# Syntenin mediates Delta1-induced cohesiveness of epidermal stem cells in culture

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## Summary

**In human interfollicular epidermis, stem cell clusters express high levels of the Notch ligand Delta1. Delta1 stimulates neighbouring cells to differentiate and also promotes stem cell clustering. Although Notch signalling is known to stimulate epidermal differentiation, little is known about the mechanism by which Delta1 promotes epidermal cell cohesiveness. This is an important issue, because the location of stem cells determines the local microenvironmental signals they receive. We now show that mutation of the Delta1 PDZ-binding domain abolishes Delta1-mediated keratinocyte cohesiveness, stimulates Notch transcriptional activity and promotes epidermal differentiation. A yeast two-hybrid screen revealed that Delta1 binds to the adaptor protein syntenin – an interaction dependent on the Delta1 PDZ-binding domain. Syntenin, like Delta1, is upregulated in the stem cell clusters of human interfollicular epidermis. Knockdown of syntenin in cells overexpressing full-length Delta1 had the same effects on Notch signalling, epidermal differentiation and adhesion as overexpressing Delta1 with a mutated PDZ-binding domain. Syntenin has previously been reported to regulate membrane traffic, and mutation of the Delta1 PDZ-binding domain or knockdown of syntenin led to rapid internalisation of Delta1. We propose that syntenin binding to Delta1 plays a dual role in promoting intercellular adhesion and regulating Notch signalling.**

Key words: Delta, Cell-cell adhesion, Epidermis, Stem cell, Syntenin

## Introduction

The epidermis, which forms the outer covering of mammalian skin, is maintained throughout life by proliferation of stem cells and differentiation of their progeny (Fuchs, 2007; Watt et al., 2006). Distinct populations of stem cells reside in the hair follicle, sebaceous gland and interfollicular epidermis (Owens and Watt, 2003; Watt et al., 2006).

Notch signalling plays an essential role in cell fate determination during embryogenesis and also in postnatal life (Artavanis-Tsakonas et al., 1999; Baron, 2003). Following binding of ligand (Delta or Jagged), Notch undergoes cleavage of its intracellular domain. The intracellular domain translocates to the nucleus, where it binds to Suppressor of Hairless Su(H) (RBP-J/CBF1 in vertebrates) to activate transcription (Baron, 2003).

In mammalian epidermis, Notch signalling regulates differentiation and has a tumour suppressor function (Lefort and Dotto, 2004). Activation of Notch in cultured interfollicular epidermal keratinocytes can stimulate terminal differentiation (Lowell et al., 2000; Rangarajan et al., 2001). Notch signalling is required for postnatal hair follicle development (Blanpain et al., 2006; Estrach et al., 2006; Pan et al., 2004; Vauclair et al., 2005) and inappropriate activation results in perturbation of tissue homeostasis (Lin et al., 2000; Uyttendaele et al., 2004). Epidermis lacking Notch1 shows increased susceptibility to developing chemically induced tumours (Nicolas et al., 2003).

In the epidermis, three Notch ligands – Delta1, Jagged1 and Jagged2 – display temporal and spatial regulation of expression (Favier et al., 2000; Powell et al., 1998). Delta1 is expressed in human interfollicular epidermis and cultured human keratinocytes (Lowell et al., 2000). In human interfollicular epidermis there is evidence for clustering of stem cells in the basal layer, and Delta1 expression is highest in the putative stem cell clusters (Jensen and Watt, 2006; Jensen et al., 1999; Lowell et al., 2000).

By overexpressing Delta1 in cultured human keratinocytes we have identified two distinct activities of Delta1 (Lowell et al., 2000; Lowell and Watt, 2001). High Delta1 expression by undifferentiated putative stem cells signals to neighbouring cells to differentiate into transit-amplifying cells. High Delta1 expression also promotes keratinocyte cohesiveness, defined as the tendency of groups of cells to remain in contact with one another; this effect requires the cytoplasmic domain of Delta1 (Lowell et al., 2000; Lowell and Watt, 2001). Although many of the events downstream of Notch activation in the epidermis have been characterised (Mammucari et al., 2005; Pan et al., 2004), little is known about the mechanism by which Delta1 promotes epidermal cell cohesiveness. This is an important issue, because the location of stem cells in particular sites within the epidermis determines the local microenvironmental signals they receive, which direct stem cells to self-renew or differentiate along distinct lineages (Fuchs, 2007; Owens and Watt, 2003; Watt et al., 2006). In the present experiments, we have established that the cohesive effects of Delta1 in keratinocytes are mediated via the PDZ-binding domain and involve the adaptor protein syntenin. We also reveal an unexpected role of syntenin in Notch activation.

## Results

We transduced human keratinocytes with zebrafish Delta (DeltaD), which shares 80% homology with mouse Delta1, so that we could use a monoclonal antibody to DeltaD to evaluate Delta expression (Itoh et al., 2003). We compared full-length DeltaD (DeltaFl) with a mutant that lacks all but 13 of the amino acids of the cytoplasmic domain (DeltaDS; equivalent to mouse Delta$^{T}$) (Henrique et al., 1997) (Fig. 1A). Delta1 has a conserved motif, ATEV*, at its C-terminus, which fits the consensus for a PDZ-domain-binding protein (Wright et al., 2004). To test the functions of this sequence, we generated a deletion (DeltaLC) and a point mutation of the PDZ-binding domain (DeltaVA) (Fig. 1A). The point mutation has been previously described to disrupt the interaction between a PDZ-binding domain and its partner (Lin et al., 1999).

All Delta constructs were introduced into primary human epidermal keratinocytes by retroviral infection. DeltaFl localised to the plasma membrane and was abundant at sites of cell-cell contact (Fig. 1B), as reported previously for endogenous human Delta1 and for overexpressed mouse Delta1 and Delta$^{T}$ (Lowell et al., 2000).

Following binding of a ligand (Delta or Serrate/Jagged), Notch undergoes cleavage of its intracellular domain and translocates to the nucleus, where it binds to RBP-J to activate transcription of HES genes (Baron, 2003). To test the ability of the Delta mutants to activate Notch signalling, we measured luciferase activity driven by the HES1 promoter (Jarriault et al., 1995). J2-3T3 cells stably transduced with the different Delta constructs were transiently transfected with the luciferase reporter and 48 hours later luciferase activity was measured (Fig. 1C). Western blotting of cell lysates established that the different Delta constructs were expressed at similar levels (Fig. 1C).

DeltaFl and DeltaDS activated the HES promoter to the same extent, whereas DeltaVA had much stronger activity (Fig. 1C). The activation of Notch observed in this experimental setting will result from the combined effects of activation in *trans* (i.e. between neighbouring cells) and inhibition in *cis* (in the same cell) (Artavanis-Tsakonas et al., 1999; Glittenberg et al., 2006). Thus, the activation of Notch induced by Delta VA could reflect an increase in the activation in trans and/or reduced inhibition in *cis*.

Colony-forming efficiency (CFE) and percentage of abortive clone formation can be used as indicators of epidermal stem cell activity in culture. Stem cells establish actively growing clones in which self-renewal and differentiation take place, whereas abortive clones are attributable to transit-amplifying cells that undergo terminal differentiation within a few rounds of division (Lowell et al., 2000). Keratinocytes were transduced with the Delta constructs and plated on wild-type (WT) feeders (Fig. 1D; Table 1). DeltaFl and DeltaDS had no effect on CFE, consistent with our previous studies (Lowell et al., 2000), although the size of clones formed by keratinocytes expressing DeltaFl was increased (Fig.

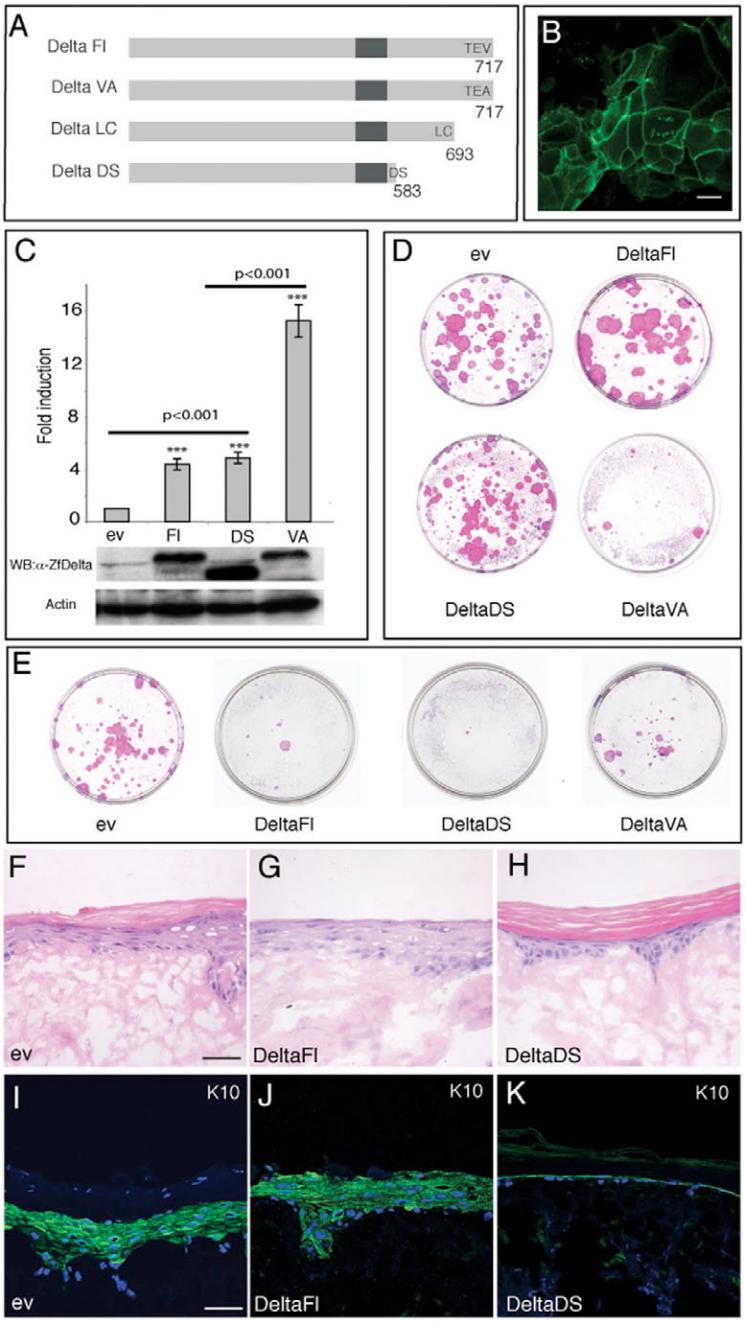


**Fig. 1.** Effects of Delta1 overexpression on Notch activation and keratinocyte differentiation. (A) Schematic representation of the Delta constructs. (B) Anti-DeltaD immunofluorescence staining of human keratinocytes transduced with DeltaFl. Bar, 20 μm. (C) Hes1 luciferase reporter activity (upper panel) in J2-3T3 cells transduced with empty vector (ev), or Delta constructs. Fold induction relative to Renilla control is shown. Mean ± s.e.m. of four experiments. Lower panels show western blot of lysates of J2-3T3 cells used in the luciferase assay, probed with anti-DeltaD and anti-actin (loading control) antibodies. (D) Clonal growth of keratinocytes transduced with empty vector or Delta constructs. (E) Clonal growth of WT keratinocytes on a feeder layer of J2-3T3 cells transduced with empty vector (ev) or Delta constructs. (F-H) Epidermis reconstituted on DED by keratinocytes transduced with empty vector (ev; F,I), DeltaFl (G,J) or DeltaDS (H,K). Sections were stained with H&E (F-H) or anti-keratin 10 antibody (green) with DAPI nuclear counterstain (blue) (I-K). Bars, 50 μm.

**Table 1. Effect of Delta1 expression on clonal growth of keratinocytes***

| | CFE | % Abortive clones |
|---|---|---|
| WT | 28.7±4.4 | 25.8±2.9 |
| Zebrafish Delta FL | 28.9±3.1 | 25.1±8.1 |
| Zebrafish Delta DS | 25.4±0.6 | 47.4±6.8 |
| Zebrafish Delta VA | 4.0±0.7 | 63.9±2.4 |
| Mouse Delta FL | 22.7±3.4 | 28.6±3.4 |
| Mouse DeltaT | 24.6±5.3 | 42.5±5.3 |

*Keratinocytes were transduced with the retroviral vectors indicated and cultured on a wild type feeder layer. CFE, colony-forming efficiency; WT, cells transduced with empty vector. Data are mean ± s.d. of triplicate dishes from at least three experiments.

1D). By contrast, keratinocytes expressing DeltaVA showed a strong reduction in CFE and an increase in the proportion of abortive clones (Fig. 1D; Table 1). Thus, the enhanced Notch activation by DeltaVA (Fig. 1C) correlated with an inhibition of clonal growth, consistent with the ability of Notch to promote epidermal differentiation (Blanpain et al., 2006; Rangarajan et al., 2001; Vauclair et al., 2005).

Boundaries between populations of cells with high and low Notch signalling activity are known to be important in *Drosophila* (Artavanis-Tsakonas et al., 1999; Glittenberg et al., 2006). As previously reported, culturing WT human keratinocytes on feeder cells (J2-3T3) transduced with Delta1 led to a reduction in CFE and an increase in the proportion of abortive clones (Lowell et al., 2000) (Table 2; see also schematic summary in Fig. 6). Expression of DeltaFl and DeltaDS in J2-3T3 had the same effects (Fig. 1E; Table 2; Fig. 6). Replacement of DeltaFl-expressing feeders with WT feeders after 5 days did not restore keratinocyte colony formation, showing that the effect was irreversible, consistent with stimulation of terminal differentiation (data not shown). WT keratinocytes cultured on DeltaVA feeders showed a small reduction in CFE and increase in abortive clones compared with cells cultured on WT feeders; nevertheless, the effects were much less dramatic than the effects of culture on DeltaFl feeders (Fig. 1E; Table 2; Fig. 6).

These results establish that a point mutation in the Delta1 PDZ-binding domain has different effects on keratinocyte differentiation to full-length Delta1 or deletion of the entire cytoplasmic domain. DeltaFl and DeltaDS can only promote differentiation in *trans* (Delta-expressing feeders, WT keratinocytes), whereas DeltaVA triggers differentiation in *cis* (Delta-expressing keratinocytes) (Fig. 6).

Although WT keratinocytes and cells transduced with DeltaFl or DeltaDS had similar clonal growth properties, they

**Table 2. Effect of Delta1-expressing feeders on clonal growth of wild-type keratinocytes***

| | CFE | % Abortive clones |
|---|---|---|
| J2 | 19.1±4.5 | 22.9±2.7 |
| J2 DeltaFl | 5.4±1.9 | 92.2±2.6 |
| J2 DeltaDS | 4.8±1.9 | 73.7±4.5 |
| J2 DeltaVA | 11.1±2 | 36.8±2.5 |
| J2 Mouse Dll1 | 7.9±4.1 | 92.4±5.1 |

*Wild-type keratinocytes were cultured on feeder layers of J2 3T3 cells transduced with the retroviral vectors shown. Data are mean ± s.d. of triplicate dishes in at least three experiments. CFE, colony-forming efficiency.

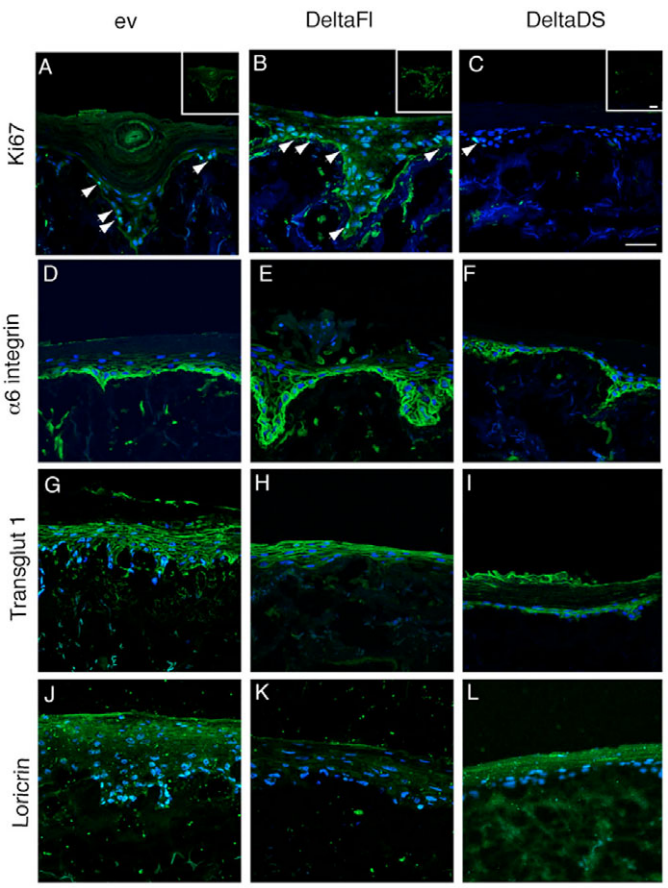


**Fig. 2.** Effects of Delta overexpression on differentiation of reconstituted human epidermis. Keratinocytes transduced with empty vector (ev,A,D,G,J), DeltaFl (B,E,H,K) or DeltaDS (C,F,I,L) were grown on DED and stained with antibodies to the proteins shown (green). DAPI was used as a nuclear counterstain (blue). Arrows indicate Ki67-positive cells in the epidermal basal layer. Inserts in A-C represent Ki67 staining only. Bars, 50 μm.

differed in their programme of terminal differentiation in an in vitro reconstituted human skin model (Fig. 1F-H). Primary human keratinocytes transduced with empty vector, DeltaFl and DeltaDS were cultured for 16 days at the air-liquid interface on a human dermal substrate (DED) (Janes et al., 2004). Keratinocytes transduced with empty vector reconstituted an epidermis comprising basal, spinous, granular and cornified layers, thereby resembling normal epidermis (Fig. 1F). Keratinocytes expressing DeltaFl also formed a multilayered epidermis, but it lacked granular and cornified layers (Fig. 1G). By contrast, the epidermis formed by keratinocytes transduced with DeltaDS had a reduced number of spinous layers, a prominent granular layer and an increased number of cornified layers (Fig. 1H). Consistent with the histological appearance, transduced epidermis of WT, Delta FL and Delta DS had normal expression of α6 integrin, a basal layer marker (Fig. 2D-F). DeltaFl promoted proliferation (Fig. 2A-C) and expression of spinous layer markers (keratin 10, Fig. 1I-K; transglutaminase 1, Fig. 2G-I), whereas DeltaDS promoted expression of granular markers (loricrin; Fig. 2J-L). The effects of DeltaDS are in good agreement with the finding that a soluble form of the Notch ligand Jagged1 stimulates

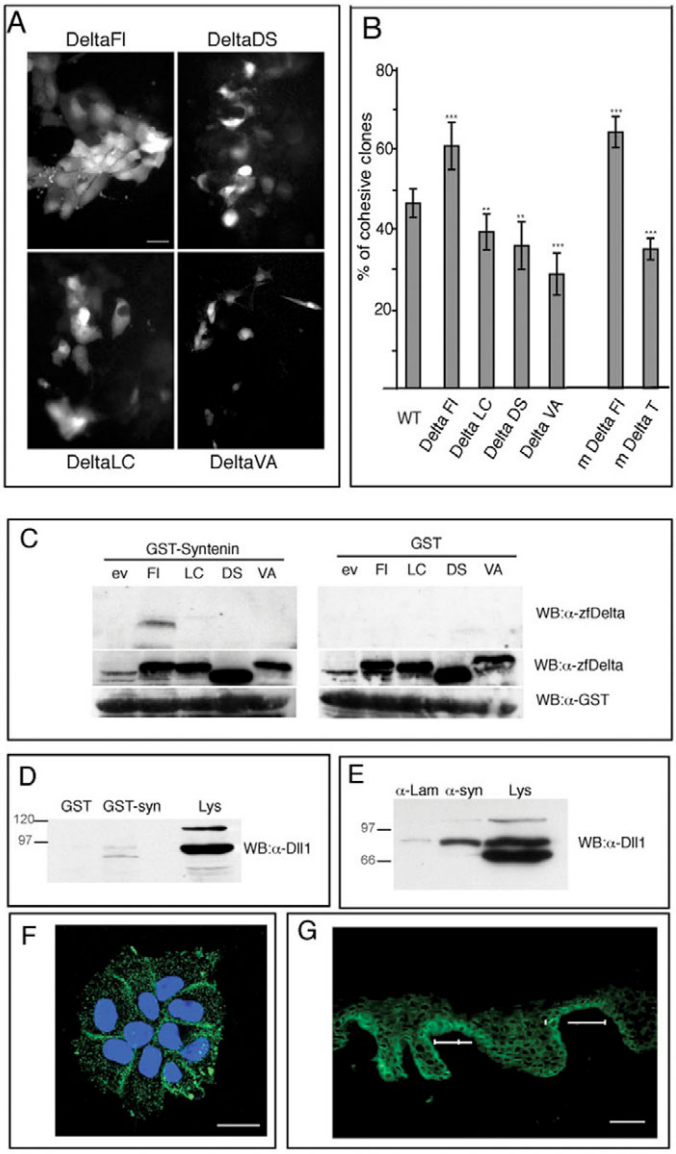


**Fig. 3.** The Delta1 PDZ domain binds syntenin and promotes keratinocyte cohesiveness. (A) GFP-labelled clones formed by keratinocytes expressing the Delta constructs shown 5 days after seeding within a confluent sheet of unlabelled WT keratinocytes. Bar, 20 μm. (B) Percentage of cohesive colonies. Data are mean ± s.d. of five experiments. Values significantly different from WT control are marked with asterisks (Student's *t*-test, ***$P$<0.001 or **$P$<0.05). (C) Western blots of A431 cells transfected with empty vector (ev) or the Delta constructs shown following pull down with GST (control; right panel) or GST-syntenin (left panel). Blots were probed with anti-ZfDelta or anti-GST antibodies. (D) Western blot of untransfected A431 cell lysates following pull down with GST or GST-syntenin (GST-syn). Blot was probed with antibody to human Delta1. Total cell lysates (Lys) served as a positive control. (E) A431 cell lysates immunoprecipitated with antibodies against syntenin (α-syn) or laminin5 (α-Lam) followed by western blotting with anti-Delta1 antibody. Lys, lysate subjected to western blotting without prior immunoprecipitation. (F) Immunofluorescence staining of human primary keratinocytes with anti-syntenin antibody (green). DAPI was used as a nuclear counterstain (blue). Bar, 10 μm. (G) Immunofluorescence staining of human interfollicular epidermis with an anti syntenin antibody. Horizontal lines mark position of putative stem cell clusters. Bar, 50 μm.

loricrin expression and cornified envelope formation (Nickoloff et al., 2002).

We next evaluated the effects of the different Delta mutants on cohesiveness of keratinocyte colonies. Cells transduced with the mutants were also transduced with a retroviral vector encoding GFP, then seeded at clonal density with confluent unlabelled WT keratinocytes (Lowell et al., 2000) (Fig. 3A). Expression of DeltaFl promoted cohesiveness (Fig. 3A,B), whereas DeltaDS promoted scattering of clones of cells, consistent with the effects of mouse Delta1 and Delta$^{T}$ (Fig. 3A,B) (Lowell et al., 2000). Deletion (DeltaLC) or mutation (DeltaVA) of the PDZ-binding domain also led to reduced cohesiveness (Fig. 3A,B). We conclude that the ability of Delta to promote keratinocyte cohesiveness is mediated by the PDZ-binding domain and that cohesiveness is regulated independently of Notch activation (compare Fig. 1C with Fig. 3B).

To investigate the mechanism by which Delta1 mediates keratinocyte cohesiveness, a two-hybrid screen was performed using the cytoplasmic domain of Delta1, including the PDZ-binding domain, as bait. Several putative partners were isolated, the most highly represented being syntenin (Table 3). Syntenin is a cytoplasmic scaffold protein, containing two PDZ domains. It interacts with the actin cytoskeleton and with cell adhesion receptors, including syndecan and integrins (Gao et al., 2000; Grootjans et al., 1997; Oh and Couchman, 2004; Zimmermann et al., 2001). Syntenin is reported to colocalise with E-cadherin and syndecan-1 at epithelial cell-cell contacts (Zimmermann et al., 2001).

To validate the association between syntenin and Delta1, a syntenin-GST fusion protein was incubated with lysates of A431 cells that had been transfected with the different Delta constructs (Fig. 3C). Only DeltaFl bound syntenin in the pull-down assay, DeltaDS, LC and VA did not, thereby establishing a requirement for the Delta PDZ domain (Fig. 3C). The interaction between syntenin and Delta1 was also observed in pull-down assays involving GST-syntenin and endogenous Delta1 protein in A431 cells (Fig. 3D; note that the upper band in the lysate lane is non-specific; compare middle lane of Fig. 3E).

To establish that endogenous syntenin bound endogenous Delta1, lysates of A431 cells were subjected to immunoprecipitation with anti-syntenin antibody or, as a negative control, anti-laminin 5 antibody, and then western blotted with an antibody to the Delta1 extracellular domain (Fig. 3E). Endogenous Delta1 was co-immunoprecipitated with endogenous syntenin. In some lysates, anti-Delta1 antibody specifically recognised two prominent bands, with only the upper band being associated with syntenin (Fig. 3E). It is likely that the lower band is the cleaved extracellular domain of Delta1. These results confirmed the direct interaction between syntenin and Delta1 and showed that the interaction is mediated by the C-terminal PDZ-binding domain of Delta1.

Syntenin is reported to be one of the genes that are highly upregulated in the reservoir of stem cells in the mouse hair follicle bulge (Tumbar et al., 2004). We therefore examined whether syntenin was upregulated in the clusters of stem cells in human interfollicular epidermis previously shown to express high levels of Delta1 (Jensen and Watt, 2006; Lowell et al., 2000). Syntenin localised to cell-cell borders in cultured

**Table 3. Putative Delta-binding partners isolated from the yeast two-hybrid screen**

| Function | Protein | Number of clones |
|---|---|---|
| Adhesion | Syntenin/Syndecan binding protein/PBP1 | 15 |
| | Synectin/SemaF cytoplasmic domain associated protein1 | 12 |
| | Discs large homolog 4 | 1 |
| Signalling | Activin receptor interacting protein Arip2 | 4 |
| | Multiple PDZ domain protein (Mpdz) | 4 |
| | SemaF cytoplasmic domain associated protein2 GIPC2 | 1 |
| | KRIT1/Ankyrin repeat-containing protein (CCM1) | 1 |
| | Kidins220/ankyrinrepeat-rich membrane-spanning protein | 1 |
| | Protein tyrosine phosphatase, non receptor type 2 (Ptpn2) | 1 |
| | Rling finger protein 2 (Rfn2) | 1 |
| Cytoskeleton | Filamin A | 1 |
| | Guanosine diphosphate dissociation inhibitor | 1 |
| Endocytosis | Ankra2/ankyrin repeat family A (RFXANK-Like) | 1 |
| Nucleus | c-Myc promoter binding protein (IRLB) | 1 |
| | Telomeric repeat binding factor 2, interacting protein (Terf2ip) | 2 |

keratinocytes (Fig. 3F) and in the basal layer of human interfollicular epidermis (Fig. 3G). Within the epidermis, syntenin expression was more abundant in the clusters of basal cells that lie closest to the tissue surface, which is the location of the putative IFE stem cells (Fig. 3G). Thus in both human and mouse epidermis there is evidence for upregulation of syntenin in stem cell populations.

**Table 4. Effect of syntenin knockdown on clonal growth of keratinocytes**

| | CFE | % Abortive clones |
|---|---|---|
| WT+Ctrl | 26.1±3.2 | 27.1±3.9 |
| WT+Rnai1 | 25.8±4.4 | 28.2±3.2 |
| FL+Ctrl | 28.3±3.2 | 22.5±7.9 |
| FL+Rnai1 | 16.6±1.4 | 57.8±8.1 |
| DS+Ctrl | 22.1±0.7 | 30.5±5.1 |
| DS+Rnai1 | 23.1±1.1 | 32.4±5.9 |

Keratinocytes were transduced with empty retroviral vector (WT), DeltaFl or DeltaDS in combination with syntenin RNAi (Rna1) or scrambled RNAi control sequence (Ctrl). Data are mean ± s.d. of triplicate dishes from at least three experiments.

To establish whether the syntenin interaction was responsible for the effect of DeltaFl on keratinocyte cohesion, we used RNAi to knock down syntenin expression. Several siRNAs against syntenin were cloned into a retroviral vector and introduced into primary human keratinocytes (Fig. 4A and data not shown). In cells transduced with the most effective siRNA sequence, Rnai1, syntenin was undetectable by western blotting (Fig. 4A) and immunofluorescence microscopy (Fig. 5B,E).

To investigate whether syntenin knockdown affected canonical Notch signalling, we performed luciferase assays in the presence of Rnai1 and the different Delta constructs (Fig. 4B). The activity induced by the truncated version of Delta (DeltaDS) was not affected by syntenin knockdown, consistent with the failure of Delta DS to bind syntenin (Fig. 3C). The knockdown of syntenin induced an increase in Notch activation in DeltaFl-expressing cells compared to cells expressing DeltaFl only. The activation obtained was almost as strong as the one obtained with DeltaVA (Fig. 4B). An independent siRNA that reduced syntenin expression less effectively also increased Notch activation, but to a lesser extent (data not shown).

We hypothesised that if the reduction in clonal growth observed with DeltaVA (Fig. 1D) is indeed due to enhanced

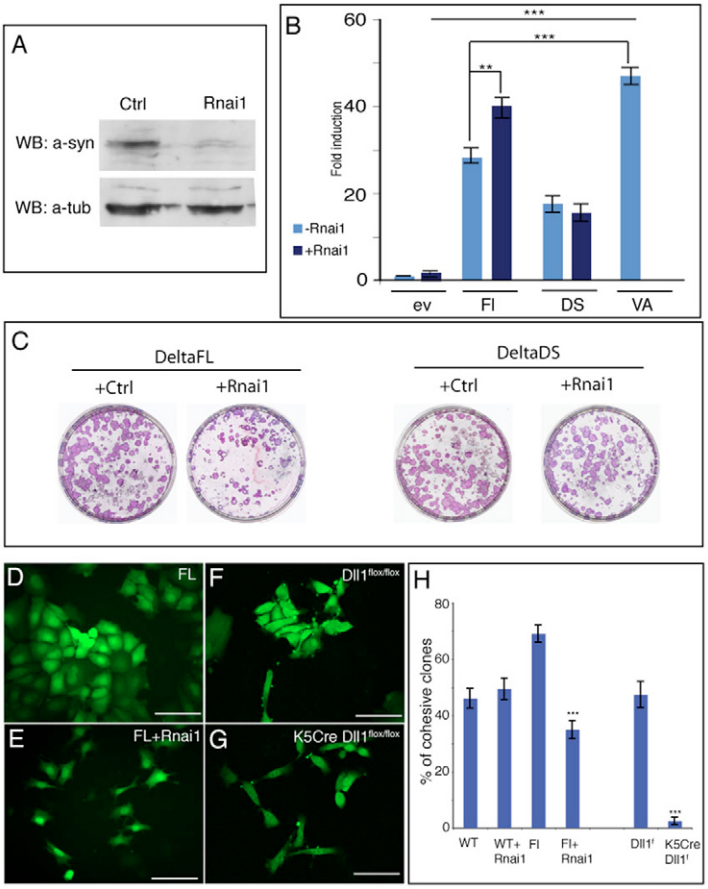


**Fig. 4.** Functional consequences of syntenin knockdown. (A) Western blot of keratinocytes transduced with empty vector (ev) or syntenin siRNA (Rnai1) probed with anti-syntenin (a-syn) or, as a loading control, anti-tubulin (a-tub). (B) Hes1 luciferase reporter activity in J2-3T3 cells transduced with empty vector (ev) or the Delta constructs shown, in the presence or absence of syntenin siRNA. Fold induction relative to Renilla control is shown. Mean ± s.e.m. of three experiments. Values significantly different from ev control or Fl are marked with asterisks (Student's *t*-test: ***$P$<0.001 or **$P$<0.05). (C) Clonal growth of keratinocytes transduced with DeltaFl or Delta DS and control (Ctrl) or syntenin Rnai1. (D-G) GFP-labelled clones formed by human keratinocytes expressing DeltaFl alone (Fl; D) or in combination with syntenin Rnai1(E), or formed by mouse keratinocytes with endogenous Delta1 levels (Dll1$^{flox/flox}$; F) or lacking Delta1 (K5Cre x Dll1$^{flox/flox}$; G). Clones were photographed 5 days after seeding within a confluent sheet of unlabelled WT keratinocytes (human or mouse). Bars, 100 μm. (H) Percentage of cohesive colonies formed by GFP-labelled keratinocytes. Data are mean ± s.d. of three experiments (Student's *t*-test, ***$P$<0.001 compared with % clones in WT).

Notch signalling, then keratinocytes transduced with DeltaFl and Rnai1 should also show reduced clonal growth. Rnai1 did not affect the CFE and the proportion of abortive clones formed by WT keratinocytes or cells transduced with DeltaDS, which cannot bind syntenin (Fig. 4C, Table 4). However, knockdown of syntenin in keratinocytes expressing DeltaFl reduced the CFE and increased the proportion of abortive clones (Fig. 4C, Table 4).

We next performed mixing experiments, comparing the behaviour of GFP labelled cells expressing DeltaFl alone, Rnai1 alone or in combination (Fig. 4D,E). Knockdown of syntenin in WT cells did not affect cell cohesion. However, knockdown of syntenin in DeltaFl-expressing keratinocytes reduced the proportion of cohesive clones to the same extent as DeltaVA (Fig. 4D,E; Fig. 3B). As independent confirmation of the role of Delta1 in promoting keratinocyte cohesion, we generated Delta1-null mouse keratinocytes from mice expressing K5Cre × Delta$^{flox/flox}$ (Hozumi et al., 2004) and found that these cells also scattered when combined with WT keratinocytes in the mixing experiments (Fig. 4F,G). Taken together, our results suggest that syntenin contributes to the effects of Delta1 on Notch activation, keratinocyte differentiation and intercellular adhesion.

Delta1 ubiquitylation and internalisation are essential for Notch signalling (Glittenberg et al., 2006; Itoh et al., 2003). The cytoplasmic domain of Delta1 is ubiquitylated via the RING ubiquitin ligase Mind bomb, which promotes Delta1 internalisation (Itoh et al., 2003). Since syntenin is involved in membrane trafficking (Fernandez-Larrea et al., 1999) and has been identified in early endosomes (Fialka et al., 1999; Latysheva et al., 2006; Thery et al., 2001) we speculated that syntenin plays a role in maintaining Delta1 at the cell surface. In support of this, in keratinocytes transduced with DeltaFl there was colocalisation of DeltaFl with endogenous syntenin at cell-cell borders (Fig. 5A-C). When Rnai1 was introduced, syntenin was no longer detectable by immunofluorescence staining (Fig. 5B,E) and there was a major reduction in the level of DeltaFl at the cell surface (Fig. 5D). The reduction in surface levels of DeltaFl correlated with an increase in DeltaFl-positive cytoplasmic vesicles, which have previously been shown to colocalise with a marker of late endosomes and lysosomes (Fig. 5D) (Lowell et al., 2000).

Based on these observations we predicted that DeltaVA, because of its inability to bind syntenin, would be cleared more rapidly from the cell surface than DeltaFl. Although DeltaDS cannot bind syntenin it also lacks the Mind-bomb-binding site (Glittenberg et al., 2006; Itoh et al., 2003) and so would not be expected to exhibit rapid internalisation. To examine this, we transduced keratinocytes with DeltaFl, DeltaDS or DeltaVA and incubated them at 4°C with the antibody to the extracellular domain of zebrafish Delta. After washing the cells extensively at 4°C to remove unbound antibody, they were transferred to 37°C, fixed after 0, 30, 90 or 240 min and the location of the antibody was

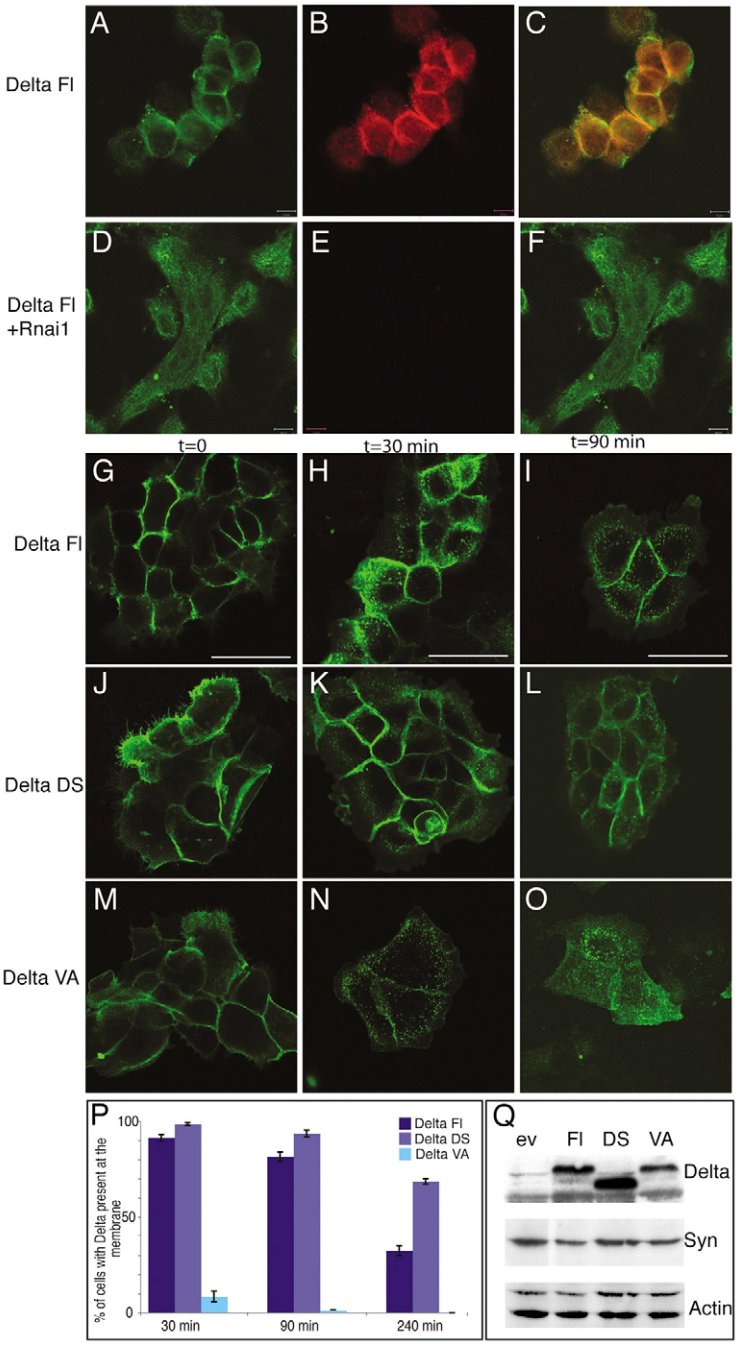


**Fig. 5.** Effects of syntenin knockdown or Delta1 mutation on cell surface expression of Delta mutants. (A-F) Keratinocytes transduced with DeltaFl and pRetrosuper control vector (A-C) or syntenin siRNA (Rnai1; D-F) were double labelled with anti-DeltaD (green A,D) and anti-syntenin (red B,E) antibodies. (C,F) Merged images of middle and left panels. Bar, 20 μm. (G-O) Primary human keratinocytes transduced with DeltaFl, DeltaDS or DeltaVA were incubated with an anti-Delta antibody at 4°C (*t*=0), then transferred to fresh medium at 37°C for the times indicated. Bars, 50 μm. (P) Percentage of cells with Delta antibody at the plasma membrane. Data are means ± s.e.m. of three independent experiments. 100 cells of each type were counted per experiment. (Q) Western blot of keratinocytes transduced with ev, DeltaFl, DeltaDS and DeltaVA constructs, probed with antibodies to Zf-Delta, syntenin and actin.

examined (Fig. 5G-P). In control keratinocytes infected with the empty retroviral vector, no binding or internalisation of antibody was detected (data not shown).

By 30 minutes, 90% of cells retained DeltaFl on the cell surface; this fell to 80% at 90 minutes and 30% at 240 minutes (Fig. 5G-I,P). DeltaDS remained at the cell surface for longer: at 240 minutes ~70% of cells still had DeltaDS on the plasma membrane (Fig. 5J-L,P). By contrast, DeltaVA was rapidly cleared from the cell surface and by 30 minutes, only 10% of cells retained surface expression of the protein (Fig. 5M-P). The vesicular localisation of DeltaVA (Fig. 5N) was reminiscent of Delta Fl localisation when syntenin was knocked down (Fig. 5D). We verified by western blotting that the overall expression levels of the different Delta constructs and endogenous syntenin were similar (Fig. 5Q). The progressive loss of Delta from the cell surface is consistent with receptor internalisation (Latysheva et al., 2006), although we cannot exclude the possibility that some Delta recycling occurred (Paterson et al., 2003; Xiao et al., 2005). These experiments suggest that syntenin binding to the PDZ domain of Delta1 promotes retention of the protein at the cell surface.

## Discussion

In the basal layer of human interfollicular epidermis the stem cells are clustered, their location being determined, at least in part, by their adhesive properties (Jensen and Watt, 2006; Jensen et al., 1999; Jones et al., 1995; Legg et al., 2003; Lowell et al., 2000). The stem cell clusters express high levels of Delta1, which promotes keratinocyte cohesiveness via a mechanism that is dependent on its cytoplasmic domain (Lowell et al., 2000; Lowell and Watt, 2001). We now show that Delta1-mediated cohesion depends on the Delta1 PDZ-binding domain and that mutation of this domain not only abolishes keratinocyte cohesion but also stimulates terminal differentiation by enhancing Notch signalling (Blanpain et al., 2006; Rangarajan et al., 2001). These results are summarised schematically in Fig. 6.

We have shown that Delta1-mediated cohesion depends on the interaction of the PDZ domain with syntenin. In addition, syntenin regulates Notch signalling by reducing Delta1 internalisation, accounting for the enhanced signalling and differentiation-promoting activity of DeltaVA. Whereas keratinocytes lacking endogenous Delta1 show reduced intercellular adhesion, syntenin knockdown in WT cells did not have the same effect. This probably reflects both heterogeneity of the WT cells (a mixture of stem and transit amplifying cells) and the ability of Delta1 to interact with additional proteins (Table 3). Since Delta1 can bind additional PDZ proteins, including synectin, Discs large and the MAGI-membrane-associated scaffolding proteins (Table 3) (Mizuhara et al., 2005; Six et al., 2003; Wright et al., 2004), there is further potential for Delta1 to participate in multiple signalling complexes at the cell surface.

Syntenin could promote Delta1-mediated cell adhesion by enabling Delta1 to remain at the cell surface for longer and/or via the ability of syntenin to interact with additional proteins. Syntenin has two PDZ domains, which are proposed to bind different proteins in the same subcellular location, resulting in assemblies of syndecans and other partners, such as ephrin-B1 and Delta1, in supramolecular assemblies (Lin et al., 1999). In keratinocytes, Delta1 colocalises with cortical actin, and overexpression of mutant Delta lacking most of the cytoplasmic domain stimulates cell spreading and motility, suggesting that the effect of Delta on cohesiveness is not solely attributable to increased intercellular adhesion (Lowell and Watt, 2001). Syntenin could potentially link Delta1 and integrins (De Joussineau et al., 2003; Julich et al., 2005), through its ability to bind syndecans, which in turn modulate integrin-mediated adhesion (Oh and Couchman, 2004).

Our observations support the view that Delta1, and indeed other Notch ligands, have two distinct functions: to initiate Notch signalling in a neighbouring cell and to initiate a PDZ-dependent signalling mechanism in the Delta1-expressing cell (Ascano et al., 2003; Itoh et al., 2003). We have shown that in cultured human interfollicular epidermis, the PDZ-binding domain of Delta1 regulates stem cell clustering and also Notch signalling through its association with syntenin. Thus, high expression of Delta1 contributes to the epidermal stem cell niche both by determining the physical location of the cells and by sending a differentiation stimulus to neighbouring transit-amplifying cells (Fig. 6).

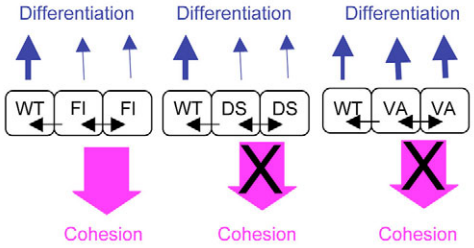


**Fig. 6.** Summary of experimental results. Keratinocytes are shown as wild type (WT) or expressing DeltaFl (Fl), DeltaDS (DS) or DeltaVA (VA). Black arrows represents signal from Delta-expressing cell to WT cell; Double-headed arrow represents reciprocal signalling between two Delta-expressing cells. The differentiation signal is represented by three sizes of blue arrow, the thinnest representing no enhanced differentiation, the thickest representing a strongly increased signal, and the medium arrow differentiation that is slightly enhanced. Intercellular adhesion (cohesion) is shown as being promoted (pink arrow) or not (crossed through arrow).

## Materials and Methods

### Constructs

Zebrafish DeltaD FL, LC and DS constructs (kind gift of G. Wright) (Itoh et al., 2003) were subcloned into the retroviral vector pBabe puro as described previously (Lowell et al., 2000). The DeltaVA point mutation construct was generated from pBabe puro-DeltaD FL using the Quikchange kit from Stratagene and the following oligos: 5′-CA ACC GAG GCG TAA CGC CAC AAC-3′ and 5′-GTT GTG GCG TTA CGC CTC GGT TGC-3′. The syntenin GST construct was a kind gift from P. Zimmermann (Zimmermann et al., 2002). Hes1 luciferase reporter was a kind gift from U. Lendhal (Blokzijl et al., 2003).

The following oligonucleotides were used for syntenin RNAi experiments: Oligo1, 5′-GAT CCC CAC TAT ATG GTG GCT CCT GTT TCA AGA GAA CAG GAG CCA CCA TAT AGT TTT TTA-3′ and Oligo 2, 5′-AGC TTA AAA AAC TAT ATG GCT CCT GTT CTC TTG AAA CAG GAG CCA CCA TAT AGT GGG-3′. The oligos were cloned in pRetrosuper-IRES-GFP according to the Manufacturer's instructions (Oligoengine).

### Antibodies

The following antibodies were used: Delta D, Zdd2 (kind gifts of J. Lewis), α6 integrin (GoH3; Serotec MCA699), keratin10 (Covance), transglutaminase1 (BC1; kind gift of R. Rice, University of California, Davis, CA), loricrin (DH11; rabbit antiserum to NH2-HQTQQKQAPTWPCK-COOH peptide), Anti-laminin5 (Polykal; kind gift of P. Marinkovitch, Stanford University, CA), Human Delta1 (biotinylated; R&D Systems; BAF1818) and syntenin (SYSY; 133002).

### Cell culture and transfection

Primary human keratinocytes were isolated from neonatal foreskins of three different individuals (strains kj, kq and kv) and cultured in the presence of a feeder layer of J2-3T3 cells in FAD medium (one part Ham's F12, three parts Dulbecco's modified Eagle's medium, $1.8\times10^{-4}$ M adenine), supplemented with 10% fetal calf serum (FCS) and a cocktail of 0.5 μg/ml of hydrocortisone, 5 μg/ml insulin, $10^{-10}$ M cholera enterotoxin and 10 ng/ml epidermal growth factor (HICE cocktail) (Lowell et al., 2000).

A431 and 293T cells were maintained in culture using Dulbecco's modification of Eagle's medium, supplemented with 10% fetal calf serum. 293T cells were transfected using lipofectamine reagent according to the manufacturer's instructions (InVitrogen) and analysed 48 hours after transfection.

### Retroviral infection

Retroviruses were prepared by a two-step packaging procedure involving transient transfection of ecotropic Phoenix cells and infection of amphotropic AM12 cells with viral supernatant from the Phoenix cultures (Janes et al., 2004). Infection of keratinocytes with pBabe puro-GFP virus was carried out by co-culture with AM12 retrovirus producer cells (Lowell et al., 2000). Retroviral infection of keratinocytes or J2-3T3 cells with all the Delta constructs was carried out using virus-containing supernatant (Janes et al., 2004). When keratinocytes were to be doubly infected with a Delta retrovirus and pBabe puro-GFP, they were grown for at least 7 days in puromycin after infection with Delta constructs before harvesting and replating on GFP retroviral producer cells.

### Clonogenicity assays

Keratinocytes were seeded at clonal density (500 cells per 60-mm-diameter dish), cultured for 14 days, then fixed and stained with Rhodanile Blue (Lowell et al., 2000). All colonies (that is, groups of ⩾2 cells) were scored on each dish and CFE was calculated as the percentage of all plated cells that formed colonies (Lowell et al., 2000). Colonies were scored as abortive if they contained fewer than 40 cells, the majority of the cells being large and terminally differentiated (Lowell et al., 2000).

### Mixing experiments

Keratinocytes were infected with pBabe puro-DeltaFl (K-DeltaFL), pBabe puro-DeltaLC (K-DeltaLC), pBabe puro-DeltaDS (K-DeltaDS), pBabe puro-DeltaVA (K-DeltaVA), or empty vector (K-WT) and subsequently with GFP. GFP-labelled cells were co-cultured with either unlabelled K-WT or K-Delta. $10^4$ GFP-labelled cells were mixed with $10^5$ unlabelled WT cells from the same strain and passage number and seeded on 35-mm-diameter tissue culture dishes (Falcon). The cultures were grown for 5 days, fixed with 4% paraformaldehyde and then the proportion of cohesive clones determined under a UV microscope, as described previously (Lowell and Watt, 2001). All experiments were carried out at least five times. Significance levels were calculated using the Student's *t*-test.

### Reconstituted human epidermis

Reconstituted human epidermis was prepared as described previously (Janes et al., 2004). 1.5 $cm^2$ pieces of de-epidermised dermis were seeded with $10^5$ retrovirally infected primary human keratinocytes and grown at the air-liquid interface in keratinocyte medium. DED cultures were harvested after 16 days and frozen immediately on dry ice for Haematoxylin and Eosin staining or immunohistochemical analysis.

### Yeast two-hybrid screen

Yeast two-hybrid protein interaction experiments and library screenings were performed using the Matchmaker GAL4 two-hybrid system 3 (BD Bioscience), according to the manufacturer's instructions. The AH109 yeast host strain was co-transformed with the bait plasmid, consisting of the intracellular domain of mouse Dll1 in pGBKT7, and a mouse E17 embryo Matchmaker cDNA library in the cloning vector pGAD1 (Groot et al., 2004).

### GST pull downs

Fusion proteins were expressed in *Escherichia coli* BL21 cells and purified from cell extracts using Glutathione-Sepharose 4B (Amersham Biosciences). Confluent cultures of A431 cells or transfected 293T cells were extracted in ice-cold buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40) containing 10 mM EDTA and protease inhibitors (Roche). Protein samples were incubated by rotation at 4°C for 40 minutes with 10 μg GST or GST-syntenin prebound to Glutathione-Sepharose 4B. Precipitates were washed in ice-cold lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40) and boiled in SDS-PAGE sample buffer before gel electrophoresis.

### Immunoprecipitation

Confluent cultures of A431 cells were extracted using ice-cold buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40) containing 10 mM EDTA and protease inhibitors (Roche). Proteins were immunoprecipitated with anti-syntenin antibody or an irrelevant antibody (anti-laminin5) and immunoblots were probed with anti-human Delta1 antibody.

### Luciferase assay

J2-3T3 cells infected with the different Delta constructs were transiently transfected with a Hes1 reporter (Jarriault et al., 1995) and a Renilla luciferase control vector (Janes et al., 2004). 24 hours after transfection, cells were extracted and the activity of the reporter was measured using the Promega dual luciferase kit, according to the manufacturer's instructions.

### Delta1 internalisation

Human primary keratinocytes infected with different Delta constructs were grown on glass coverslips. Cells were incubated with 10 μg/ml mAb Zdd2 in serum-free FAD medium for 1 hour at 4°C. Cells were rinsed three times with ice-cold serum free FAD (zero time point) and then transferred to pre-warmed complete growth medium at 37°C for different length of time (30 minutes, 90 minutes and 240 minutes). At each time-point, cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature. Cells were subsequently permeabilised in 0.1% Triton X-100, rinsed in PBS and blocked for 1 hour with blocking buffer (10% fetal calf serum and 0.25% fish skin gelatin). After blocking, cells were incubated with Alexa Fluor 488-conjugated secondary antibody, rinsed in PBS and water and analysed using a Zeiss confocal microscope. The percentage of cells that retained Delta1 on the plasma membrane at each time point was determined. At least 100 cells were counted per experimental condition and three independent experiments were performed.

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