 276-282.

Betschinger, J., Mechtler, K. and Knoblich, J. A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. *Cell* **124**, 1241-1253.

Bilder, D., Schober, M. and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat. Cell Biol.* **5**, 53-58.

Bryant, P. J. (1997). Junction genetics. *Dev. Genet.* **20**, 75-90.

Capaldo, C. T. and Macara, I. G. (2007). Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. *Mol. Biol. Cell* **18**, 189-200.

Chalmers, A. D., Pambos, M., Mason, J., Lang, S., Wylie, C. and Papalopulu, N. (2005). aPKC, Crumbs3 and Lgl2 control apicobasal polarity in early vertebrate development. *Development* **132**, 977-986.

Chen, X. and Gumbiner, B. M. (2006). Crosstalk between different adhesion molecules. *Curr. Opin. Cell Biol.* **18**, 572-578.

Gimond, C., van Der Flier, A., van Delft, S., Brakebusch, C., Kuikman, I., Collard, J. G., Fassler, R. and Sonnenberg, A. (1999). Induction of cell scattering by expression of beta1 integrins in beta1-deficient epithelial cells requires activation of members of the rho family of GTPases and downregulation of cadherin and catenin function. *J. Cell Biol.* **147**, 1325-1340.

Gumbiner, B., Stevenson, B. and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* **107**, 1575-1587.

Harris, T. J. and Peifer, M. (2004). Adherens junction-dependent and -independent steps in the establishment of epithelial cell polarity in Drosophila. *J. Cell Biol.* **167**, 135-147.

Horne-Badovinac, S., Lin, D., Waldron, S., Schwarz, M., Mbamalu, G., Pawson, T., Jan, Y., Stainier, D. Y. and Abdelilah-Seyfried, S. (2001). Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr. Biol.* **11**, 1492-1502.

Humbert, P., Russell, S. and Richardson, H. (2003). Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *BioEssays* **25**, 542-553.

Hutterer, A., Betschinger, J., Petronczki, M. and Knoblich, J. A. (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during Drosophila embryogenesis. *Dev. Cell* **6**, 845-854.

Kane, D. A., McFarland, K. N. and Warga, R. M. (2005). Mutations in half baked/E-cadherin block cell behaviors that are necessary for teleost epiboly. *Development* **132**, 1105-1116.

Knaut, H., Blader, P., Strahle, U. and Schier, A. F. (2005). Assembly of trigeminal sensory ganglia by chemokine signaling. *Neuron* **47**, 653-666.

Knust, E. and Bossinger, O. (2002). Composition and formation of intercellular junctions in epithelial cells. *Science* **298**, 1955-1959.

Maderspacher, F. and Nüsslein-Volhard, C. (2003). Formation of the adult pigment pattern in zebrafish requires leopard and obelix dependent cell interactions. *Development* **130**, 3447-3457.

Musch, A., Cohen, D., Yeaman, C., Nelson, W. J., Rodriguez-Boulan, E. and Brennwald, P. J. (2002). Mammalian homolog of Drosophila tumor suppressor lethal (2) giant larvae interacts with basolateral exocytic machinery in Madin-Darby canine kidney cells. *Mol. Biol. Cell* **13**, 158-168.

Nebenführ, A., Ritzenthaler, C. and Robinson, D. G. (2002). Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiol.* **130**, 1102-1108.

Ohshiro, T., Yagami, T., Zhang, C. and Matsuzaki, F. (2000). Role of cortical tumour-suppressor proteins in asymmetric division of Drosophila neuroblast. *Nature* **408**, 593-596.

Pellinen, T. and Ivaska, J. (2006). Integrin traffic. *J. Cell Sci.* **119**, 3723-3731.

Peng, C. Y., Manning, L., Albertson, R. and Doe, C. Q. (2000). The tumour-suppressor genes lgl and dlg regulate basal protein targeting in Drosophila neuroblasts. *Nature* **408**, 596-600.

Plant, P. J., Fawcett, J. P., Lin, D. C., Holdorf, A. D., Binns, K., Kulkarni, S. and Pawson, T. (2003). A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nat. Cell Biol.* **5**, 301-308.

DEVELOPMENT

Shin, K., Fogg, V. C. and Margolis, B. (2006). Tight junctions and cell polarity. *Annu. Rev. Cell Dev. Biol.* **22**, 207-235.

Sonawane, M., Carpio, Y., Geisler, R., Schwarz, H., Maischein, H. M. and Nuesslein-Volhard, C. (2005). Zebrafish penner/lethal giant larvae 2 functions in hemidesmosome formation, maintenance of cellular morphology and growth regulation in the developing basal epidermis. *Development* **132**, 3255-3265.

Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. *J. Cell Sci.* **119**, 979-987.

Tanentzapf, G. and Tepass, U. (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat. Cell Biol.* **5**, 46-52.

Tinkle, C. L., Lechler, T., Pasolli, H. A. and Fuchs, E. (2004). Conditional targeting of E-cadherin in skin: insights into hyperproliferative and degenerative responses. *Proc. Natl. Acad. Sci. USA* **101**, 552-557.

Tinkle, C. L., Pasolli, H. A., Stokes, N. and Fuchs, E. (2008). New insights into cadherin function in epidermal sheet formation and maintenance of tissue integrity. *Proc. Natl. Acad. Sci. USA* **105**, 15405-15410.

Tunggal, J. A., Helfrich, I., Schmitz, A., Schwarz, H., Gunzel, D., Fromm, M., Kemler, R., Krieg, T. and Niessen, C. M. (2005). E-cadherin is essential for *in vivo* epidermal barrier function by regulating tight junctions. *EMBO J.* **24**, 1146-1156.

van Eeden, F. J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A. et al. (1996). Genetic analysis of fin formation in the zebrafish, Danio rerio. *Development* **123**, 255-262.

Vasioukhin, V., Bauer, C., Degenstein, L., Wise, B. and Fuchs, E. (2001). Hyperproliferation and defects in epithelial polarity upon conditional ablation of alpha-catenin in skin. *Cell* **104**, 605-617.

Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A. and Ohno, S. (2003). Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr. Biol.* **13**, 734-743.

Zhang, X., Wang, P., Gangar, A., Zhang, J., Brennwald, P., TerBush, D. and Guo, W. (2005). Lethal giant larvae proteins interact with the exocyst complex and are involved in polarized exocytosis. *J. Cell Biol.* **170**, 273-283.

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