# Zebrafish *penner/lethal giant larvae 2* functions in hemidesmosome formation, maintenance of cellular morphology and growth regulation in the developing basal epidermis

**Mahendra Sonawane[1,*], Yamila Carpio[1,2], Robert Geisler[1], Heinz Schwarz[1], Hans-Martin Maischein[1] and Christiane Nuesslein-Volhard[1]**

[1]Max-Planck Institut für Entwicklungsbiologie, Department of Genetics, Spemannstrasse 35, Tuebingen, D-72076, Germany
[2]Centre for Genetic Engineering and Biotechnology, Animal Biotechnology Department, La Habana, CP 10 600, Cuba
*Author for correspondence (e-mail: mahendra.sonawane@tuebingen.mpg.de)

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## Summary

**Epithelial cells are equipped with junctional complexes that are involved in maintaining tissue architecture, providing mechanical integrity and suppressing tumour formation as well as invasiveness. A strict spatial segregation of these junctional complexes leads to the polarisation of epithelial cells. In vertebrate epithelia, basally localised hemidesmosomes mediate stable adhesion between epithelial cells and the underlying basement membrane. Although components of hemidesmosomes are relatively well known, the molecular machinery involved in governing the formation of these robust junctions, remains elusive. Here, we have identified the first component of this machinery using a forward genetic approach in zebrafish as we show that the function of *penner* (*pen*)/*lethal giant larvae 2* (*lgl2*) is necessary for hemidesmosome formation and maintenance of the tissue integrity in the developing basal epidermis. Moreover, in *pen/lgl2* mutant, basal epidermal cells hyper-proliferate and migrate to ectopic positions. Of the two vertebrate orthologues of the *Drosophila* tumour suppressor gene *lethal giant larvae*, the function of *lgl2* in vertebrate development and organogenesis remained unclear so far. Here, we have unravelled an essential function of *lgl2* during development of the epidermis in vertebrates.**

Key words: Epidermis, Hemidesmosomes, *penner/lgl2*, Cell proliferation, Zebrafish

## Introduction

The epidermis, a stratified epithelium, acts as a barrier against pathogens, chemicals and physical agents, and prevents the loss of body fluids. The basal layer of the epidermis harbours hemidesmosomes, which are essential for stable adhesion between basal epidermal cells and the underlying basement membrane formed of extracellular matrix. Hemidesmosomes are multi-protein complexes with α6β4 integrins as the trans-membrane core components, which interact with laminin 5 in the basement membrane (reviewed by Fuchs and Raghavan, 2002). The loss or impaired formation of hemidesmosomes, owing to mutations in genes that code for hemidesmosomal components, leads to detachment of the epidermis from the underlying basement membrane under mechanical stress (reviewed by Pulkkinen and Uitto, 1999). This results in blistering of the skin, a disease condition called epidermolysis bullosa. As hemidesmosomes mediate stable adhesion, their disassembly may be a prerequisite for the migration of basal epidermal cells during wound healing and metastatic invasion (Mariotti et al., 2001; Rabinovitz et al., 1999). Although components of hemidesmosomes have been described relatively well in recent years (reviewed by Borradori and Sonnenberg, 1996; Fuchs and Raghavan, 2002), it is not clear how the formation of these junctions is regulated during the epidermal development to specify the basal domain of basal epidermal cells.

How polarised distribution of various cellular junctions occurs during development is one of the most fundamental questions in cell biology. In the embryonic epidermis of *Drosophila*, the zonula adherens demarcates the boundaries of the apical domain, whereas septate junctions are formed in the basolateral domain. Genetic analysis of zonula adherens and septate junction formation in the *Drosophila* embryonic ectoderm (epidermis) has revealed genes and pathways that are involved in polarisation of epithelia. The competitive interaction between *crumbs*, *lethal giant larvae* and *bazooka/par3* pathways have been shown to be crucial for the apical localisation of zonula adherens in the embryonic epidermis (Bilder et al., 2003; Tanentzapf and Tepass, 2003). In absence of a functional *lgl* pathway, which along with *lgl* also comprises *scribble* and *discs large*, the apical domain expands at the expense the basolateral domain, indicating essential function of this pathway in the maintenance of the basolateral domain (Bilder et al., 2000; Bilder et al., 2003; Tanentzapf and Tepass, 2003). Consistent with this, the function of *scribble* and *discs large* is also

necessary for the formation of septate junctions (Bilder and Perrimon, 2000; Woods et al., 1996; Woods et al., 1997).

The polarised distribution of phosphorylated *Drosophila* Lgl has been suggested to be necessary for the basal targeting of proteins such as Miranda, which determine the fate of ganglion mother cells (Betschinger et al., 2003; Ohshiro et al., 2000; Peng et al., 2000). Vertebrates have two *Drosophila lgl* orthologues, *lgl1* and *lgl2*. Similar to *Drosophila* Lgl, both Lgl1 and Lgl2 interact with the Par3/Bazooka pathway and are phosphorylated at conserved serine residues by aPKC (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). The phosphorylation of Lgl1 and Lgl2 is also necessary for their localisation exclusively at the basolateral membrane, as nonphosphorylatable Lgl1 and Lgl2 also localise to the apical domain (Musch et al., 2002; Yamanaka et al., 2003). At the basolateral membrane, Lgl1 binds to syntaxin 4, a t-SNARE that is involved in the fusion of post-Golgi transport vesicles to the target membranes, and has been proposed to link the establishment of cell polarity to the polarised exocytosis (Musch et al., 2002).

In addition to defects in cell polarity, *lgl* mutant larvae exhibit neoplastic growth of neuroblasts as well as imaginal disc epithelium in *Drosophila*, and in the absence of *lgl* function, non-invasive tumours expressing activated Ras develop into aggressive cancers (reviewed by Bilder, 2004; Gateff, 1978; Pagliarini and Xu, 2003). In mouse, *lgl1* is expressed almost ubiquitously in adults and during embryogenesis (Klezovitch et al., 2004). The *lgl1* knockout mouse exhibits disorganisation of the apical junctional complex and disruption of apicobasal polarity in neuroepithelium, resulting in hyper-proliferation of neuroblasts and brain dysplasia (Klezovitch et al., 2004). Consistent with its role in regulating proliferation, transcripts of *lgl1* have been shown to be absent in various human carcinomas (Grifoni et al., 2004). At present, it is not clear whether *lgl2* has any functions in regulating growth during development or organ formation.

Owing to the unavailability of an appropriate model system, a forward genetic analysis of hemidesmosome formation has never been performed. We envisage that a forward genetic analysis of hemidesmosome formation in the zebrafish would uncover developmental cues regulating the assembly of these robust structures, pathways involved in establishing the basal domain of epidermal cells and targeting hemidesmosomal components to the basal cortex, as well as novel components of hemidesmosomes. Here, we confirm the feasibility of using zebrafish as a model system to perform a forward genetic analysis of hemidesmosome formation. We screened an existing collection of zebrafish skin mutants (van Eeden et al., 1996) and show that *penner* (*pen*) function is necessary for hemidesmosome formation in basal epidermal cells. Genetic mapping and molecular cloning reveals that *penner* is zebrafish *lgl2*, and functions in hemidesmosome formation and maintenance of the basal domain in basal epidermal cells. We further show that in *penner* mutant larvae basal epidermal cells migrate to ectopic places, and hyper-proliferate, indicating that *lgl2* is also involved in regulating growth of the basal epidermis. Thus, our approach has unravelled a previously unknown function of *lgl2* during development of the basal epidermis of vertebrates.

## Materials and methods

### Fish strains

The morphological and immunohistological analysis of *pen* mutant was carried out in Tübingen (Tü) and WIK background. For in situ hybridisations *albino* larvae in Tü background were used. For transplantations, embryos from actin-GFP transgenic fish were used as donors and from *albino* fish as recipients.

### Histology: light and electron microscopy

Wild-type and mutant larvae were fixed overnight in 4% PFA in PBS at 4°C, washed in PBS, upgraded in ethanol series (30% to absolute) and embedded in Technovit 7100. Blocks were sectioned (3 μm) using an automated Leica RM 2165 microtome, stained using Giemsa (Kiernan, 2001) and embedded in permount. For electron microscopy (EM), after fixation in 4% PFA, specimens were post-fixed with 1% $OsO_4$ in $H_2O$ for 10 minutes on ice, followed by fixation and contrast with 1% uranyl acetate for 1 hour on ice, then dehydrated by increasing concentrations of ethanol and embedded in Epon. Ultrathin sections made from the head, trunk and tail region (60-90 nm) were stained with lead citrate and uranyl acetate.

### BrdU labelling

For labelling, 5-day-old wild-type and *penner* mutant larvae were incubated with 10 mM BrdU solution in 2% DMSO in embryonic medium (E3) for 2 hours. After treatment, larvae were washed several times. Fixation and staining was carried out as follows.

### Immunohistochemistry

Three- to five-day-old wild-type and mutant larvae were fixed overnight in 4% PFA in PBS at 4°C followed by permeabilisation in absolute methanol at –20°C, to stain for actin using C4 mouse anti actin (Cedarlane), β-catenin using P14L antibody, BrdU using ab6326 (abcam) antibody. For staining cytokeratin using Ks pan 1-8 (Progen Biotechnik) antibody, larvae were fixed in Dent's fixative at –20°C. For staining GFP using anti-GFP antibody (Torrey Pines), embryos were either fixed in 4% PFA or in Dent's fixative. After downgrading the larvae to 0.1 M phosphate buffer (PB), they were washed with PBT (PB+0.8% Triton X-100) five times and blocked in 10% normal goat serum. For BrdU staining, larvae were treated with 4 N HCl for 20 minutes, washed extensively in PB and blocked in 1% BSA for 1-3 hours. Antibodies were diluted as: anti actin, ab 6326 (1:100); Ks pan 1-8 (1:10); P14L (1:50); anti GFP (1:200). Samples were incubated at room temperature for 4 hours or overnight at 6-8°C. Afterwards, larvae were washed five times in PBT and incubated with Cy3-, Alexa 488- and alkaline phosphatase (AP)-conjugated or biotinylated anti-mouse, anti-rat or anti-rabbit antibodies. Larvae were washed, developed in NBT-BCIP or using ABC elite kit (Vectastain) along with DAB (for AP conjugated and biotinylated secondary antibodies only), post fixed in 4% PFA and either upgraded in glycerol for fluorescence/light microscopy or embedded in technovit for sectioning or processed for EM analysis. For fluorescence microscopy, sections were counterstained using DAPI and mounted in 70% glycerol.

### Genetic mapping, mutation analysis and analysis of conserved domains

Heterozygous fish (Tü background) carrying mutation in *penner* ($pen^{t06}$) were crossed with wild-type WIK fish. F2 mutant larvae were used for meiotic mapping using SSLP markers, as described previously (Geisler, 2002; Shimoda et al., 1999). Subsequent fine mapping was carried out using SNP markers identified in the BAC ends. The sequence of zC148E17 was analysed for ORFs using GENSCAN at: http://genes.mit.edu/GENESCAN.html

For mutational analysis, cDNA was prepared using AMV Reverse Transcriptase (Invitrogen) from mRNA isolated from 5-day-old Tü, WIK and *penner* mutant larvae using Oligotex spin columns. For sequencing, cDNA was amplified using gene-specific primers (designed

using accession number BC052919) and Pfu DNA polymerase (MBI Fermentas), purified over QIAquick coloumns (Qiagen) and directly sequenced or cloned in PCR4-TOPO vector (Invitrogen). To detect mutations, sequence analysis was performed using Lasergene software from DNASTAR. The sequences from two different amplification reactions were examined to confirm the mutation.

The wild-type Lgl2 sequence was used to search for conserved domains with reverse position specific blast using databases CDD v2.00-11382PSSMs and KOG v1.00-4825PSSMs available at http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi

### In situ hybridisation

In situ hybridisation was performed as described (Schulte-Merker, 2002) using a *lgl2* probe from nucleotides 1621-2615 of BC052919. *lgl1* probe was derived from a partial zebrafish cDNA clone equivalent to amino acids 7-334 of *Xenopus* Lgl1.

### BAC rescue, morpholino injections and cell transplantations

BAC zC242O14 was injected in embryos obtained from *pen*$^{+/-}$ parents (with WIK background) at the one-cell stage at a concentration of 10 ng/μl. Embryos showing deformities were removed on d1. After 5 dpf, larvae were fixed in Dent's fix and processed for keratin staining.

Antisense morpholino oligos 5′G-CCCATGACGCCTGAACCTCTTCAT3′ directed against translational start site and a five-base mismatch (underline) control morpholino 5′GCACATAACGCCTCAA-CCTGTTAAT3′ (Gene Tools, Corvallis) were dissolved in sterile water to obtain 4 mM stocks. These stocks were diluted to 200 μM with sterile water for injections at the one- to four-cell stage. For transplantations, morpholino oligos were injected in donor embryos obtained from actin-GFP transgenic line, embryos were grown up to the 1000-cell stage at which cells from these were transplanted to albino embryos that were at the same stage. Recipient larvae with epidermal clones (usually 20-25% of total transplanted) were fixed after 3.5 or 5.5-6.0 days post fertilisation (dpf) in 4% PFA or in Dent's fixative for further immunohistological analysis. For electron microscopic analysis of clones, GFP was detected using anti GFP antibody (rabbit) and biotinylated anti-rabbit antibody along with elite ABC system (Vectastain) and DAB.

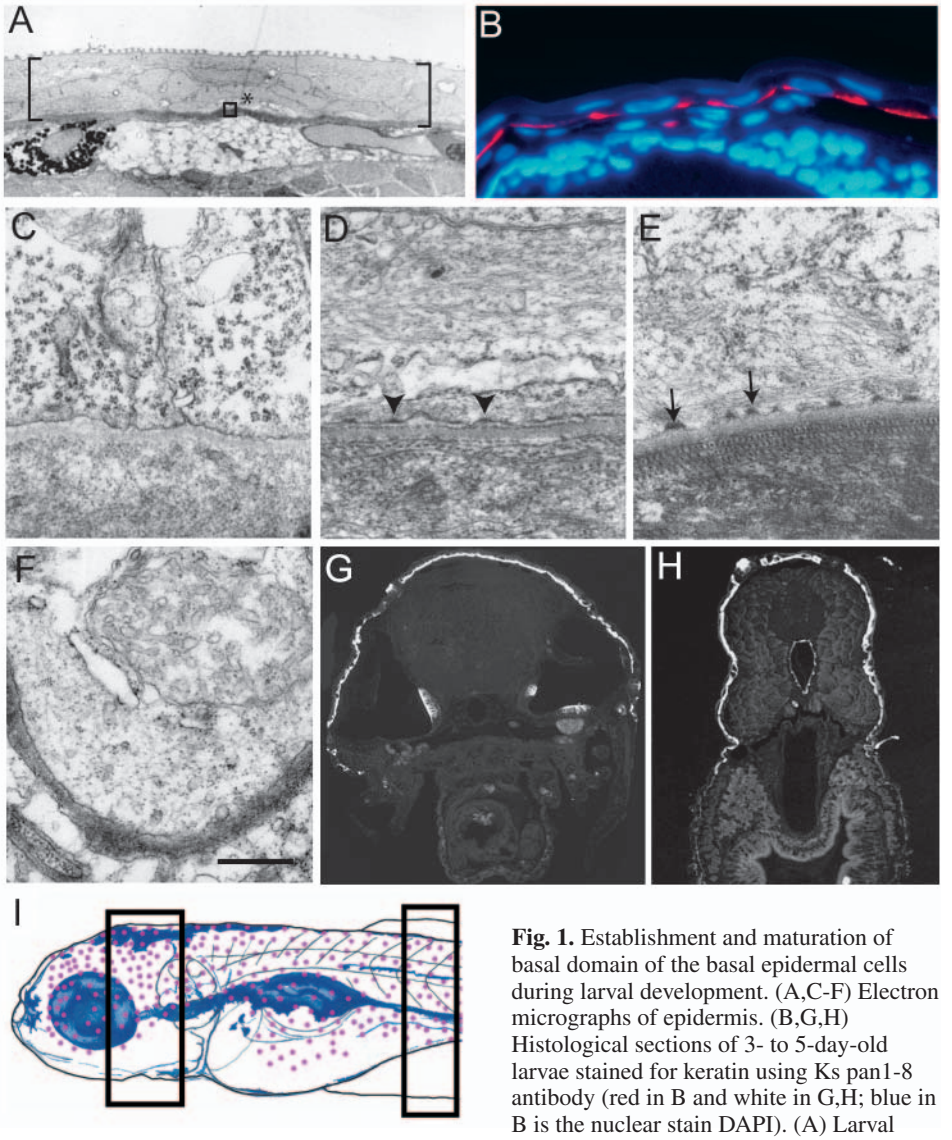


**Fig. 1.** Establishment and maturation of basal domain of the basal epidermal cells during larval development. (A,C-F) Electron micrographs of epidermis. (B,G,H) Histological sections of 3- to 5-day-old larvae stained for keratin using Ks pan1-8 antibody (red in B and white in G,H; blue in B is the nuclear stain DAPI). (A) Larval epidermis, marked by brackets, is bilayered. Asterisk indicates the basal epidermal cell and the area equivalent to that included in the box was analysed from 3- to 5.5-day-old larvae to assess the formation of hemidesmosomes in C-E. (B) Keratin is localised basally in the basal epidermis of 3-day-old larvae. (C-E) Hemidesmosomes are not present in 3-day-old larvae (C); however, premature hemidesmosomes (arrowheads in D) and mature hemidesmosomes (arrows in E) are present in 4.5- and 5.5-day-old larva, respectively. (E,F) Although hemidesmosomes are present in the dorsal epidermis (E), they are absent in the ventral epidermis covering the lower jaw of 5.5-day-old larvae (F). Keratin is also absent in the ventral or ventrolateral epidermal cells of 5-day-old larvae (G,H). Schematic drawing (I) of 5.5-day-old larvae (based on whole-mount keratin staining, immunohistology and electron microscopy data), indicating the epidermal domain (red dots) marked by keratin expression and presence of hemidesmosomes. Sections analysed by electron microscopy were made from the body region marked with rectangles in I. Scale bar: 8.9 μm in A; 40 μm in B; 543 nm in C-F.

## Results

### Hemidesmosome formation during development of the epidermis

We performed electron microscopy and immunohistological analysis using an anti-keratin Ks pan 1-8 antibody to characterise the events during epidermal development in zebrafish larvae. We found that the larval epidermis consists of an outermost periderm and the basal epidermis (Fig. 1A). During development, basally localised cytokeratin becomes apparent in basal epidermal cells by 3 days post fertilisation (dpf) and remains localised thereafter (Fig. 1B; data not shown). In electron micrographs, hemidesmosomes become visible from 4.5 dpf (Fig. 1C,D) and matured into well-

developed electron dense structures by 5.5 dpf (Fig. 1E). Thus, the basal domain is clearly defined in basal epidermal cells by 3 dpf and undergoes a subsequent maturation process, which involves formation of hemidesmosomes. However, not all the epidermal cells possess a basally localised keratin cytoskeleton and hemidesmosomes in 5-day-old larvae. Instead, these cellular features are restricted to the dorsal epidermis covering brain/spinal chord as well as the lateral epidermis covering somites (Fig. 1E-I). We also observed a considerable overlap between keratin expression and hemidesmosome forming region of the epidermis, which is represented in a schematic (Fig. 1I). The absence of keratin and hemidesmosomes in the ventral epidermis covering the lower jaw, heart, yolk/gut as well as in the fin fold epidermis indicates significant differences in the basal epidermis across body of the larvae (Fig. 1F-I).

### The *penner* function is essential for hemidesmosome formation and maintenance of the tissue integrity as well as cellular morphology in the basal epidermis

We screened an existing collection of zebrafish larval skin mutants (van Eeden et al., 1996) to identify genes involved in the process of hemidesmosome formation. Among these mutants, *pen* exhibits a late phenotype that is apparent by 4.5 to 5 dpf, coinciding with the time of hemidesmosome formation. The phenotype is marked by an overgrowth in the ventral epidermis and rounded up cells in fin folds (Fig. 2A,B) (van Eeden et al., 1996) and most of the larvae die on the 6th developmental day. Histological analyses of 5-day-old *pen* mutant larvae revealed that the epidermis of these larvae detaches from the underlying tissues (Fig. 2C,D). We have analysed ultra-structural details of the basal epidermis and the underlying basement membrane zone (BMZ) using electron microscopy. We found that in an undetached dorsal and lateral mutant epidermis, hemidesmosomes were missing, basal lamina appeared normal and, although formed, collagen lamella underlying the basal lamina appeared disorganised (Fig. 2E). The formation of collagen lamella indicated that dorsal and lateral epidermis differentiated normally in the mutant. Other junctions, such as desmosomes and tight junctions, were present and showed normal ultra-structural appearance in the mutant epidermis (Fig. 2F-H). We then performed immunohistological analysis using anti β-catenin antibody on mutant and wild-type larvae (Fig. 2I,J). A weak but significant β-catenin staining was observed in the mutant epidermis (Fig. 2J), indicating the presence of adherens junctions. Clearly, the *pen* function is essential only for the process of hemidesmosome formation and maintenance of the epidermal integrity.

In whole mounts of 5-day-old *pen* mutant larvae, stained for keratin, basal epidermal cells also appeared spindle shaped or round in contrast to polygonal in wild type (Fig. 3A,B). Further immunohistological analysis showed that keratin is not localised to the basal cortex as in wild-type larvae (Fig. 3C,D). We then asked if organisation of the actin cytoskeleton is also affected in *pen* mutant larvae. Our analysis revealed that, in contrast to the basal localisation of keratin, f-actin is localised in a punctate manner at the apical and lateral border in wild-type basal epidermal cells (Fig. 3C,E). Thus, domains of keratin and actin localisation are mutually exclusive in the wild-type basal epidermis. Interestingly, in *pen* mutant larvae, we often observed f-actin in the basal domain of basal epidermal cells but punctate staining at the apical and lateral borders remained unaffected (Fig. 3F). Thus, in the basal epidermis of the *pen* mutant, changes in the cellular

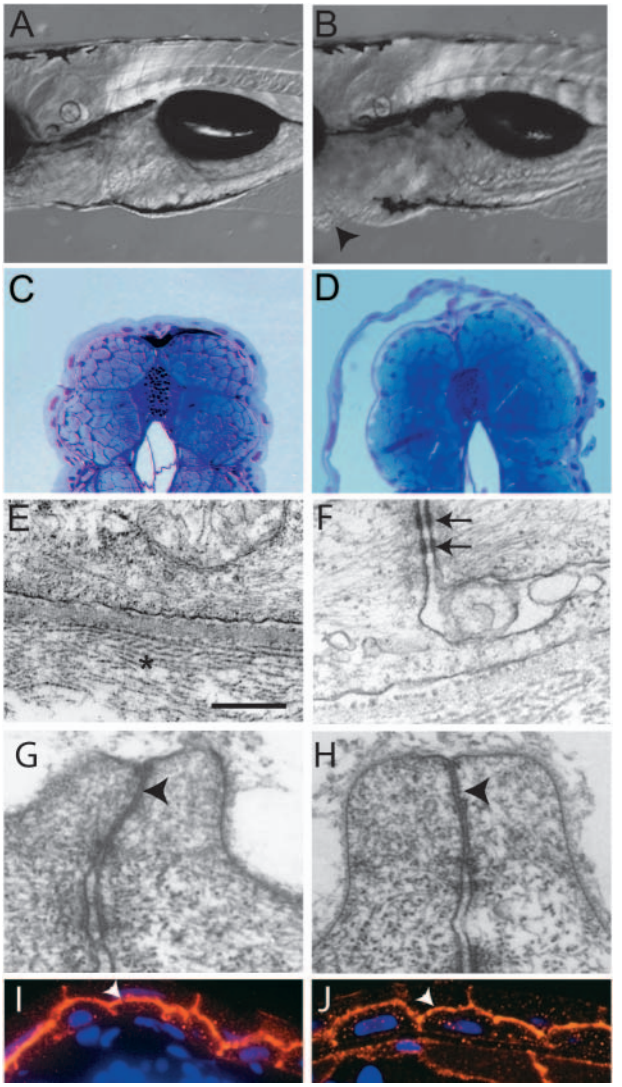


**Fig. 2.** Analysis of the *pen* mutant phenotype. (A,B) DIC images of 5-day-old wild type (A) and *pen* mutant larvae (B). The *pen* mutant larvae exhibit small ventral epidermal growths (arrowheads in B). (C,D) Giemsa stained thin sections of 5-day-old wild-type and mutant larvae. Detachment of epidermis is observed in mutant larvae (D). (E-H) Electron microscopic analysis of the epidermis. Hemidesmosomes are absent and collagen lamella is non-coherent (star) but the basal lamina in between appears normal in the epidermis of *pen* mutant larvae (E; also compare with Fig. 1E). Desmosomes (arrows in F), however, are present in the mutant epidermis. Tight junctions in the periderm of wild-type and *pen* mutant (arrowheads in G and H, respectively) show no ultra-structural differences. (I,J) Immunostaining with anti-β-catenin antibody to mark adherens junctions. In wild type (I) as well as in mutant (J), β catenin (arrowheads) is localised at the apical and lateral borders of basal epidermal cells. Staining intensity is lower in the *pen* mutant. Scale bar: 0.3 mm in A,B; 160 μm in C,D; 543 nm in E; 405 nm in F; 297 nm in G,H; 30 μm in I,J.

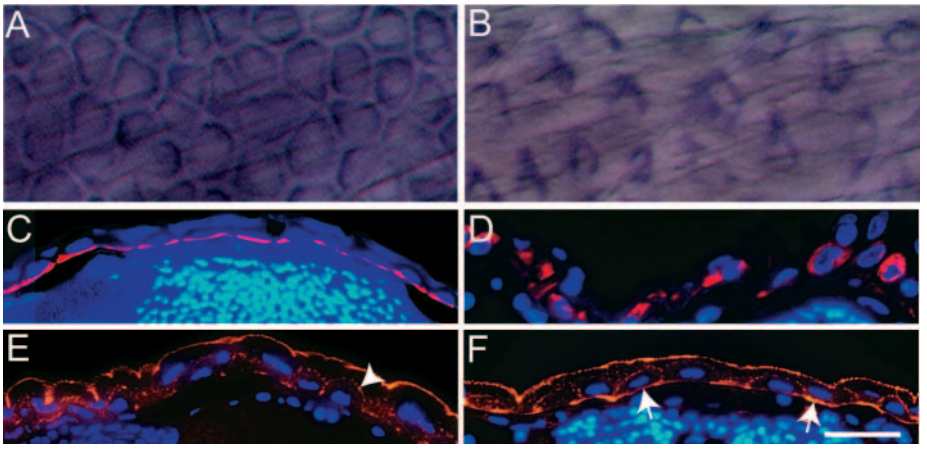


**Fig. 3.** *pen* function is essential for the maintenance of the organisation of cytoskeletal elements in basal epidermal cells. (A-D) Immunohistological staining in 5-day-old wild-type (A,C) and *pen* mutant larvae (B,D) using anti keratin (Ks pan-8) antibody. In whole mounts, basal epidermal cells appear polygonal in wild type (A) and spindle shaped in mutants (B). Histological sections of 5-day-old wild-type and *pen* mutants stained for keratin counterstained by DAPI (C,D), show that in contrast to wild type (C), keratin (red) does not remain localised to the basal cortex in mutant larvae (D). (E,F) Immunohistological staining with anti-actin antibody followed by counterstaining with DAPI indicates punctate localisation of actin (red) at apical and lateral borders of basal epidermal cells in wild type (arrowhead in E). By contrast, actin is also localised basally in mutants (arrows in F). Scale bar: 40 μm in A,B; 60 μm in C-F.

morphology are coupled to the changes in the localisation of cytoskeletal elements.

We further investigated if the mutation in *pen* is specifically affecting the process of hemidesmosome formation and maturation of the basal domain or if early defects in the keratin and actin localisation result in the subsequent loss of hemidesmosomes in *pen* mutant larvae. For this, 3.5-day-old progeny ($n$=40) of wild type as well as $pen^{+/-}$ parents were analysed for localisation of keratin and actin. If the mutation had affected the localisation of cytoskeletal elements before a visible onset of the phenotype, then 25% of the progeny would reflect this fact. But localisation of these components (analysed in whole mounts for keratin or sections for actin) in all of the progeny obtained from $pen^{+/-}$ parents looked similar to the wild type (data not shown). Moreover, there were no differences in the morphology of basal epidermal cells in mutant and wild-type larvae at this time point (data not shown). This clearly indicated that lack of hemidesmosome in the *pen* epidermis is not a consequence of early defects in the organisation of the keratin and actin cytoskeleton.

To conclude, the *pen* function is specifically required for the process of hemidesmosome formation and maintenance of the organisation of cytoskeletal elements, as well as cellular morphology in the basal epidermis.

## *penner* is zebrafish *lethal giant larvae2* and is expressed in the developing basal epidermis

We mapped (1872 meioses) *pen* on linkage group 12 (49.1 cM from the top) between two simple sequence length polymorphic (SSLP) markers (Fig. 4A). An EST cluster (wz 5928) maps to this region and allowed us to screen libraries in order to find BACs representing this region. The end of one of these BACs (zC209I1) was used as a length polymorphic marker and also mapped on the distal end of the sequence contig ctg10155 (zebrafish assembly zV2, Fig. 4A). We placed *pen* between this marker and a single nucleotide polymorphic (SNP) marker identified at the proximal end (Fig. 4A) of the sequence contig. A third SNP marker in between these two, showed very close linkage (0 recombinants) with *pen*. This region harbours sequences that show homology to the second zebrafish orthologue of the *Drosophila* gene *lethal giant larvae* (Fig. 4A). We then examined the sequence of a BAC (zC148E17) spanning this region to find that it harbours *lgl2* sequence along with *myosin XV* and an unknown ORF (Fig. 4A). We reconfirmed the linkage of these three genes with the mutation using SNP markers at both the ends of this BAC (0 recombinants). Previously, it has been shown that the *Drosophila lethal giant larvae* gene is involved in maintaining cell polarity in epithelia (Bilder et al., 2000; Manfruelli et al., 1996; Tanentzapf and Tepass, 2003). Therefore, we sequenced *lgl2* cDNA from 5-day-old mutant and wild-type (tü and WIK) larvae. Our sequencing analysis demonstrated that *lgl2* bears a nonsense mutation (Trp 399 to stop) eliminating ~60% of the C terminal part of the protein in mutants, which harbours a part of putative syntaxin binding domain (KOG1983) and conserved serine residues (Fig. 4B,B′,D) that have been shown to be phosphorylated by aPKC (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). To confirm that the mutation in *lgl2* is responsible for the phenotype observed in *pen*, we asked whether a BAC carrying a wild-type copy of *lgl2* and upstream regulatory sequences could complement the mutation and rescue the *pen* mutant phenotype. We selected zC242O14 for injections as it harboured *lgl2* (confirmed by PCR using gene specific primers; data not shown) and possibly contained upstream regulatory sequences. Moreover, one of the end of this BAC falls in the middle of zC148E17 suggesting only partial presence of *Myosin XV* ORF. In uninjected 5-day-old *pen* mutant larvae in WIK background ($n$=63), all the basal epidermal cells, stained for keratin, exhibited spindle or round shapes (data not shown). After BAC injections in zygotes, around 7% mutant larvae ($n$=42) showed clones of compactly arranged wild-type polygonal epidermal cells, indicating partial rescue (Fig. 4C).

The *pen/lgl2* gene is expressed in the entire epidermis of 24-

hour-old zebrafish larvae and the expression declines thereafter (Fig. 5A; data not shown). Using cell size as a criterion, we confirmed that *lgl2* transcripts are indeed present in the basal cells of the epidermis, including fin fold epidermis (Fig. 5B-E). Developing nasal placodes, otic vesicles and presumptive gut also exhibited strong expression of *lgl2* (Fig. 5A; data not shown). Although it declined during the larval development, we could detect *lgl2* expression in 5-day-old larvae using RT-PCR (data not shown). Our in situ expression analysis of embryos undergoing early cleavages also revealed the maternal contribution of *lgl2* transcripts (see Fig. S1A,B in the supplementary material). We further asked if *lgl1* is expressed in the epidermis and other epithelia. Interestingly, no *lgl1* transcripts were detected in the epidermis as well as in the gut and epithelia of otic vesicles and nasal placodes (see Fig. S1C in the supplementary material; data not shown).

To further confirm that the loss of *lgl2* function is indeed responsible for the epidermal phenotype observed in *pen* mutants and to test whether maternal *lgl2* transcripts have any role in maintaining the integrity and epithelial organisation of the basal epidermis prior to the hemidesmosome formation, we knocked down the function of maternal and zygotic *lgl2* using antisense morpholino oligos (lgl2MO) directed against the translational start site. Although the injections of control morpholino oligos did not result in any phenotype, embryos injected with lgl2MO resulted in severe abnormalities and shortened body axis (data not shown). We assumed that this early phenotype is due to the depletion of maternal transcripts. As these embryos remained retarded, to assess the effect of the lgl2MO on developing basal epidermal cells, we transplanted cells from morpholino injected embryos (marked by GFP) into the uninjected recipient *albino* embryos and obtained mitotic clones in the epidermis. The analysis of 6-day-old recipient larvae, using anti-GFP and anti-keratin antibodies, revealed that basal epidermal clones, derived from lgl2MO-injected embryos, comprised spindle-shaped or rounded-up cells similar to those in the *pen* mutant (Fig. 6B). Of the 543 clones analysed from 52 larvae, 73% showed these characteristic shape changes (Fig. 6J). The histological sectioning of a few of these clones revealed that, similar to the *pen* mutant, keratin cytoskeleton was no longer restricted to the basal cortex (Fig. 6G-I). We then performed the electron microscopy analysis of clones derived from lgl2MO-injected embryos to analyse the hemidesmosome formation. Of the three clones analysed from three randomly chosen larvae, two showed either the complete lack of hemidesmosomes (Fig. 6K,L) or near absence with very few premature hemidesmosome-like structures formed (data not shown). In contrast to lgl2MO-injected embryos, only 13% of clones derived from uninjected

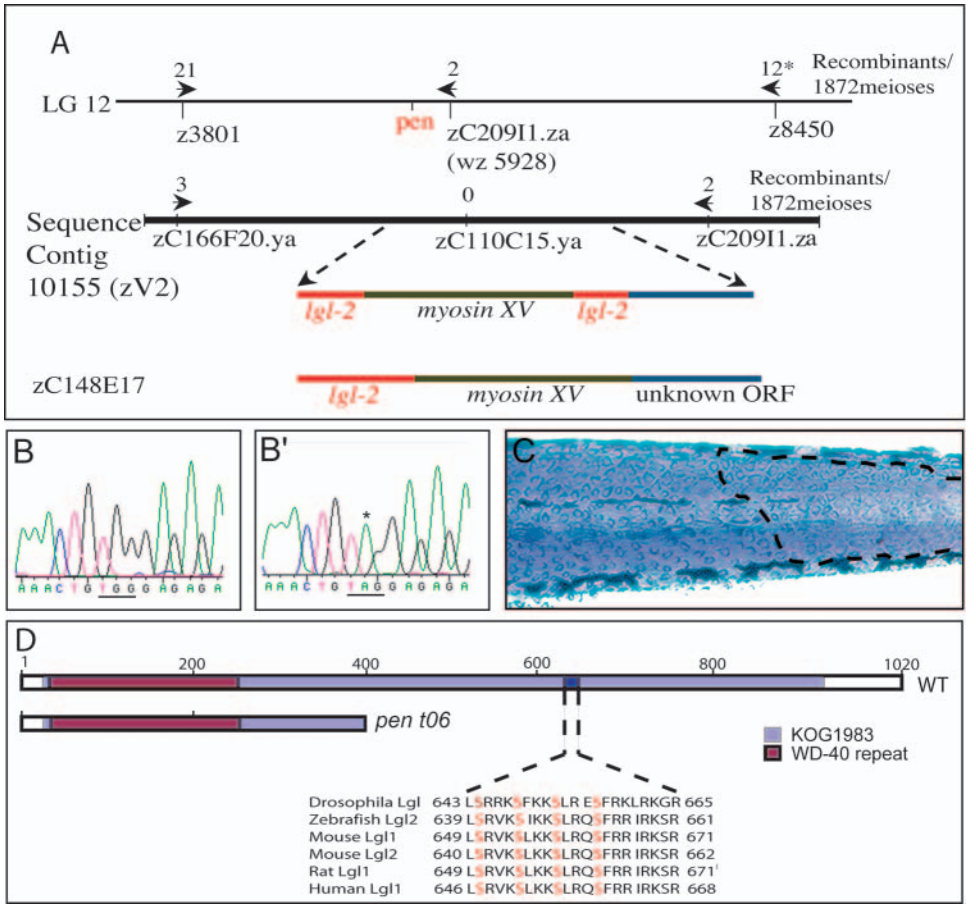


**Fig. 4.** *penner* is the second zebrafish orthologue of *D-lgl* and is expressed in the basal epidermis. (A) *pen* maps in between SSLP markers z3801 and z8450 on linkage group 12. A BAC (zC209I1.za) that maps very close to *pen* also maps on sequence contig 10155 (zV2). *pen* shows tight linkage with a SNP marker present in zC110C15.ya (0 recombination). This region harbours putative *myosin XV* flanked by partial sequences homologous to *lgl2*. BAC zC148E17 maps to the same region and sequence analysis of this BAC revealed the presence of an uninterrupted *lgl2* sequence. (B-B′) The partial sequence of *lgl2* from wild-type (B) and mutant (B′) larvae. Asterisk in B′ indicates the transition event leading to conversion of a codon for Trp (TGG in b) into a stop codon (TAG) in mutant. (C) *pen* mutant larvae injected with BAC zC242O17 containing wild type *lgl2* and stained for keratin. BAC injections result in the partial rescue of *pen* mutant phenotype, as indicated by the presence of wild-type polygonal cells (marked by broken line) next to rounded up and loosely organised mutant cells. (D) Schematic of wild-type Lgl2, depicting WD-40 repeat and KOG 1983 domain. The sequence between amino acids 639 and 661 contains aPKC phosphorylation sites (serine residues in red) and is similar or identical to that in *Drosophila* Lgl, and Lgl 1 and Lgl2 of mouse, rat and human. There is a partial loss of KOG 1983 domain and a lack of aPKC phosphorylation sites in the pen$^{t06}$ allele. Asterisk indicates 776 meioses were analysed with SSLP marker z 8450.

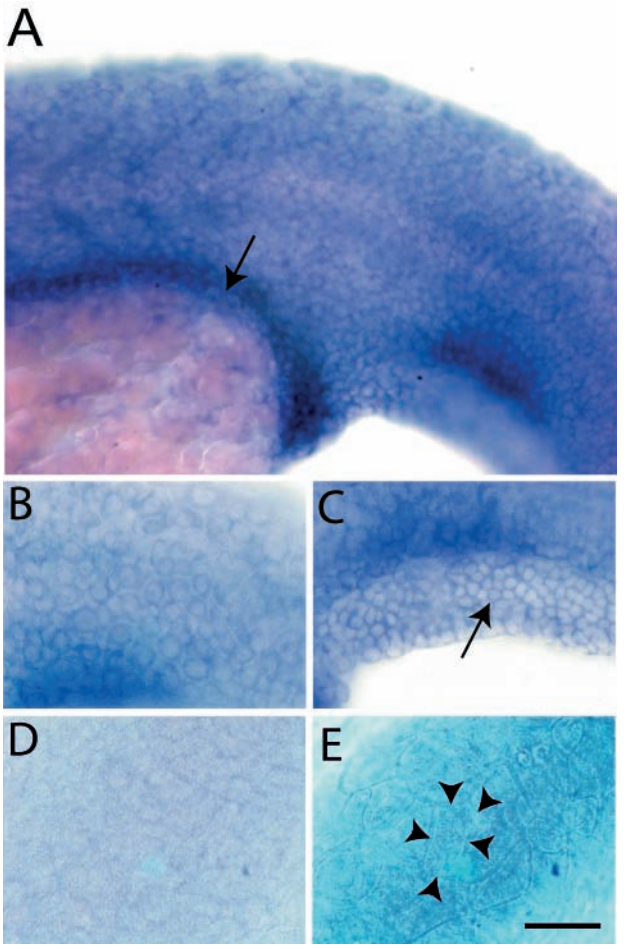


**Fig. 5.** Expression analysis of *lgl2* by in situ hybridisation. Epidermal cells and presumptive gut (arrow) of a 24-hour-old larva exhibit *lgl2* expression (A). The basal cells of epidermis (B) including fin fold epidermis (C, arrow) express *lgl2*. Basal epidermis (D) and periderm (E) of control embryo hybridised with sense probe. The peridermal cells (cell borders marked by arrowheads in E) are bigger than the basal epidermal cells (B,C). Scale bar: 50 μm in A; 33 μm in B-E.

embryos ($n$=564 from 31 larvae; data not shown) and 20% derived from control morpholino-injected embryos ($n$=437 from 25 larvae) exhibited spindle-shaped or rounded-up cells (Fig. 6J). Thus, more than 80% of the control clones displayed wild-type-like flattened and polygonal shapes with basal localisation of keratin, excluding any necessity for further EM analysis (Fig. 6A,D-F). To assess the effect of the depletion of maternal Lgl2, we asked whether clones carrying lgl2MO would exhibit any early phenotypes with respect to cell shape changes at 3.5 dpf. Interestingly, similar to clones carrying control morpholino, ~85% clones carrying lgl2MO showed flattened polygonal cells, characteristic of the wild-type basal epidermis (Fig. 6C,J).

We conclude that *pen* is the second zebrafish orthologue of the *Drosophila* gene *lethal giant larvae* and its function is essential for hemidesmosome formation. Moreover, maternally derived *lgl2* transcripts are not essential for maintenance of the cellular morphology prior to hemidesmosome formation. In fact, it appears that, subsequent to 5 dpf, absence of hemidesmosomes triggers changes in the cellular morphology and mislocalisation of cytoskeletal elements.

### Basal epidermal cells become migratory and hyperproliferate in the *pen/lgl2* mutant larvae

The loss of hemidesmosomes has been correlated with metastasis (Bergstraesser et al., 1995; Herold-Mende et al., 2001; Schenk, 1979). Moreover, loss of *lgl* function leads to the disruption of cell polarity and neoplastic growth in the brain and imaginal discs of *Drosophila* larvae (reviewed by Bilder, 2004). In *pen* mutant larvae, basal epidermal cells undergo shape changes that are indicative of epithelial to mesenchymal transition, which is a characteristic of invasive or migrating cells (Fig. 3A,B). Therefore, we asked whether basal epidermal cells become migratory in *pen/lgl2* mutants. The keratin antibody we used (Ks pan1-8) labels basal cells in dorsal and lateral epidermis in wild-type larvae (Fig. 1G,H; Fig. 7A). However, in 97% *pen* mutant larvae ($n$=65) we observed labelled cells in either the fin fold epidermis and/or ventral epidermis covering lower jaw (Fig. 7B; data not shown), suggesting that basal cells in the epidermis become migratory in the absence of *lgl2* function. As the loss of stable adhesion to the basal lamina is a pre-requisite for basal epithelial cells to acquire migratory behaviour, it is possible that *lgl2* may be suppressing migratory behaviour by exerting its effect through the formation and maintenance of hemidesmosomes in the basal epidermis.

To further investigate whether basal epidermal cells also hyperproliferate, we performed a bromo-deoxyuridine (BrdU) incorporation assay on 5-day-old wild type and *pen* mutant larvae. Although in wild-type larvae, proliferating cells were mainly restricted to the epidermis covering lower jaw, pectoral fins, swim bladder and surrounding neuromasts (Fig. 7C), we observed variation in the distribution of proliferating cells in the mutant epidermis (data not shown). Nevertheless, relatively consistent hyper-proliferation in the epidermis covering yolk/gut/swim bladder and/or around the base of the pectoral fin was observed in mutant larvae when compared with wild-type larvae (Fig. 7D). The quantification of proliferating epidermal cells in a specified area of 0.06 $mm^2$ in this region (Fig. 7C,D) revealed an almost fourfold increase ($t$-test, $P$<0.01) in mutant larvae (42±21, $n$=11) when compared with wild-type larvae (11±4, $n$=10). Furthermore, histological analysis of these larvae showed that BrdU mainly labelled the basal cells of the epidermis in wild-type and *pen* mutant larvae (data not shown). Thus, the presence of overgrowth in the ventral epidermis (Fig. 2B) could be the effect of the increase in the cell number either because of hyper-proliferation or the migration of epidermal cells from dorsal/ lateral epidermis into this region, or both. We conclude that the loss of *pen/lgl2* function leads to the acquisition of migratory behaviour and hyper-proliferation of the basal epidermis.

## Discussion

A genetic analysis of the human blistering disorder epidermolysis bullosa and a reverse genetic approach in mice has revealed the functional importance of various components of hemidesmosomes (reviewed by Borradori and Sonnenberg, 1996; Fuchs and Raghavan, 2002). However, it is still unclear how the formation of hemidesmosomes is developmentally

regulated to specify the basal domain of epithelial cells. To address this issue, here we have used zebrafish as a model system and performed forward genetic analysis of hemidesmosme formation during development of the zebrafish larval epidermis.

In an attempt to understand when hemidesmosomes develop in the zebrafish larval epidermis, we analysed electron microscopic changes occurring during development of the epidermis. In the larval epidermis, hemidesmosomes begin to appear at 4 to 4.5 dpf and develop in size and shape by 5.5 dpf (Fig. 1C-E). During epidermal development, keratin is expressed and localised in the basal cortex in 3-day-old larvae (Fig. 1B) and remains localised at the basal cortex thereafter (Fig. 3C; data not shown). Furthermore, hemidesmosomes and keratin are absent in the ventral and fin fold epidermis (Fig. 1F-I). At present, it is not clear how cells of the ventral or fin fold epidermis adhere to the basement membrane. The alternative mechanisms of cell to basement membrane adhesion may exist and develop in these parts of the epidermis, while hemidesmosomes are developing in the dorsal and lateral epidermis.

The *pen* mutant was identified in the first Tuebingen mutagenesis screen based on overgrowth of the ventral epidermis (Fig. 2B) (van Eeden et al., 1996). Here, we have described the previously unknown epidermal detachment phenotype, which is a consequence of the lack of hemidesmosomes in the basal epidermis of mutant larvae (Fig. 2D,E). This detachment phenotype resembles that seen in individuals suffering from junctional or hemidesmosomal form of epidermolysis bullosa (reviewed by Pulkkinen and Uitto, 1999). We also show that *pen* function is essential for maintenance of the morphology of basal epidermal cells and organisation of cytoskeletal elements (Fig. 3). Furthermore, we have positionally cloned *pen* to reveal that it encodes for zebrafish Lethal giant larvae 2 (Fig. 4).

Vertebrates have two orthologues of the *Drosophila* gene *lethal giant larvae.* Of these, *lgl1* knockout mice exhibit brain dysplasia without any defects in any other tissues (Klezovitch et al., 2004). In these mice, components of the apical junctional complex such as β-catenin, myosin II-B and f-actin are disorganised, indicating loss of polarity in the neuroepithelium (Klezovitch et al., 2004). Here, our analyses have revealed that *pen/lgl2* functions in the formation of basally localised hemidesmosomes and maintenance of the basal localisation of keratin cytoskeleton. The localisation of actin and β-catenin at lateral and

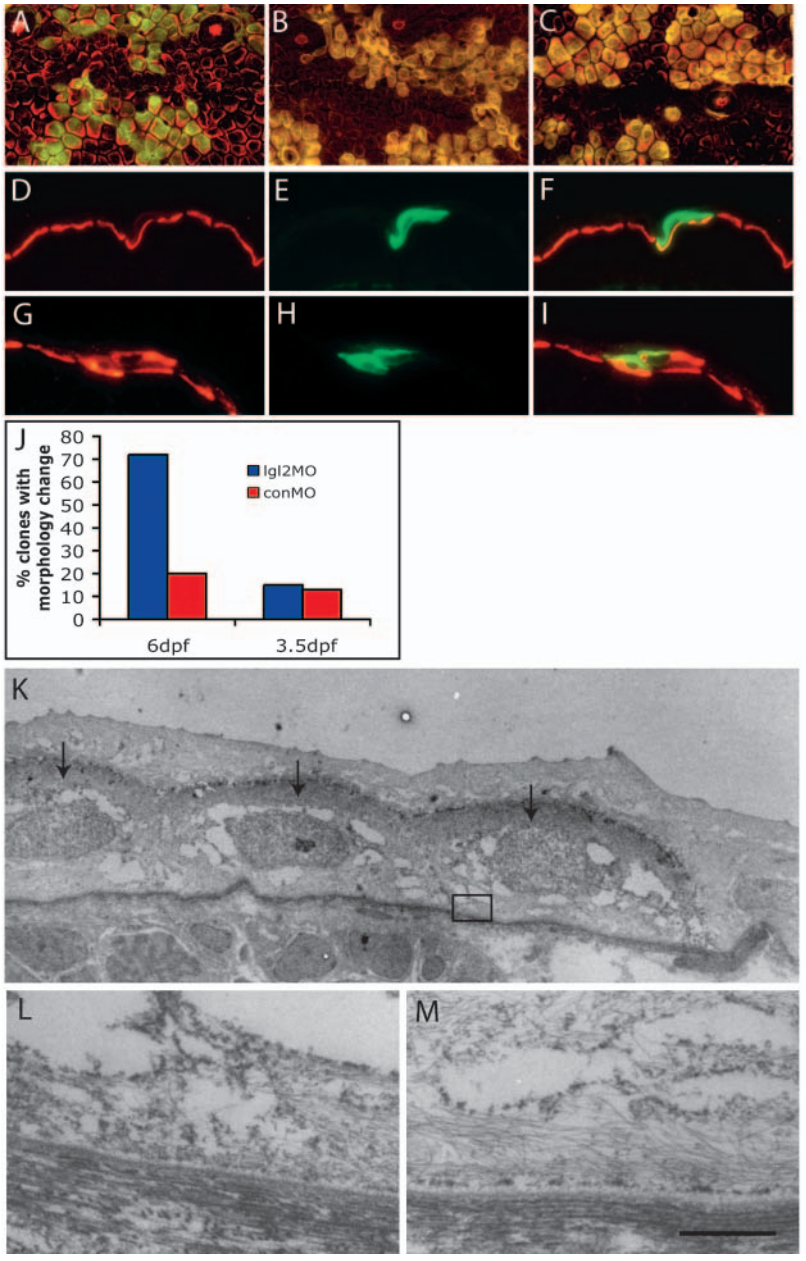


**Fig. 6.** Clones derived from antisense *lgl2* morpholino (lgl2MO)-injected embryos recapitulate the *pen* phenotype. (A-I) Confocal and immunohistological analysis of recipient embryos with epidermal clones labelled with GFP (green) and keratin (red). In overlays (A-C,F,I), clones appear yellow. At 6 dpf, clones derived from control morpholino (conMO)-injected embryos (A) comprise polygonal and flattened cells, similar to wild-type epidermal cells, while those derived from lgl2MO-injected embryos (B) showed changes in cellular morphology, becoming spindle or round shaped, similar to *pen* mutant. By contrast, on 3.5 dpf, clones containing lgl2MO (C) did not show any changes in cellular morphology. Immunohistological analysis of clones (6dpf) with conMO (D-F) reveals basal localisation of keratin, whereas clones containing lgl2MO exhibit mislocalisation of keratin (G-I). (J) Quantification of clones containing conMO or lgl2MO and exhibiting *pen*-like phenotype. Small proportions of clones carrying conMO comprise cells that deviate from usual morphology or show spindle shapes. There is a vast increase in the proportion of clones exhibiting *pen*-like phenotype at 6 dpf but not at 3.5 dpf (when, instead, they carry lgl2MO). (K-M) Electron microscopic analysis of hemidesmosome formation. Clone marked with GFP is detected with electron-dense DAB (arrows in K). Hemidesmosomes are absent in the clone (L represents boxed region in K) in contrast to the control recipient epidermis (M). Scale bar: 7 μm in K; 543 nm in L,M.

apical borders of basal cells remains unaffected before and after the onset of hemidesmosomal phenotype (Fig. 2J, Fig. 3F; data not shown). We do observe some decrease in the intensity of β-catenin staining as cells express hemidesmosomal phenotype but it does not seem to be the primary effect of the mutation because even clones carrying lgl2MO, which would also knockdown maternal *lgl2* expression, did not show any phenotype on 3.5 dpf that was indicative of loss of β-catenin at the apical or lateral borders. Thus, *lgl2* is primarily involved in hemidesmosome formation, a process that is involved in the maturation of the basal domain during epidermal development. Involvement of *lgl1* in maintenance of apical junctional complex in the brain (Klezovitch et al., 2004) and that of *lgl2* in the formation of hemidesmosomes and maintenance of the cellular morphology in basal epidermal cells (Figs 2, 3 and 6) indicate that these two *Drosophila lgl* orthologues may have evolved tissue specific functions during vertebrate development. This statement is further supported by the fact that *lgl1* transcripts are absent in developing epithelia wherein *lgl2* transcripts are abundant (see Fig. S1C-E in the supplementary material).

In *Drosophila*, a pathway comprising *lgl*, *scribble* (*scrib*) and *discs large* (*dlg*) has been shown to mediate its function at septate junctions to establish the basal domain in epithelial cells (Bilder et al., 2000) (reviewed by Humbert et al., 2003; Tanentzapf and Tepass, 2003). Septate junctions are located basolaterally in epithelial cells in *Drosophila* and are functionally analogous to the apically localised tight junctions in vertebrate epithelia. The loss of *scrib* and *dlg* function leads to the disruption of septate junctions (Bilder and Perrimon, 2000; Woods et al., 1996; Woods et al., 1997). However, *Drosophila* embryos lacking *lgl* activity exhibit only a transient loss of cell polarity without affecting the formation of septate junctions in the embryonic epidermis (Tanentzapf and Tepass, 2003). This suggests that the function of *lgl* alone is not essential for establishment or maintenance of the basal domain in the embryonic epidermis of *Drosophila*. In vertebrates, Lgl2 localise basolaterally and involved in defining the basolateral domain in epithelial cells of the frog blastula (Chalmers et al., 2005). Furthermore, overexpression of *lgl2* in these cells has been shown to result in the loss of tight junctions (Chalmers et al., 2005). Similarly, in MDCK cells, an in vitro model to study apicobasal polarity in vertebrate epithelia, overexpression of *lgl2* inhibits tight junction formation through enhanced colocalisation of Lgl2 with apical Par 6β (Yamanaka et al., 2003). However, this effect of *lgl2* overexpression on tight junctions has been proposed to be secondary to the expansion of basolateral domain and loss of the apical membrane domain (Chalmers et al., 2005). Our loss-of-function analysis has now clearly revealed that in vertebrate epidermis, *lgl2* has evolved an essential function specifically in the formation of basally localised hemidesmosomes (Fig. 2).

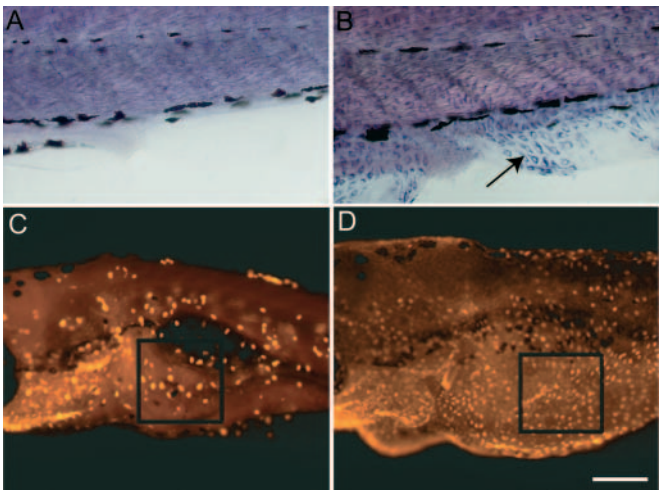


**Fig. 7.** The loss of *lgl2* function leads to acquisition of migratory behaviour and epidermal cell proliferation. (A,B) Immunohistological staining in wild-type (A) and *pen* mutant (B) using anti keratin (Ks pan-8) antibody. Epidermal cells exhibiting keratin staining are never present in fin fold of wild-type larvae (A). By contrast, mutant larvae show presence of keratin-containing cells in fin folds (arrow in B). These cells are spindle shaped and appear to migrate from the lateral epidermis into fin folds. (C,D) Assessment of cell proliferation using BrdU incorporation assay. The number of proliferating cells in ventral epidermis is higher in *pen* mutant (D) than in wild type (C). BrdU-labelled cells in the boxed region (in C,D) were counted from 10 different larvae to quantify the increase in *pen* larvae (see text for details). Scale bar: 40 μm in A,B; 0.2 mm in C,D.

In neuroblasts, apically localised aPKC phosphorylates Lgl protein, resulting in an inactivation of Lgl at the apical cortex (Betschinger et al., 2003). This leads to a polarised distribution of active Lgl, which is essential for the basal targeting of proteins such as Miranda, which are essential to determine the fate of ganglion mother cells (Betschinger et al., 2003). Vertebrate orthologues of the *Drosophila lgl* genes *lgl1* and *lgl2* share around 55% sequence identity at the protein level in humans. Both Lgl1 and Lgl2 belong to a family of proteins (KOG1983) with an ability to bind to syntaxins, which mediate the fusion of transport vesicles with the plasma membrane during exocytosis. Indeed, mammalian Lgl1 does interact with the SNARE protein syntaxin 4 (Musch et al., 2002). *Drosophila* Lgl/Lgl1 has also been shown to bind to non-muscle myosin II in various species (Kagami et al., 1998; Musch et al., 2002; Strand et al., 1994; Strand et al., 1995). Therefore, the polarised distribution of active (non-phosphorylated) *Drosophila* Lgl/Lgl1, may translate into the establishment of cell polarity through basal targeting of protein components by polarised exocytosis- or actomyosin-dependent mechanism (Betschinger et al., 2003; Musch et al., 2002; Peng et al., 2000; Plant et al., 2003). As phosphorylation of Lgl by aPKC is a key event in generating cellular asymmetry, we analysed the sequence of Lgl2 for the presence of conserved serine residues that are phosphorylated in *Drosophila* Lgl/Lgl1. We found that zebrafish Lgl2 also possesses these conserved serine residues at positions 640, 644, 648 and 652 (Fig. 4D). Lgl2 indeed is a substrate for aPKC (Yamanaka et al., 2003) and could be involved in the basal targeting of proteins through polarised exocytosis. The mutation in zebrafish *pen/lgl2* reported here eliminates the two-thirds of the Lgl2 protein that contains these aPKC phosphorylation sites and may also abrogate the interaction of Lgl2 with syntaxins (Fig. 4B,B′,D). As hemidesmosomes are not formed in the *pen* mutant epidermis or in epidermal cells in which *lgl2* is knocked down (Fig. 2D,E; Fig. 6K,L), we propose that the basal targeting of proteins, regulated by Lgl2, is essential for hemidesmosome formation in basal epidermal cells during development. Further investigation is necessary to understand whether the formation of hemidesmosomes depends on maintenance of the basal

membrane domain mediated through Lgl2, or whether Lgl2 is directly involved in the formation of hemidesmosomes, which in turn is necessary for maintenance of the basal membrane domain.

The loss of *lgl* function results in neoplastic growth of the brain as well as imaginal discs in *Drosophila* larvae and also promotes invasiveness (reviewed by Bilder, 2004; Gateff, 1978; Pagliarini and Xu, 2003). These studies have led to the proposal that *lgl* function would be essential in preventing cancer (reviewed by Humbert et al., 2003). Consistent with this proposal, *lgl1* knockout mice exhibit hyper-proliferation of neuroblasts and the expression of *lgl1* is downregulated in various human carcinomas (Grifoni et al., 2004). The function of *lgl2* in tumour suppression or tissue growth regulation has remained unknown. Our analysis has revealed that basal epidermal cells hyperproliferate and seem to acquire migratory potential in the absence of *lgl2* function (Fig. 7). It is not clear whether epidermal cells migrate as a sheet in *pen* mutant or whether they detach from each other and migrate. However, the presence of desmosomes and adherens junctions in the mutant epidermis favours the former possibility. Further in vivo imaging studies would shed more light on the migratory behaviour and whether the migration is directional or random. As *pen* phenotype is lethal, we could not test whether the mutation in *lgl2* would lead to tumour formation in the epidermis or any other tissues. Interestingly, *pen* mutant larvae exhibit overgrowth of the ventral jaw epidermis along with rounded up cells in the fin fold (Fig. 2B). The reason for this phenotype is not yet clear. It may be a consequence of infiltration of the ventral jaw region by keratin containing hyperproliferating basal epidermal cells of the dorsal and lateral epidermis. Alternatively, this overgrowth may be a result of the hyper-proliferation of ventral epidermal cells (Fig. 7D) or it could also be a combined effect of these two processes. Indeed, *lgl2* is expressed in the ventral epidermis as well as in the fin fold epidermis (Fig. 5C; data not shown), leading to the possibility that *pen/lgl2* could also be involved in maturation of the basal domain in basal cells of the ventral epidermis and its loss may trigger the proliferative response. However, owing to the unavailability of basally localised ultra-structural and/or protein markers, we could not test this possibility. In any case, the *pen* mutant would be an excellent model to analyse how and why the loss of *lgl2* function leads to hyper-proliferation and acquisition of migratory potential, displayed by cancer cells.

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### Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/14/3255/DC1

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