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Comparative Analysis of the Functions of Integrin Adaptor Molecules ILK and PINCH1 in the Skin Epithelium

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Prof. Dr. Reinhard Fässler betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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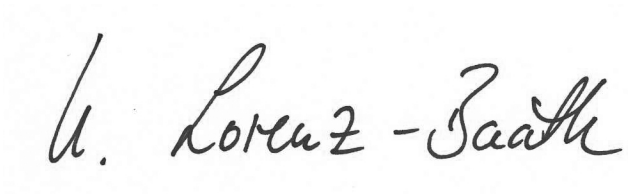

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Meinen Eltern, meinem Mann Veikko und meinem Sohn Ole

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Publication 1

Integrin-linked kinase: integrin's mysterious partner

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Publication 3 in preparation

PINCH-1 works in an ILK-dependent as well as independent manner in keratinocytes

Curriculum Vitae

Abbreviations

BM	Basement membrane
DP	Dermal papilla
E	Embryonic day
ECM	Extracellular matrix
EM	Electron microscopy
EPU	Epidermal proliferative unit
FA	Focal adhesion
FAK	Focal adhesion kinase
FC	Focal complex
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factors
HF	Hair follicle
HM	Hair matrix
HS	Hair shaft
ILK	Integrin-linked kinase
IRS	Inner root sheath
K	Keratin
MAPK	Mitogen-activated protein kinase
ORS	Outer root sheath
P	Postnatal day
PAK	P21-activated kinases
PH	Pleckstrin-homology
PINCH	Particularly interesting new cysteine-histidine-rich protein
RTK	Receptor tyrosine kinase
vWFA	von Willebrand factor A

Summary

The adhesion receptor family of β1 integrins is crucial during development and tissue homeostasis. Nonetheless, an important and still largely unanswered question is how integrins mediate intracellular functions. Numerous integrin adaptor molecules have been identified but the precise function of them, especially *in vivo* remains to be clarified. During homeostasis of the epidermis and its appendages β1 integrins regulate the adhesion to the basement membrane (BM) and its assembly, the tightly regulated programmes of differentiation, maintenance of stem cells, migratory processes and proliferation.

Integrin-linked kinase (ILK) is part of the tri-molecular ILK-PINCH-Parvin (IPP) complex and directly binds integrins. Within the IPP-complex the stability of all three members is interdependent. We investigated whether ILK and PINCH1 are required for integrin-mediated functions in skin epithelium and re-evaluated the current concept of protein interdependence.

Conditional ablation of PINCH1 or ILK in keratinocytes in mice caused epidermal defects and hair loss reminiscent of the β1 integrin-deficiency. In the epidermis integrin-mediated adhesion was decreased leading to blistering, BM disruption and epidermal hyperthickening accompanied by abnormal differentiation and proliferation. The mutant hair follicles were highly distorted and stunted and failed to initiate epithelial outgrowth pointing towards impaired keratinocyte migration. *In vitro* studies attributed the migration defect to impaired focal adhesion formation and actin assembly. This, in turn, affected cell spreading and the formation of stable lamellipodia protrusions which impaired directional and persistent migration. However, both phenotypes differed as loss of PINCH1 also affected cell-cell adhesion and compromised actin-dependent processes more severely.

In summary our data show that ILK and PINCH1 play important roles downstream of β1 integrin in keratinocytes. However, PINCH1 has also functions that are independent from the IPP-complex.

Introduction

The integrin receptor family

Integrins have been discovered in the 1980's as cell surface receptors specific for all metazoa. They physically link the extracellular matrix (ECM) to the cytoskeleton and different cell signaling machineries. The property to physically link structural proteins of the ECM with structural proteins of the intracellular (actin, intermediate filaments) compartments was the reason why they were named integrins (Hynes, 1987). Integrins are heterodimeric transmembrane receptors, composed of an α and a β subunit

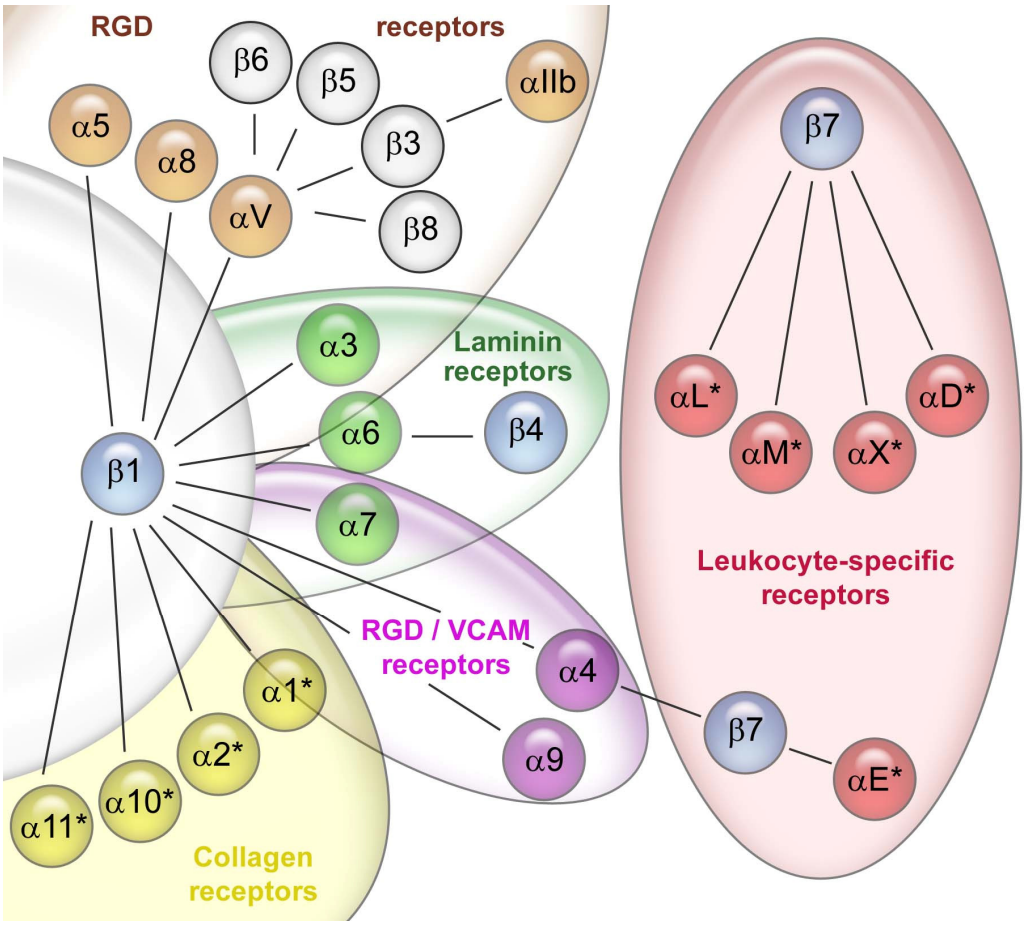

Figure 1: Integrin receptor family

Integrin heterodimers grouped by their main recognition or cell type specificities. The nine αA-domain containing α subunits are indicated (*).

that bind in addition to ECM proteins a variety of other ligands such as soluble proteins, cell surface receptors of neighboring cells or pathogens. Both subunits are type I transmembrane proteins with a large extracellular domain, a transmembrane domain and short cytoplasmic domains. The only exception is the long cytoplasmic tail of the β4 integrin subunit. In vertebrates 8 β and 18 α subunits exist and the non-covalent association of αβ pairs leads to the formation of 24 heterodimers known to date which can be grouped by their main ligand specificities (Figure 1) (Hynes, 2002).

Each particular integrin can bind several ligands and most ligands can be bound by more than one integrin. Ligand-binding of integrin receptors requires preceding activation in order to prevent undesirable cell adhesion. This activation depends on signaling cues, requires an intramolecular, conformational switch and the presence of divalent cations.

Despite the overlapping ligand preferences it has become clear that each integrin has a specific function for the organism. This is demonstrated by the large variety of phenotypes of knock-outs or integrin mutations in mice. The defects range from peri-implantation lethality as in the case of β1 integrin, through developmental defects, perinatal lethality and tissue-specific postnatal abnormalities (Table 1).

These studies indicate how crucial integrins are for development, immune response, leukocyte traffic and homeostasis. Accordingly many integrins are at the heart of human diseases and exploited as targets for therapeutic approaches.

Integrins have been studied extensively over the last 20 years which has improved the understanding of integrin structure, regulation and function. Yet, the signal transmission triggered by integrins and their regulation is a field of ongoing investigations (reviewed by Arnaout et al., 2005; Hynes, 2002; Luo et al., 2007).

Integrin	Viability	Phenotype	Reference
α1	V	Reduced tumor vascularization	Gardner et al., 1996
α2	V	Delayed platelet aggregation and reduced mammary gland branching	Holtkotter et al., 2002 Chen et al., 2002
α3	PEL	Kidney tubule defects, reduced branching in lungs and mild skin blistering	Kriedberg et al., 1996 DiPersio et al., 1997
α4	E11/14	Chorioallantoic fusion defect and heart defects	Yang et al., 1995
α5	E10/11	Defects in mesodermal and vascular development and neural crest apoptosis	Yang et al., 1993
α6	PEL	Severe skin and sqamous epithelia blistering	Georges-Labouesse et al., 1996
α7	V	Muscular dystrophy	Mayer et al., 1997
α8	PEL	Small or absent kidneys	Muller et al., 1997
α9	POL	Lymphatic duct defect	Huang et al., 2000
α10	V	Mild skeletal abnormalities	Bengtsson et al., 2005
αv	EL10/PEL	EL10: placental defects; PEL: cerebral vascular defects, cleft palate	Bader et al., 1998
αIIb	V	No platelet aggregation	Tronik-Le Roux et al., 2000
αL	V	Impaired leukocyte recruitment	Schmits et al., 1996
αM	V	Defective phagocytosis and apoptosis of neutrophils	Coxon et al., 1996
αE	V	Reduced intraepithelial lymphocytes	Schon et al., 1999
β1	EL6.5	Peri-implantation lethality	Fässler and Meyer, 1995
β2	V	Leukocytosis and skin infections	Scharffetter-Kochanek et al., 1998
β3	V	No platelet aggregation, osteosclerosis	Hodivala-Dilke et al., 1999
β4	PEL	Severe skin and sqamous epithelia blistering	van der Neut et al., 1996
β5	V	No obvious defects	Huang et al., 2000
β6	V	Skin inflammation and impaired lung fibrosis	Huang et al., 1996
β7	V	No Peyer's patches and reduced intraepithelial lymphocytes	Wagner et al., 1996
β8	EL10/PEL	EL10: placental defects; PEL: cerebral vascular defects	Zhu et al., 2002

Table 1: Integrin gene knock-out phenotypes

For almost all integrins, except α11, αD and αX knock-out mice have been generated; almost each of them displays a specific phenotype. References that are listed are not included in the refrence list. EL, embryonic lethal; V, viable; PEL, perinatal lethality; POL, postnatal lethality (Table modified from Hynes, 2002).

1.1. Structure of the integrins

1.1.1. Integrin extracellular domains and ligand binding

The large extracellular domains of integrins are responsible for ligand binding and comprise over 100 kDa for α subunits and more than 75 kDa for β subunits. The extracellular parts associate within an αβ dimers and both subunits contribute to the ligand specificity, although not necessarily in direct ligand binding. Half of the α subunits contain an additional, extracellular αA domain involved in ligand binding.

The αA domain (~190 kDa) from αM was the first integrin structure that was resolved and shown to be a core of parallel β sheets surrounded by α helices, resembling von Willebrand factor A (vWFA) domains known to be involved in protein-protein interactions. Indeed, the ligand binding of αA domain containing integrins occurs through this domain. Divalent cations are further required for ligand binding of integrins. A metal ion binding site was identified within the αA ligand binding domain (Lee et al., 1995) and was termed metal ion-dependent adhesion site (MIDAS). Since the integrin and the ligand contribute to the coordination of the metal ion, ligand binding leads to changes in the metal ion coordinating residues of the integrins. This triggers conformational alterations within the entire domain. Indeed, the αA domain was shown to take either a “closed” or “open” conformation depending on the absence or presence of the ligand (Emsley et al., 1997; Emsley et al., 2000; Hynes, 2002). The open form has a high affinity to bind ligands and can therefore be termed “active” conformation (Xiong et al., 2000).

The first crystal structure of an entire extracellular domain was the αA domain-lacking αVβ3 integrin (Xiong et al., 2001). The structure revealed 12 distinct domains within the integrin ectodomain - four in the αV subunit and eight in the β3 subunit - assembling into a globular head with two legs. The ligand binding head is composed of a β-propeller domain of the α subunit

complexed with the βA domain that is homologous to the αA domain. In addition to the MIDAS cation binding site two adjacent metal ion binding sites were characterized: ADMIDAS (adjacent to metal-ion dependent adhesion site) and LIMBS (ligand-induced metal ion-binding site). The domain structure of the extracellular integrin parts is represented in Figure 2.

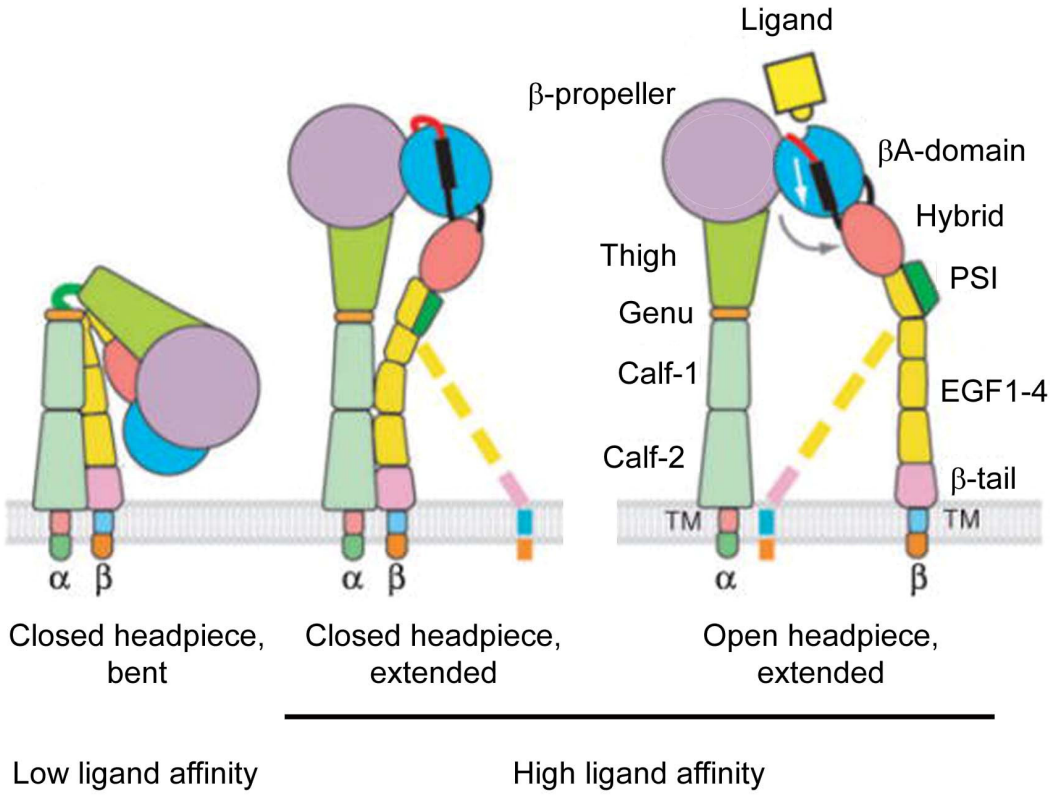

Figure 2: Integrin architecture

Integrin domain structure and rearrangements during activation are represented. The β subunit lower legs are flexible and separate during activation. They are shown in what may be the predominant (*solid representation*) and less predominant (*dashed lines*) orientations (Cartoon modified from Luo et al., 2007).

Surprisingly and in contrast to previous electron microscopy (EM) images (Nermut et al., 1988), this first structure revealed that the head was bent over by 135° towards the legs. Since the elucidation of this first structures, several studies showed that the bent conformation represents the low affinity, “inactive” state and that activation and ligand-binding is associated with both

conformational changes within the ligand binding head and metal ion coordination sites ("closed" and "open" similar to αA) (Xiong et al., 2002) and the opening of the integrin extracellular domain towards an extended conformation in a switch-blade-like fashion (Figure 2) (Takagi et al., 2003; Xiao et al., 2004).

Thus, three conformations of the integrin extracellular part have been resolved by crystallography and EM. The bent conformation with a closed headpiece and the extended conformation with either closed or open head conformations with high ligand affinities (reviewed by Luo et al., 2007).

The conformational switches associated with the high affinity state of integrins can also be triggered by the exchange of the physiological cations Ca^{2+} and Mg^{2+} against Mn^{2+}, which triggers a change in the metal ion coordination of the ligand binding domains.

1.1.2. Integrin transmembrane and cytoplasmic domains

In their inactive conformation integrin transmembrane domains are associated with each other. Consistently, in the bent αVβ3 structure, the α and β subunit ectodomain C-termini were only a few angstroms apart (Xiong et al., 2001). Upon activation or after Mn^{2+}-induced extension of the extracellular domain they undergo a spatial separation. This concept is supported by studies that artificially inactivated integrins in solution with a C-terminal clamp (Takagi et al., 2001) or by mutation- and FRET-based assays proving that the association is disrupted during activation (Kim et al., 2003).

Integrin cytoplasmic tails are very small compared to the extracellular parts, usually less than 50 amino acids - with the exception of β4 cytoplasmic tail that contains over 1000 amino acids. The cytoplasmic tails of integrins interact with each other with low affinity. This was first shown by surface plasmon resonance for recombinant αIIb and β3 integrin cytoplasmic peptides (Vallar et al., 1999). The interactions take place between membrane-proximal helices of both subunits (Vinogradova et al., 2002) which are highly conserved in the vast majority of subunits. Interactions of the cytoplasmic domains of α and β

subunits have been proposed to restrain integrin in an inactive state. Abrogation of the cytoplasmic domain interactions by deletions or mutations within membrane-proximal regions generally results in integrin activation.

Finally, integrin cytoplasmic tails link to the cytoskeleton and represent binding sites for intracellular molecules. Talin is one of the best studied direct binding partners of integrin β subunit cytoplasmic tail.

Talin binding sites in the β subunits overlap with the interaction sites for α subunits. Due to the high affinity for the talin head, talin head binding abrogates integrin cytoplasmic domain interaction and separates the tails, thereby leading to integrin activation (Calderwood et al., 2002; Vinogradova et al., 2004; Vinogradova et al., 2002). Talin binding, therefore, is the first example of how integrin cytoplasmic parts can regulate the activation and function of the extracellular domain. The interactions of other cytoplasmic proteins with integrin tails will be discussed in a separate chapter (*Chapter 2.1*).

1.2. Bidirectional regulation of integrin function and signaling

Aberrant activation or failure of integrin activation can be detrimental to an organism and is at the heart of many human diseases. Activation of integrins is a tightly and multi-dimensionally controlled process.

Based on the structural informations of integrin extracellular and intracellular domains, the following model for integrin regulation became widely accepted:

The different conformational states of integrins exist in equilibrium. The bent conformation has a low affinity for ligand binding and is characterized by the tight association of the α and β transmembrane and cytoplasmic domains. Pertubations of cytoplasmic domain interaction induce separation of both cytoplasmic and transmembrane domains resulting in integrin extension and favors the activated state with an open, ligand-binding site exposed headpiece. Generally, this model is compared with a switch-blade-like mechanism.

1.2.1. Inside-out signaling

The control of the ligand binding affinity of the extracellular domain from within the cell via integrin cytoplasmic domains is generally referred to as “inside-out” signaling. It is currently believed that the binding of talin to the membrane proximal NPxY/F motifs in the β subunit cytoplasmic domain is a final step in switching integrins into the high affinity state, as downregulation of talin ablated integrin activation by integrin activators such as CD98 or activated R-Ras (Tadokoro et al., 2003) and talin1-deficient platelets lack integrin activation *in vitro* and *in vivo* (Nieswandt et al., 2007). However, several studies show that the talin requirement for integrin activation might not fit to the entire integrin family, i.e. β2 and β7 integrins seems to be distinct as β2 is constitutively linked to talin in resting neutrophils and β7 binds talin only poorly (Calderwood et al., 2001; Sampath et al., 1998). Obviously, talin binding as a last common step in activation is preceeded by other physiological regulators. The role of serine/threonine and of tyrosine phosphorylation of the α and β subunit cytoplasmic tails with respect to integrin activation remains unclear. Transient integrin activation additionally requires rapid deactivation and may involve downregulatory integrin binding partners.
Activation of integrins by the non-physiological induction via Mn^{2+}, however, decouples the requirement for inside-out signaling from activation.

1.2.2. Integrin avidity

Lateral association of integrins within the plasma membrane or clustering are critical for linking the ligand-bound integrin to the cytoskeleton (Hato et al., 1998). However, it remains unclear whether changes in integrin lateral distribution contribute to integrin priming or represent an important step for strengthening integrin adhesions.
Polar distribution of integrins in the lamellipodium of migrating cells is achieved by vesicular trafficking and represents an important step during cell migration (Katagiri et al., 2003; Shimonaka et al., 2003). It is also believed that clustering is dependent on the presence of multi-valent ligand clusters

(Kim et al., 2004). This idea is further supported by recent data restricting the integrin lateral association by distributing RGD ligands on nanoscale patterns, High inter-ligand distances do not abrogate cell attachment but impair cell spreading and migration (Cavalcanti-Adam et al., 2007) suggesting that regulation of integrin affinity and avidity may occur sequentially. Important to note is that changes in integrin affinity do not influence integrin avidity and vice versa, indicating that the regulation of both processes occurs independently from each other.

The mechanisms resulting in integrin redistribution during initial stages of activation are not known. Oligomerization of transmembrane domains, or implication of lipid rafts were proposed, but their exact involvement remains to be discovered.

1.2.3. Outside-in signaling

Extracellular ligand binding by integrins regulates a large variety of biological responses within the cell including cytoskeletal re-organization, gene expression, survival, differentiation, adhesion and motility. Individual integrin receptors transmit extracellular cues to the cytoplasm via conformational changes. Extracellular engagement triggers the separation of transmembrane and cytoplasmic domains which in turn enables interactions with a large variety of intracellular binding partners that will be discussed in the following paragraph. The essential requirement for the separation between the α and β subunit for outside-in signaling has been recently demonstrated (Zhu et al., 2007). Additional modes for modifying intracellular responses might include integrin clustering or the interaction with other cell surface proteins such as tetraspanins or uPAR (Hemler, 2003; Wei et al., 1999).

Integrin function in signaling and matrix adhesion

Integrin functions such as actin binding and signaling mediated by integrin-binding proteins touch imortant areas of the presented research and will therefore be discussed in-depth in the following chapters.

1.3. Cytoplasmic integrin binding proteins

As described above, integrin cytoplasmic tails play an important role in regulating the bi-directional signaling of integrins. The recruitment of proteins that bind the integrin cytoplasmic domain triggers changes in integrin ligand affinities and is also responsible for all signals transduced from the outside towards the cytoplasm. Integrin cytoplasmic tails lack intrinsic enzymatic activities. β subunit tails regulate essential functions for integrins, such as subcellular localization and activation of signaling pathways (Liu et al., 2000). To date more than 20 proteins have been identified to bind integrin β tails (Table 2). Many of them are involved in cytoskeletal interactions and dynamics (mostly actin-binding) or in signaling.

The cytoplasmic α tails between different integrins are less conserved. Therefore, α subunits are involved in the regulation of unique integrin receptors functions either by directly initiating signaling events or by modulating β subunit signaling. However, only few proteins that bind to α subunit tails have been elucidated thus far. They include F-actin, caveolin-1, paxillin and calcium-binding proteins (Liu et al., 2000).

Intriguingly, the binding sites of various cytoplasmic tail binding proteins are overlapping, excluding their simultaneous binding to the same integrin molecule. This implies an increased complexity for the functions triggered by integrin-binding partners (Geiger et al., 2001; Liu et al., 2000).

A profound discussion of the diverse functions of all integrin cytoplasmic tail interacting proteins would reach far beyong the scope of this introduction. The interested reader is therefore referred to excellent reviews on the integrin interactome and downstream signaling (Liu et al., 2000; Schwartz and Ginsberg, 2002; Wiesner et al., 2005; Zaidel-Bar et al., 2007).

Binding partner	Integrin subunit	Reference
Actin binding proteins		
Talin	β1, β2, β3	Horwitz et al., 1986; Knezevic et al., 1996; Pfaff et al., 1998; Goldmann, 2000
Filamin	β1, β2, β3, β7	Pavalko et al.,1989; Loo et al., 1998; Pfaff et al., 1998; Goldmann, 2000
α-actinin	β1, β2	Otey et al., 1990; Pavalko et al., 1991; Cattelino et al., 1999
F-actin	α2	Kieffer et al., 1995
Myosin	β3	Jenkins et al., 1998; Sajid et al., 2000
Skelemin	β1,β3	Reddy et al., 1998
Tensin-1	β1, β3, β5, β7	Calderwood et al., 2003
Signaling proteins		
ILK	β1,β3	Hannigan et al., 1996
FAK	β1,β2,β3	Schaller et al., 1995; Chen et al., 2000
Cytohesin-1	β2	Kolanus et al., 1996
Cytohesin-3	β2	Hmama et al., 1999
Dok-1	β1, β3, β5, β7	Calderwood et al., 2003
c-src	β3	Arias-Salgado et al., 2003
Other proteins		
Paxillin	β1, β3, α4	Schaller et al., 1995; Chen et al., 2000; Liu et al., 1999
Grb2	β3	Law et al., 1996
Shc	β3	Law et al., 1996
β3-endonexin	β3	Shattil et al., 1995; Eigenthaler et al., 1997
TAP-20	β5	Tang et al., 1999
CIB	αIIb	Naik et al., 1997; Shock et al., 1999;
Calreticulin	α	Rojiani et al., 1991; Leung-Hagesteijn et al., 1994; Coppolino et al., 1995
Caveolin-1	α	Wary et al., 1998
Rack1	β1, β2, β5	Liliental et al., 1998
WAIT-1	β7	Rietzler et al., 1998
JAB1	β2	Bianchi et al., 1998
Melusin	β1	Brancaccio et al., 1999
MIBP	β1	Li et al., 1999
ICAP-1	β1	Chang et al., 1997; Zhang and Hemler, 1999
CD98	β1, β3	Zent et al., 2000
DRAL/FHL2	α3, α7, β2	Wixler et al., 2000
Dab1	β1, β3	Calderwood et al., 2003
Dab2	β3, β5	Calderwood et al., 2003
Eps8	β1, β3, β5	Calderwood et al., 2003

Table 2: Integrin cytoplasmic domain-binding proteins

The α subunits are highlighted in red. References that are listed are not found in the reference list (Table modified from Liu et al., 2000).

1.4. Signaling via integrins

Physiological responses following integrin adhesion depend on integrin tail associated proteins, as integrin cytoplasmic tails lack enzymatic activities.
Interestingly, the focal-adhesion kinase (FAK), integrin-linked kinase (ILK) and c-src are to date the only proteins with ascribed kinase activity among the direct binding partners. Others have been directly involved in cell cycle regulation (β3 endonexin) or transcriptional co-activation (JAB1). More importantly however, integrin-binding proteins such as Paxillin, Shc, Grb2, FAK and ILK contribute to a further recruitment of signaling molecules and adaptors to the sites of integrin adhesion. This leads to the assembly of a large signaling hub upon integrin engagement and clustering (Liu et al., 2000).
Integrins regulate mitogenic signaling, survival, differentiation and cytoskeletal remodelling by affecting a variety of intracellular pathways. The efficiency of the MAP (mitogen-activated protein) kinase pathways is regulated by integrins at several levels, i.e. through recruitment of Grb2 and son-of-sevenless (Sos) complex, p21-activated kinases (PAKs) or Rap1 (Juliano, 2002). Other important signaling pathways that are downstream of integrin-mediated adhesions include PI3K and NF-κB.
Another significant aspect of integrin function is the cross-talk between integrins and other signaling receptors, such as receptor tyrosine kinases (RTKs), G-coupled receptors and cytokine receptors that converge at the level of MAP kinase or PI3K signaling. This coupling allows integrins to integrate ECM cues with growth or differentiation signals. Finally, the regulation of Rho-GTPases (particularly Rac, Rho and Cdc42) affects cellular key processes, but most importantly the dynamic of the actin cytoskeleton.
For detailed information on integrin signaling the reader is referred to several reviews (Giancotti and Ruoslahti, 1999; Guo and Giancotti, 2004; Juliano, 2002; Schwartz and Ginsberg, 2002).

1.5. Integrin-actin connections

1.5.1. Anatomy and complexity of integrin adhesions

Adhesions with the ECM are formed by all types of adherent cells, but differ in morphology, cellular localization and molecular composition. They are very dynamic structures that bring the cell membrane in close contact with the substrate and build around ligand-activated integrins. At present, focal complexes (FCs), focal adhesions (FAs), fibrillar adhesions and podosomes are known as classical matrix adhesion structures. FCs are small dot-like adhesion sites that are mainly found at the edges of cellular protrusions. FCs are highly dynamic, often transient and upon maturation transform into FAs. Mature FAs are less dynamic, have an elongated shape and are often found in the periphery of cells. Through a complex of various accessory proteins, they anchor actin stress fibers to the cell membrane. Fibrillar adhesions are found in the central parts of cultured cells, are alined with extracellular fibronectin fibrils and contain $\alpha 5\beta 1$ and tensin. Podosomes are the typical adhesion structures of osteoclasts and hematopoetic cells and are composed of an actin core surrounded by a cylindrical matrix adhesion structure (Berrier and Yamada, 2007; Geiger et al., 2001; Wiesner et al., 2005).

While their morphology seems well defined, the molecular composition of matrix adhesions is more complex. Over 50 proteins are transiently or stably found in matrix adhesions and many can affect these structures without being directly associated with them. It is known that their molecular composition differs among the types of adhesions. In addition, their degree of complexity is likely to be even higher due to several reasons (Berrier and Yamada, 2007; Wiesner et al., 2005; Zamir and Geiger, 2001a; Zamir and Geiger, 2001b). First, the intracellular pool of available FA components differs between cell types and tissues. Second, numerous modular proteins with several binding sites are found in matrix adhesions allowing a multitude of possibilities for protein-protein interactions. Finally, simultaneous association of several

partners is often excluded due to overlapping binding sites or allosteric modulation of binding site exposure.

Current models believe that different integrin interactors and FA components may be present within one large adhesion site or that different proteins may bind consecutively (Geiger et al., 2001). The molecular and functional differences among integrin adhesions will certainly still provide intriguing areas of profound research in the future.

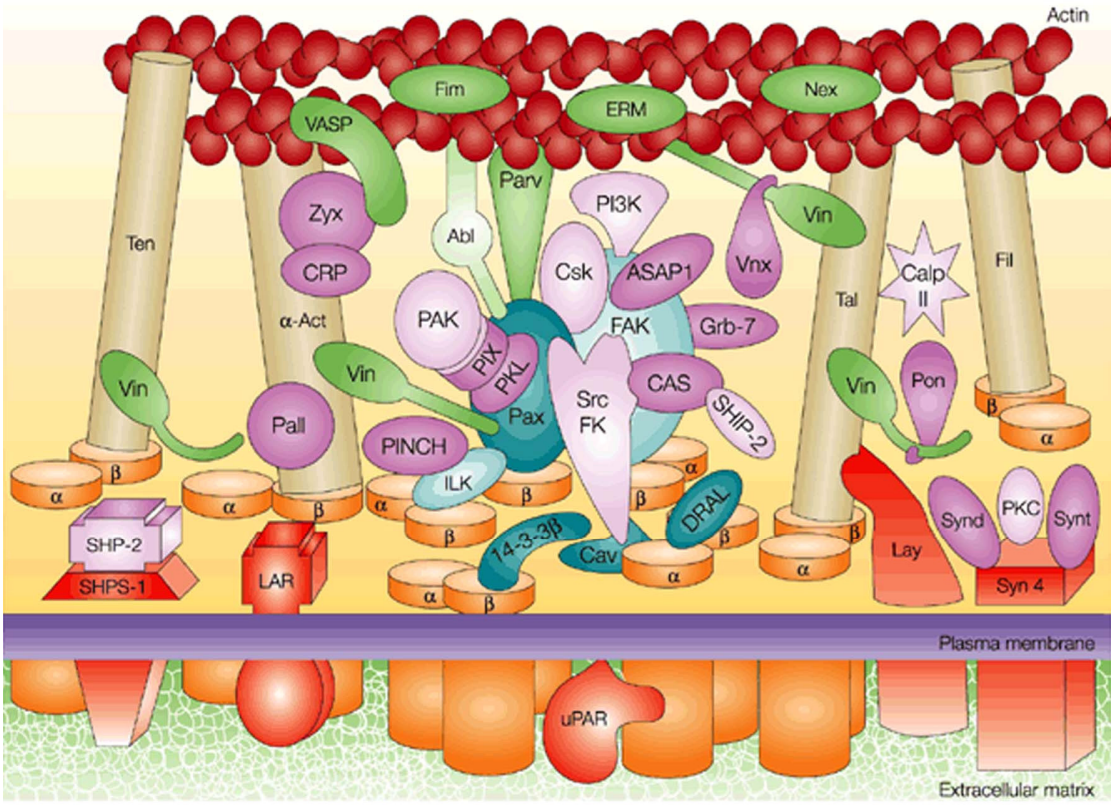

Figure 3: Molecular complexity of cell-matrix adhesion

Various proteins can localize to integrin-adhesion sites. Proteins that directly bind integrin and actin are depicted as golden rods, other direct integrin binding patners are shown in blue. Actin associated proteins are presented in green. Additional signaling molecules and adaptor proteins are also found (drawn in violet) (Cartoon is taken from Geiger et al., 2001).

Given the high degree of complexity within the field of matrix adhesion it is important to emphasize that the following paragraphs of actin binding and regulation cannot claim to be exhaustive and universally valid but demonstrate the current principles for ECM–cytoskeletal connections.

1.5.2. Actin binding via integrins

The major structural function of matrix adhesions is to mediate the connection to the actin cytoskeleton. This can be achieved by a variety of integrin-binding proteins that bind actin either directly or indirectly.

Direct actin binding

Talin1,2 form antiparallel homodimers of two 270 kDa subunits that play a key role in integrin activation. Integrin β subunit binding is mainly mediated by the FERM domain in the head region of the molecule whereas the rod-shaped tail contains two binding sites for F-actin and G-actin. Talin binds and activates vinculin that additionally promotes and stabilizes F-actin crosslinks (Critchley, 2005).

α-actinins are a family consisiting of four 100 kDa proteins that form homodimers and bind to β integrins and actin. FAK dependent phosphorylation decreases its association with actin *in vitro* (Otey and Carpen, 2004).

Tensin is a 220 kDa protein that contains three actin binding domains. Binding to the integrin β1 subunit is only weak, but it strongly binds to β3, β5 and β7. A Src Homology 2 (Sh2) domain in the C-terminus mediates binding to other tyrosine-phosphorylated adhesion plaque proteins such as FAK or p130Cas (Lo, 2004). Recently three additional family members have been identified (Lo, 2006).

Filamins, a family of three members, are 280 kDa modular and dimeric proteins that crosslink F-actin. In addition to integrins filamins associate with other receptors and numerous actin regulatory proteins (Popowicz et al., 2006).

Indirect actin binding

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that binds via its N-terminal FERM domain to integrin cytoplasmic tails *in vitro*. Although FAK is one of the most prominent adhesion plaque proteins, the *in vivo* relevance of the direct integrin binding is still unclear. Via its FA targeting (FAT) sequence it can bind to talin and paxillin and thereby link to and regulate F-actin (Mitra et al., 2005).

Integrin-linked kinase (ILK), the 52 kDa putative non-receptor serine/threonine kinase is composed of four N-terminal ankyrin repeats, a pleckstrin-homology domain and a C-terminal kinase domain that mediates the binding to integrin β1 and β3 subunits. ILK connects to the actin cytoskeleton via binding to the parvins, paxillin or kindlins. ILK and its binding partners as well as their functional relevance for actin regulation and signaling will be presented in the following chapter.

The **kindlin** family of 3 three proteins binds to integrin β1 and β3 and regulates the actin matrix adhesions and actin anchorage via ILK, migfilin and vinculin (Montanez, Ussar, Moser and Fässler, unpublished observation).

Paxillin binds with high affinity to the α4 integrin subunit and also the β1 integrin. Its direct interaction partners vinculin and α-parvin bind to actin. Paxillin also binds ILK and FAK and seems to be one of the earliest proteins – together with talin - recruited to nascent adhesion sites (Turner, 2000).

In addition there are actin binding proteins known to be associated with integrin adhesion sites that are not closely connected to integrins. Many of them are multi-domain proteins wich mediate the coordinated recruitment of actin binding and regulatory proteins as well as signaling molecules (Brakebusch and Fässler, 2003; Geiger et al., 2001; Lo, 2006; Wiesner et al., 2005).

1.5.3. Actin regulation via integrins

Many of the actin-binding proteins not only bind, but are also involved in the regulation of the actin turnover. Indeed the integrin-actin connection is highly

dynamic and subject to many regulatory processes. Rapid actin turnover is required for the dynamic processes during cell motility.

The Arp2/3 complex is the key protein in actin network assembly whose actin-nucleating activity depends on the presence of activators. The best characterized Arp2/3 activator family is the WASp/Scar family (Mullins et al., 1998). Current models describe N-WASp as the main Arp2/3 activator in filopodia and WAVE2 as the predominant activator in lamellipodia even though this is known to be a simplified concept (Vartiainen and Machesky, 2004; Wiesner et al., 2005).

Integrins recruit or activate various activators of the Arp2/3 complex. WASp and N-WASp can be phosphorylated by Src-family kinases and FAK has recently been reported to bind and phosporylate N-WASp. Alternatively, N-WASp is recruited to integrin adhesions through the adaptor proteins Nck2 and PINCH ('particularly interesting new cysteine-histidine-rich protein')-ILK complex (Tu et al., 1998) or vinculin (DeMali et al., 2002). Cortactin, present in matrix adhesions such as podosomes, directly binds and activates Arp2/3 (Uruno et al., 2001).

The global control of the actin cytoskeleton through the concerted action of Rho-family GTPases has become a universal paradigm (Etienne-Manneville and Hall, 2002). Therefore, the regulation of Rho-GTPases or Rho-GTPase effectors – guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs) – by matrix adhesion components represents an important axis of integrin-actin signaling. However, Eps8 is the only GEF identified so far that is recruited to matrix adhesions via direct integrin interaction (Calderwood et al., 2003). Many GEFs are recruited to integrin adhesion sites via binding to other adhesion plaque proteins. The Rac-GEF Dock180 can be recruited through Crk and p130Cas by activated FAK or through Nck2 to the PINCH-ILK complex (Hsia et al., 2003; Tu et al., 2001). Paxillin can recruit the Rac/Cdc42 GEF β-PIX whereas β-parvin binds to α-PIX (Rosenberger et al., 2003; Turner et al., 1999).

Negative or positive regulation of Rho activity occurs though p190RhoGAP recruited by Src (Arthur et al., 2000) or p190RhoGEF through FAK (Zhai et al., 2003).
The recruitment of GEFs to matrix adhesions via polyphosphoinositides (PIPs) represents another mechanism of regulating GEF activity (Schmidt and Hall, 2002).
Recently it became evident that not only the activation *per se* but also localized targeting of active GTPases Rac or Rho is required for the intracellular spatial regulation of actin dynamics (Del Pozo et al., 2004; Del Pozo et al., 2002; Watanabe et al., 1999).

ILK, PINCH and Parvin

Integrin-associated proteins are required for regulating intracellular key functions of integrins. Among them, three proteins have emerged as important regulators of integrin-mediated functions, namely ILK, PINCH and parvin.

1.6. ILK

ILK was first identified in 1996 in a yeast two-hybrid screen for proteins that could bind to the cytoplasmic tail of β1 integrin (Hannigan et al., 1996). The 52 kDa protein was named based on the described kinase activity towards the integrin β1 integrin cytoplasmic tail. Subsequently, ILK binding to β3 integrin was also shown (Pasquet et al., 2002; Yamaji et al., 2002). The domain structure of ILK reveals the presence of three N-terminal ankyrin repeats and an additional putative ankyrin module, followed by a pleckstrin-homology (PH) – like domain and a putative serine/threonine kinase domain located at the C-terminus.
Cell biological studies revealed that changes in ILK protein levels are crucially affecting both cell morphology and function. Most strikingly ILK could be implicated in the regulation of cell spreading, cell-ECM adhesion, ECM

assembly, cell proliferation and cell-cell adhesion (Novak et al., 1998; Radeva et al., 1997; Sakai et al., 2003; Vespa et al., 2005; Wu et al., 1998).

Many of these cellular functions are integrin- or actin-dependent processes and indeed, ILK interaction partners implement several actin regulatory functions.

PINCH binds to ILK via its first ankyrin repeat and links to important actin regulators, such as DOCK180 and PAK via binding to Nck2 (Tu et al., 1999; Tu et al., 1998). MIG2/kindlin-2 is binding to ILK via its kinase domain. MIG2/kindlin-2 in turn binds via migfilin and filamin to actin (Tu et al., 2003) and Monatez et al., submitted). Also the parvins, a family of actin-binding proteins, bind the kinase domain of ILK (Nikolopoulos and Turner, 2000; Yamaji et al., 2001). β-parvin was shown to interact with the GEF α-PIX, which may activate Rac1 and Cdc42 (Rosenberger et al., 2003). Finally, the ILK kinase domain binding to paxillin recruits further actin binding proteins (Nikolopoulos and Turner, 2001) (Figure 4).

The requirement for ILK in the regulation of the integrin-actin connection has been further validated *in vivo*. In *C. elegans* loss of pat-4/ILK leads to severe adhesion defects with muscle detachment and embryonic lethality (Mackinnon et al., 2002). In *D. melanogaster* loss of ILK leads to muscle detachment due to a detachment of F-actin from the cell membrane containing βPS integrin (Zervas et al., 2001). Constitutive ablation of ILK in mice leads to peri-implantation lethality around E5.5, as early as the loss of β1 integrin. Studies in embryoid bodies, mimicking early embryogenesis, revealed defects in polarity of the epiblast leading to abnormal actin distribution (Sakai et al., 2003).

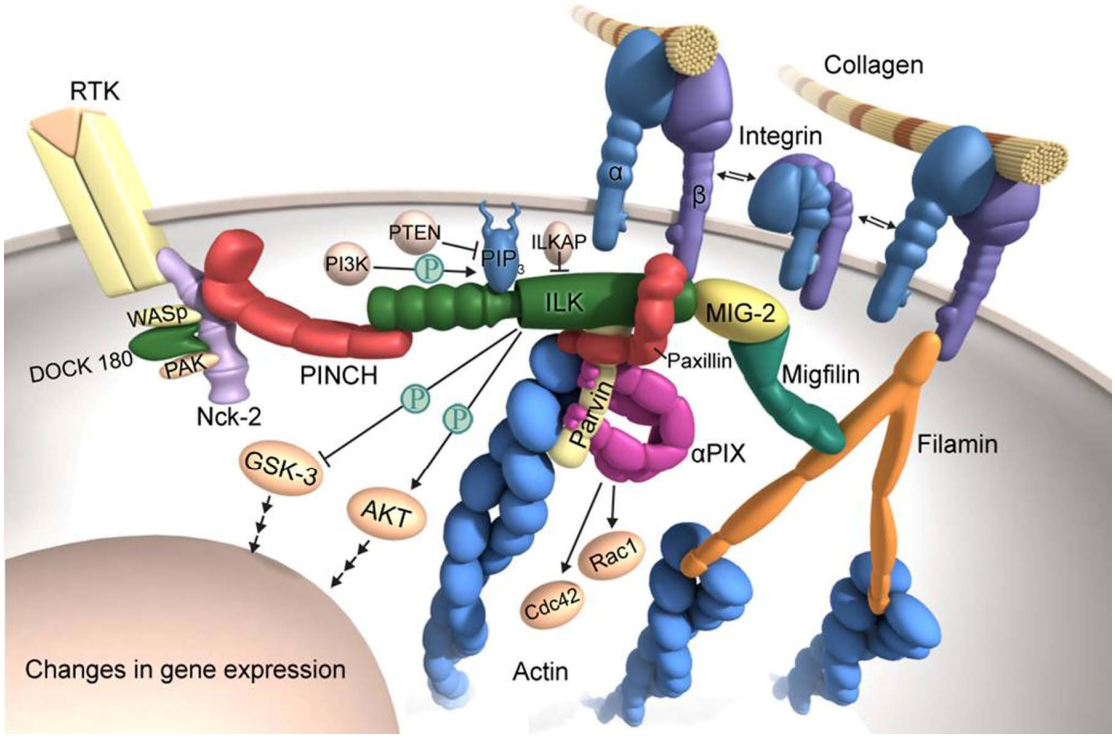

Figure 4: ILK-PINCH-Parvin complex in FAs

ILK interacts with several proteins that link to the actin cytoskeleton. ILK-PINCH-Parvin form the heterotrimeric IPP complex. PINCH also regulates the actin cytoskeleton via additional adaptors and links to RTK signaling. Newly identified PINCH interactors such as Thymosin β4 and RSU-1 are not depicted (Cartoon taken from Grashoff et al., 2004).

In addition to the regulation of the integrin-actin connection, ILK is involved in downstream signaling events via its kinase function. Although ILK C-terminus shows important homology to canonical serine/threonine kinases, it lacks conserved residues within the catalytic kinase domain responsible for ATP- and peptide binding and phosphotransfer (Hannigan et al., 1996).

Nonetheless, both immunoprecipitated and recombinant ILK has been shown to phosphorylate several substrates *in vitro*, such as Akt/PKB and GSK3β (Delcommenne et al., 1998; Persad et al., 2001) and others (Table 1; (Legate et al., 2006)). In many cell types overexpression of ILK also leads to increased Akt/PKB and GSK3β phosphorylation, while expression of a dominant-negative mutant of ILK - E359K - reduces the phosphorylation (Delcommenne et al., 1998; Novak et al., 1998). However, other studies did

not detect defects in kinase activity upon this mutation. Instead the interaction with paxillin and α-parvin was disrupted, affecting ILK recruitment to FAs (Nikolopoulos and Turner, 2002; Yamaji et al., 2001). In effect, changes in the ILK protein-protein interactions were observed for the majority of ILK mutants studied so far (Attwell et al., 2003; Filipenko et al., 2005; Nikolopoulos and Turner, 2002; Persad et al., 2001).

In vivo, the evidence for ILK kinase activity is similarly controversial. In both *C. elegans* and *D. melanogaster* dominant negative ILK mutants are able to fully rescue the loss of ILK (Mackinnon et al., 2002; Zervas et al., 2001). In mice conditional ablation of *ILK* does not impair PKB/Akt and GSK3β phosphorylation in many cell types such as chondrocytes, fibroblasts, hepatocytes and keratinocytes (Gkretsi et al., 2007; Grashoff et al., 2003; Lorenz et al., 2007; Sakai et al., 2003; Terpstra et al., 2003). However, in many other cell types such as macrophages, endothelial cells, neurons or leukocytes Akt/PKB or GSK3β signaling was altered (Friedrich et al., 2004; Friedrich et al., 2002; Gary et al., 2003; Troussard et al., 2003).

These discrepancies might reflect cell-type-specific differences in the requirement for ILK in Akt/PKB or GSK3β activation or suggest the possibility that ILK might affect Akt/PKB signaling rather indirectly.

The reader is referred to the first publication presented in this PhD thesis and further excellent reviews for additional information about the recent concepts concerning the function of this exciting molecule (Grashoff et al., 2004; Legate et al., 2006)

1.7. PINCH

PINCH1 was initially described as a marker for senescent erythrocytes (Rearden, 1994). A second isoform, PINCH2, was subsequently identified in vertebrates (Braun et al., 2003; Zhang et al., 2002a), while invertebrates such as *C. elegans* or *D. melanogaster* possess only one PINCH orthologue (Clark et al., 2003; Hobert et al., 1999). The 37kDa PINCH proteins are composed of five tandemly repeated LIM domains, each composed of two cysteine-rich zinc-fingers that mediate protein-protein interactions, and a short C-terminal

tail that comprises a nuclear localization sequence. PINCH1,2 both bind to ILK via the first LIM domain in a mutually exclusive manner (Tu et al., 1999; Zhang et al., 2002a).

In vitro studies showed that PINCH1 – together with ILK – is required for actin-dependent processes such as spreading and migration and that PINCH2 can compensate for the loss of PINCH1 (Fukuda et al., 2003; Stanchi et al., 2005). Depletion of PINCH1 also triggered apoptosis via impairing Akt phosphorylation on Ser473 and Thr 308, both required for full Akt activation. Loss of ILK only affected Ser473 phosphorylation (Fukuda et al., 2003). The molecular mechanism of this regulation remains unclear.

In addition to ILK, PINCH1 has been shown to bind Nck2, a SH3- and SH2-containing adaptor protein that binds to the fourth LIM domain on PINCH1. Nck2 interacts with growth factor receptors and key components of small GTPase signaling (Tu et al., 1998). Nck2 regulates actin dynamics through several pathways: via the WASp family members and the Arp2/3 complex and via small GTPases and PAK or DOCK180 (Buday et al., 2002). The disruption of the PINCH1-Nck2 interaction severly affects cell spreading, emphasizing its importance for actin-dynamic processes (Vaynberg et al., 2005; Velyvis et al., 2003). However, it is currently unclear whether the PINCH1-Nck2 interaction is of any relevance *in vivo*, as mice with a genetic deletion for either Nck1 or Nck2 are phenotypically normal, suggesting their functional compensation. PINCH1, however, has been demonstrated not to bind Nck1 (Tu et al., 1998).

An additional link to the actin cytoskeleton stems from the interaction of PINCH with Thymosin β4, a small peptide sequestering G-actin monomers known to regulate actin-driven cellular processes (Bock-Marquette et al., 2004; Sun and Yin, 2007). Furthermore, PINCH1 has been shown to interact with Ras-suppressor protein RSU-1 in both vertebrates (Dougherty et al., 2005) and *D. melanogaster* (Kadrmas et al., 2004) which negatively regulates the JNK activity (Figure 4).

Several studies *in vivo* and *in vitro* have also revealed nuclear localization of PINCH (Hobert et al., 1999; Li et al., 2005; Zhang et al., 2002a). Although other LIM domain proteins have been shown to shuttle between adhesion

sites and the nucleus (Hervy et al., 2006), no nuclear function has so far been attributed to PINCH.

Genetic studies in *C. elegans* revealed that Unc-97/PINCH colocalizes with integrins and is required for the integrity of integrin attachment sites, therefore being essential for development (Hobert et al., 1999; Norman et al., 2007). Similar requirements of PINCH for integrin-dependent processes such as cell attachment and actin organization were also shown in the *D. melanogaster* (Clark et al., 2003). In the adult mouse, PINCH1 and PINCH2 are widely (co)expressed in a large number of tissues, whereas during early embryonic development only PINCH1 is expressed (Braun et al., 2003). PINCH2 deficient mice do not show any overt phenotype, but the upregulation of PINCH1 in tissues with most prominent PINCH2 expression, suggests the possibility of functional compensation of the two isoforms *in vivo* (Stanchi et al., 2005). Genetic ablation of PINCH1 in mice, however, leads to early embryonic lethality during implantation. Analysis of embryoid bodies, which model peri-impantation, revealed defects in endodermal and epiblast adhesion to an abnormal basement membrane (BM) and in addition abnormal cell-cell adhesion and increased apoptosis (Li et al., 2005; Liang et al., 2005) which were not observed upon ablation of β1 integrin and ILK in the same system.

1.8. ILK-PINCH-Parvin complex

The heterotrimeric ILK-PINCH-Parvin (IPP) complex is formed through simultaneous binding of ILK to PINCH and parvin family members (Figure 4). Assembly of the IPP complex occurs in the cytoplasm prior to the recruitment into FAs (Fukuda et al., 2003; Zhang et al., 2002b) and complex formation is critical for stability and recruitment of each individual IPP member into FAs.

In vitro studies showed that downregulation of one member results in a concomitant degradation of the other components. The reduction occurs on protein level and can be rescued by inhibition of the proteasome (Fukuda et al., 2003). However, the expression of PINCH1 LIM1-domain in PINCH2-deficient cells leads to a stabilization of ILK levels (Stanchi et al., 2005) or

expression of the N-terminal ankyrin-repeat domain of ILK in ILK-deficient cells rescues PINCH (Grashoff and Fässler, unpublished data).
Nevertheless, localization of the IPP complex to FAs critically depends on the presence of the full-length proteins within the complex, indicating that additional IPP interactions are required for FA targeting. Those could potentially include binding to integrins, Paxillin or Mig-2/kindlin-2, as mutation of the Paxillin binding site of ILK leads to IPP complex displacement from FAs in cells (Nikolopoulos and Turner, 2001) and in *C. elegans* the Mig-2/Kindlin-2 orthologue UNC-112 is essential for ILK localization to integrin adhesion sites (Mackinnon et al., 2002).
The significance of the intact IPP complex for cell-matrix adhesion and actin organization is well established, while it is still unclear whether IPP complex members can exert IPP-independent functions. Degradation of the IPP components upon deletion of one member is not always complete (Fukuda et al., 2003; Li et al., 2005), opening up the possibility for functions of individual IPP members outside of the complex.
PINCH1 might have a role in the formation or stabilization of cell-cell adhesions independently from ILK as suggested by studies in embroid bodies (Li et al., 2005; Sakai et al., 2003). ILK however was also detected in cell-cell contacts in keratinocytes where it contributed to cell-adherens junction formation (Vespa et al., 2005; Vespa et al., 2003). Nuclear shuttling is described for PINCH1 in several systems (Campana et al., 2003; Hobert et al., 1999; Li et al., 2005), but recently also ILK was shown to translocate to the nucleus in a phosphorylation-dependent manner (Acconcia et al., 2007).
Despite first hints towards seperate functions of ILK, PINCH and parvins, the evidences are still puzzling and will require further investigations in the upcoming years.

Skin

The skin is a multilayered organ which covers the outer surface of the mammalian body. It is composed of an epithelial compartment, the epidermis,

and a mesenchymal compartment, the dermis which are separated by a BM and the adipose subcutis underneath. Appendages such as hair follicles (HFs) and sweat glands are interspersed in the epithelium.
The skin provides a barrier which prevents loss of body fluids from the inside and protects against infectious agents, temperature changes, and trauma or substance uptake from the outside. The epidermal potential for continuous self-renewal and wound repair following injury due to the presence of stem cells is crucial for the maintenance of its vital functions.

1.9. Epidermal architecture and homeostasis

The epidermis is a stratified squamous epithelium that is composed of several layers of keratinocytes. Only the basal layer that is in direct contact with the underlying BM contains proliferating cells. Basal keratinocytes express integrins and can be distinguished through the specific expression of keratin 5/14 (K5/14). During stratification cells leave the basal layer and move towards suprabasal locations, where they withdraw from cell cycle, switch off integrin expression and enter a specific differentiation programme. This process gives rise to spinous and granular layers and culminates with the production of terminally differentiated dead corneocytes of the stratum corneum that will eventually be shed from the epidermal surface. Structural properties of suprabasal keratinocytes depend to a large extend on keratins therefore keratinocytes also alter the pattern of keratin expression during differentiation. K1/10 are characteristic for the intermediate spinous layer, whereas granular layer and the outermost stratum corneum can be distinguished by the expression of loricrin and filaggrin, respectively (Figure 5) (Blanpain and Fuchs, 2006; Fuchs and Raghavan, 2002).
Interestingly, it is still unclear how the formation of a multilayered epithelium – stratification – is really achieved. Experimental evidence from *in vitro* studies suggests a process referred to as delamination that is effectively leading to the formation of a multilayered epithelium. Basal keratinocytes weaken their adhesion to the BM and are pushed off into the spinous layers by their

neighboring cells in a process that likely requires apical actin dynamics (Vaezi et al., 2002; Watt and Green, 1982).

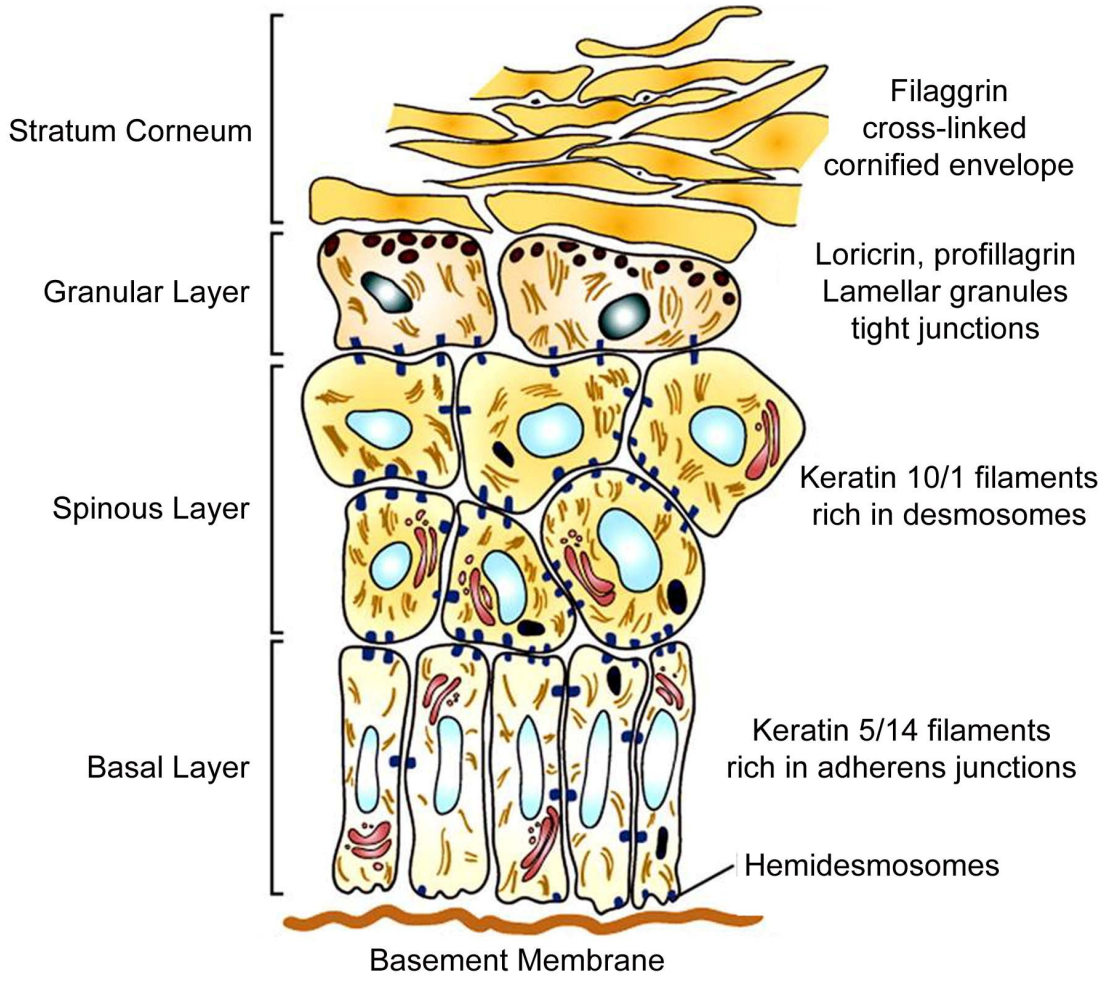

Figure 5: Architecture of the stratified epidermis

The program of epidermal differentiation is shown in this schematic, illustrating the BM at the base, the proliferative basal layer, and the three differentiation stages: spinous layer, granular layer, and outermost stratum corneum. At the right key markers for differentiation are indicated (Cartoon modified from Fuchs, 2008).

Alternatively, mitotic spindle orientation perpendicular to the BM during basal cell division might directly place one of the two daughter cells into suprabasal position. During stratification of murine skin the majority of cell divisions was shown to be asymmetric with a spindle orientation perpendicular to the BM and this preferential spindle orientation was dependent on the presence of β1

integrin (Lechler and Fuchs, 2005). More recently however, this model has been challenged by a report showing that only three percent of spindles lie perpendicular to the basal layer in mouse postnatal epidermis (Clayton et al., 2007). Therefore, more studies are now needed to determine the importance of cell division and/or delamination during epidermal stratification (Figure 6A).

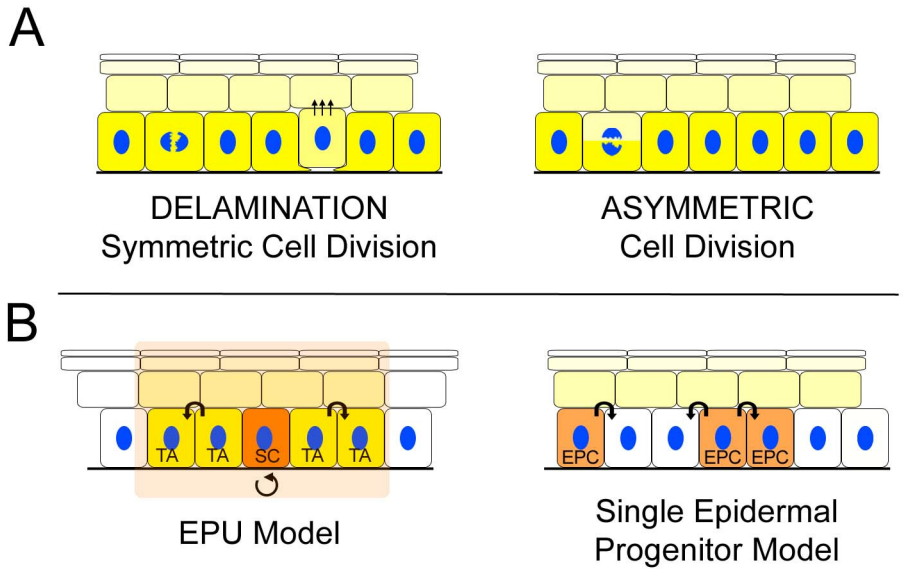

Figure 6: Models of epidermal stratification and self-renewal

(A): During delamination basal cells weaken the adhesion to the BM upon commitment to suprabasal cell fate. Commited cells are pushed towards spinous layers. Asymmetric cell division of basal cells leads to the direct localization of a commited daugther cell in suprabasal position. (B) The epidermis is composed of distinct epidermal proliferative units (EPUs) with each one stem cell per unit. Transit-amplifying (TA) cells divide up to 4-5 times. In the single progenitor model the epidermis is maintained by one type of epidermal progenitors (EPCs) that may undergo an unlimited number of divisions.

Constant epidermal regeneration capacity is ensured by the presence of epidermal stem cells. According to the classical epidermal proliferative unit (EPU) model the basal layer of keratinocytes contains stem cells, that are very slowly cycling, transit-amplifying cells that are able to undergo a limited number of cell divisions and post-mitotic basal cells. Each EPU, one descrete epidermal segment, is maintained by a single stem cell and is constant in size (Potten, 1981). However, also the presence of two types of epidermal progenitor cells has lately been questioned by a single-type progenitor model

for clonal populations of the epidermis (Clayton et al., 2007) (Figure 6B). This debate has currently led to live discussions (Fuchs, 2008; Jones et al., 2007), which will set the stage for upcoming investigations. High expression of β1 integrin is speculated to be a hallmark of human epidermal stem cells (Jones et al., 1995), however this finding has not been confirmed ever since for murine epidermis and it is unclear how β1 integrin would determine the properties of stem cells in the epidermis.

1.10. HF morphogenesis and cycling

The development of HFs from the fetal epidermis during morphogenesis involves a tigthly controlled signaling exchange with the underlying mesenchyme (Blanpain and Fuchs, 2006; Hardy, 1992; Paus et al., 1999; Schmidt-Ullrich and Paus, 2005). Following inductive signals the first morphological signs of HF formation become evident at embryonic day (E) 14 when epidermal keratinocytes get organized into an easily recognizable *hair placode* accompanied by the condensation of specific fibroblasts in the underlying mesenchyme (Figure 7).

During epithelial downward extension, the keratinocytes enwrap the dermal condensate, so-called *dermal papilla* (DP) at their base. Inductive signals from the DP maintain a high proliferation of the adjacent *hair matrix* (HM) keratinocytes. During later stages of morphogenesis, HM daughter cells move upwards the HF and differentiate into the six cylindric layers of *inner root sheath* (IRS) and *hair shaft* (HS) layers. The outer layer of the follicle is then called *outer root sheath* (ORS) which is continuous with the interfollicular epidermis, expresses integrins and K5/14 and is surrounded by a BM.

HF morphogenesis lasts until postpartum and occurs in asynchronous manner. By postnatal day (P) 3 approximately 30 % of the HFs have morphologically completed morphogenesis, downgrowth however still continues (Paus et al., 1999). By P14 both downgrowth and differentiation are completed. Throughout postnatal life HF growth is cyclic.

By the onset of the regression phase – *catagen* – keratinocyte proliferation in the HF rapidly decreases and the lower HF portion degenerates through

apoptosis. The DP is dragged towards the permanent portion of the HF through an epithelial strand surrounded by the retracting BM.

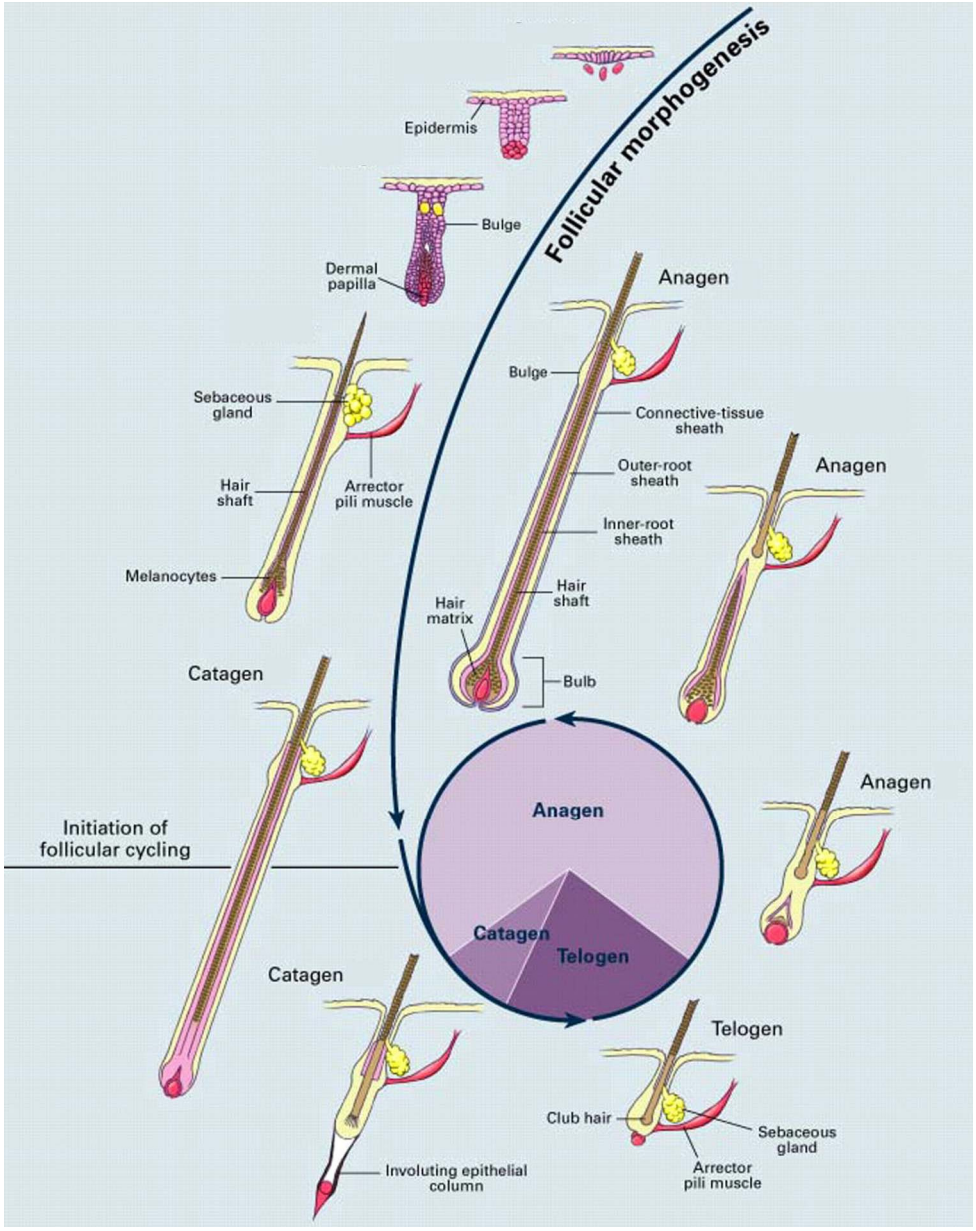

Figure 7: Development and cycling of HFs

Morphology of HFs during selected stages of HF morphogenesis and cycling (Cartoon modified from Paus and Cotsarelis, 1999).

The quiescent phase is referred to as *telogen*. Each new hair cycle begins during *anagen* with the induction of a proliferative hair germ at the bottom of the HF in response to signals from the DP and subsequent progression to a

mature HF involving downgrowth and differentiation (Figure 7) (Muller-Rover et al., 2001; Paus and Cotsarelis, 1999). In contrast to human HFs, the murine hair coat is particular in undergoing synchronized hair cycling during the first postnatal cycles.

The *bulge*, a specific region within the proximal ORS just below the sebaceous gland, is the natural niche for HF stem cells and represents the permanent portion of the HF during cycling. During growth periods transit-amplifying cells constantly migrate from the *bulge* along the ORS to maintain the proliferative cell pool of the HM.

The molecular mechanisms underlying HF development and cycling are still poorly understood, but genetic studies in mice reveal the importance of signaling pathways involving Wnts, bone morphogenic proteins (Bmps), sonic hedgehoc (Shh), fibroblast growth factor (FGF), epidermal growth factor (EGF), NFκB and Notch signaling (reviewed in Blanpain and Fuchs, 2006 and Schmidt-Ullrich and Paus, 2005).

1.11. The role of integrins in the epidermis and HFs

Integrins are the main receptors for cell-BM attachment *in vivo*, therefore several integrins are found to be expressed in and to be of essential importance for the function of keratinocytes.

1.11.1. Integrin expression in skin epithelium

Integrins are predominantly found in basal and ORS keratinocytes that are in direct contact with a BM. Constitutively expressed integrins are $\alpha 2\beta 1$ integrin (collagen receptor), $\alpha 3\beta 1$ (laminin receptor), $\alpha 9\beta 1$ (tenascin receptor), $\alpha 6\beta 4$ (laminin receptor) and to lower amounts $\alpha V\beta 5$ (vitronectin receptor). Certain integrins, mainly fibronectin receptors (α5β1, αVβ6 and α9β1), are expressed or upregulated during pathological conditions such as skin wounding or tumor development (Watt, 2002).

In undamaged skin $\alpha 6\beta 4$ is concentrated at the BM zone and is the core component of hemidesmosomes anchoring the keratin filaments to the BM.

α3β1 integrin might be involved in hemidesmosome nucleation at the BM zone (Litjens et al., 2006). The role of α2β1 integrin *in vivo* is still unclear, even though it is expressed at very abundant levels in the epidermis. α2β1 integrin localizes both to the baso-lateral and apical surfaces and is required for keratinocyte adhesion to type 1 collagen *in vitro* (Zhang et al., 2006). Interestingly, the expression of α2β1, α3β1 and α6β4 integrins along the ORS varries in distinct subdomains of the HF (Commo and Bernard, 1997).

1.11.2. Analysis of integrin function in epidermal and HF biology

The importance of integrins and their ligands for epidermal and HF integrity *in vivo* is most directly demonstrated by the generation of integrin knock-out mice.

In order to circumvent embryonic or early postnatal lethality as observed in several constitutive integrin knock-out mice, such as β1 or β4 integrin (see Table 1) different genetic and technical tools have been employed to study integrin function in the skin epithelium *in vivo*. The Cre-loxP system has been commonly used to target gene deletion specifically to keratinocytes. The promoters from the K14 or K5 genes direct Cre recombinase expression to basal keratinocytes, sebaceous gland and ORS cells and thereby also to all differentiated keratinocytes. Endogenous K5 and K14 are known to be expressed starting around E9.5 during embryogenesis when epidermal and HF morphogenesis begins (Byrne et al., 1994). However, it is of importance to consider that different mouse lines expressing the Cre recombinase under exogenous K5- or K14-promotors only show effective Cre activity at slightly later stages and might delete target genes with varying efficiencies or kinetics influencing the observed phenotypes. This has been the case for the keratinocyte-specific deletion of β1 integrin (Brakebusch et al., 2000; Raghavan et al., 2000). Additional possibilities for gene function analysis during adulthood are given through the availability of inducible Cre-expressing mouse lines or grafting methods using immuno-compromised mice (Conti et al., 2003; Lopez-Rovira et al., 2005).

Constitutive deletion of either α6 or β4 integrin in mice leads to neonatal lethality due to severe detachment of the epidermis and other sqamous epithelia through absence of hemidesmosomes, reminiscent of the human disorder epidermolysis bullosa (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). Mice carrying a constitutive α3-integrin null mutation die also shortly after birth (Kreidberg et al., 1996) due to kidney and/or lung failure. Skin analysis at birth revealed milder blistering at the dermal-epidermal junction due to BM disruption, suggesting a role for α3β1 in the establishment of BM integrity (DiPersio et al., 1997). Epidermal morphogenesis *per se* was, however, not altered in mice lacking α3 and α6 integrins (DiPersio et al., 2000). Grafting experiments using α3 integrin-deficient epidermis revealed stunted HF growth and differentiation accompagnied by an abnormal actin cytoskeleton in HF keratinocytes upon lack of α3 integrin (Conti et al., 2003).

1.11.3. β1 integrin function in epidermis and HF

Conditional ablation of β1 integrin in keratinocytes leads to early lethality either shortly after birth due to severe blistering and dehydration when K14-Cre is used (Raghavan et al., 2000) or later due to impaired food intake and developmental retardation following K5-Cre mediated deletion (Brakebusch et al., 2000).

In the interfollicular epidermis β1 integrin was responsible for hemidesmosome formation and BM integrity, leading to epidermal detachment and reduced expression of α6 and β4 integrins in mutant keratinocytes. Terminal differentiation of epidermal keratinocytes was not affected, suggesting that integrin downregulation is not the central molecular switch for initiation of differentiation. However, in postnatally surviving mice the epidermis became hyperplastic even though keratinocyte proliferation was decreased. Keratinocyte migration both *in vitro* and upon wounding *in vivo* was impaired.

Most strikingly the loss of β1 caused progressive hair loss in mice leading to an almost complete alopecia by an age of four weeks. The reduced number of hairs was caused by severe HF malformations ranging from premature growth arrest during morphogenesis to hyperthickened ORS and severe distortions accompanied by a reduced HM proliferation. Concomitant with the loss of hair, infiltrating macrophages accumulated around abnormal HFs, pro-inflammatory cytokines became upregulated and the mice developed a dermal fibrosis (Brakebusch et al., 2000; Grose et al., 2002; Lopez-Rovira et al., 2005; Raghavan et al., 2000; Watt, 2002). Recent data showed that LM511 (laminin-10) whose major receptor in keratinocytes is α3β1 is particularly enriched in the specialized BM around elongating hair germs and that its deletion impaired HF development.

Altogether these data suggest that integrins and their ligands play crucial roles in skin epithelium that reach far beyond simply anchoring keratinocytes to the underlying BM. Particularly β1 integrins are required for keratinocyte migration, HF development and BM integrity. They maintain the balance between proliferation and differentiation.

Aim of the thesis

The adhesion receptor family of β1 integrins is essential for embryonic development, as embryos lacking the β1 integrin die during implantation. The use of tissue-specific gene deletion strategies in mice showed that this integrin family is of crucial importance for BM integrity, cell adhesion, migration, proliferation and differentiation in several tissues, such as skin, cartilage or muscle *in vivo*. Nonetheless, an important and still largely unanswered question is how integrins mediate these functions. Numerous integrin adaptor molecules have been identified but the precise function of each especially *in vivo* remains to be clarified.

During homeostasis the skin epithelium and its appendages require the controlled deposition of, and adhesion to BM molecules, tightly regulated programmes of differentiation, maintenance of stem cells, migratory processes and sustained proliferation. All above mentioned processes strongly depend on β1 integrin signaling via integrin-associated molecules.

AIM1

Analysis of the functional relevance of the integrin-adaptor molecule ILK for β1 integrin-dependent processes in skin epithelium via gene targeting in keratinocytes.

ILK forms a heterotrimeric IPP complex with PINCH and parvin family members and this complex formation is required for protein stability. Downregulation of one member concomitantly triggers proteasomal degradation of the others. However, it is unclear whether also IPP-independent functions might exist for each of the binding partners.

AIM2

Analysis of PINCH1 functions in keratinocytes by conditional gene targeting in keratinocytes to gain insight into shared and independent functions of ILK and PINCH1.

Short summaries of publications

Publication 1: Integrin-linked kinase: integrin's mysterious partner

This review article summarizes the present knowledge about the functions of ILK, a direct interaction partner of β1 and β3 integrins. We address cell biological properties as well as ILK functions *in vivo* with a particular focus on the present controversy regarding its postulated kinase activity.

ILK is one member of the intracellular cytoplasmic plaque recruited to integrin adhesion sites which is present as a ternary complex together with PINCH and parvin family members. ILK-PINCH-parvin- (IPP-) complex formation is vital for the protein stability of each complex member and necessary for focal adhesion (FA) targeting through possible candidates such as integrins, paxillin and kindlins. One core function of the IPP-complex is to provide a binding platform for actin regulatory proteins. Thereby ILK is implicated in cell spreading, FA formation, cell adhesion and ECM assembly. On the other hand, ILK has been associated with cell proliferation drawing special attention to the kinase activity due to its potential implication in tumor growth. Among the kinase substrates are GSK3β and PKB/Akt implicated in cell growth and survival. During development, ILK loss causes severe muscle attachment defects in worms and flies that were fully rescued by kinase-dead ILK mutants. Loss of ILK in mice caused early embryonic lethality. Conditional ablation in chondrocytes reduced skeletal growth but did not affect GSK3β and PKB/Akt, whereas PKB/Akt phosphorylation was decreased in ILK-deficient macrophages.

In summary, ILK is essential during development due to its essential role in actin organization. The kinase activity, however, remains controversial.

Publication 2: Integrin-linked kinase is required for epidermal and HF morphogenesis

Integrin β1 is crucial for epidermal and hair follicle (HF) integrity. ILK binds to integrins, links to the actin cytoskeleton and is thought phosphorylate several substrates, among them GSK3β. In this present article we report the keratinocyte-restricted ablation of the ILK gene in mice and addressed the functional requirement for ILK downstream of integrin β1 *in vivo*.

ILK ablation caused epidermal defects and progressive hair loss. In the epidermis integrin-mediated adhesion was reduced resulting in blister formation at the dermal-epidermal junction, basement membrane (BM) disruption and altered keratinocyte polarity. Additionally, epidermal differentiation and proliferation were altered, characterized by abundant integrin-expressing and proliferating keratinocytes in suprabasal layers.
HF differentiation strongly depends on active wnt-signaling stabilizing β-catenin through GSK3β phosphorylation. ILK-deficient HFs displayed wnt-signaling activity excluding a functional importance for ILK activity function in this signaling pathway. However, mutant HFs accumulated proliferating cells along the ORS during morphogenesis and failed to initate outgrowth during cycling. This suggested a migration defect of ILK-mutant keratinocytes as neither stem cell maintenance nor proliferation were compromised. *In vitro* studies confirmed a defect in directional and persistent migration due to reduced focal complex and focal adhesion formation and thus impaired stabilization of lamellipodia in ILK-deficient cells.

We conclude that ILK is crucial for epidermal and HF morphogenesis due to its functions in integrin adhesion and actin dynamics and is dispensable for wnt signaling.

Publication 3: PINCH-1 works in an ILK-dependent as well as independent manner in keratinocytes

PINCH1 is a LIM-only domain protein that is recruited to integrin adhesion sites in a heterotrimeric complex together with ILK and parvin family members (IPP-complex). Within the IPP-complex protein stabilities of all three members are interdependent. In order to address whether PINCH1 and ILK are indeed obligate partners we generated mice with a keratinocyte-restricted deletion of PINCH1.

PINCH1 ablation resulted in epidermal defects and progressive hair loss reminiscent of the phenotypes of ILK-deficient skin epithelium. Impaired integrin-mediated adhesion resulted in epidermal blistering and basement membrane disruption. The hyperthickened epidermis contained abnormally located integrin-expressing, proliferating suprabasal cells. Yet, abnomal cell polarity as observed by basal actin accumulation did not result in an altered orientation of mitotic spindles during epidermal cell division. In striking contrast to ILK-deficient keratinocytes cell-cell adhesions were weakened and lacked vinculin recruitment. The mutant hair follicles (HFs) were highly distorted and shortened. During morphogenesis proliferating cells accumulated along the ORS. Later, at the initiation of cycling, the outgrowth of mutant HF keratinocytes failed resulting in organ destruction by inflammatory macrophages. Impaired keratinocyte migration was associated *in vitro* with severe failure in focal adhesion formation and actin cytoskeleton assembly.

In summary, our data show that PINCH1 plays important roles for epidermis and HF biology. With regard to cell-cell adhesion, actin assembly and integrin adhesion the defects observed upon PINCH1 loss exceed the keratinocyte-specific ILK-null phenotype. This implies that PINCH1 also acts independently through the recruitment of other interaction partners or though IPP-independent stabilization in novel cellular compartments.

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APPENDIX

Publication 1

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Integrin-linked kinase: integrin's mysterious partner

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Integrin-linked kinase: integrin's mysterious partner

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Integrin-mediated cell adhesion regulates a vast number of biological processes including migration, survival and proliferation of cells. It is therefore not surprising that defects in integrin function are often rate-limiting for development and profoundly affect the progression of several diseases. The functions of integrins are mediated through the recruitment of cytoplasmic plaque proteins. One of these is integrin-linked kinase, which connects integrins to the actin cytoskeleton and transduces signals through integrins to the extracellular matrix and from integrins to various subcellular compartments.

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Abbreviations

αPIX	PAK-interactive exchange factor-α
BM	basement membrane
CH	calponin homology
CPI-17	protein-kinase-C-dependent phosphatase inhibitor of 17 kDa
Dock180	180-kDa protein downstream of CRK
EB	embryoid body
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
FA	focal adhesions
GSK-3	glycogen synthase kinase 3
ILK	integrin-linked kinase
ILKAP	ILK-associated phosphatase
Mig-2	mitogen inducible gene-2
MLC	myosin light chain
PAK	p21-activated serine/threonine kinase
PDK	3-phosphoinositide-dependent kinase
PH	pleckstrin homology
PHI-1	phosphatase holoenzyme inhibitor 1
PI3K	phosphatidylinositol-3-kinase
PINCH	particularly interesting new cysteine-histidine-rich protein
PIP3	phosphoinositol trisphosphate
PTEN	protein tyrosine phosphatase and tensin homolog

Introduction

Cell adhesion is mediated by multiprotein complexes composed of adhesion receptors, extracellular matrix (ECM) proteins and cytoplasmic plaque proteins. The cell adhesion receptors determine the specificity of the cell–cell or the cell–ECM interaction and recruit cytoplasmic plaque proteins to the cell adhesion site. The cytoplasmic plaque proteins transduce signals initiated by the adhesion receptor, link the adhesion receptors to the cytoskeleton and regulate the functional properties of the adhesion receptors themselves.

Integrins are a large family of adhesion receptors comprising >20 members that mediate highly dynamic cell–cell and cell–ECM interactions. The association and the release of integrin–ligand interactions are achieved by the ability of integrins to adopt different conformations. The active conformation is triggered by intracellular signals and cytoskeleton assembly and results in ligand binding, integrin clustering and recruitment of cytoplasmic plaque proteins into integrin attachment sites called focal adhesions (FAs) [1,2]. One protein that plays a central role in integrin activation and signaling is integrin-linked kinase (ILK) [3]. ILK is composed of three structurally distinct domains: three ankyrin repeats near the N terminus (a fourth ankyrin repeat was identified in human ILK but lacks well-conserved residues), a short linker sequence, and a kinase domain at the C terminus. The linker domain, together with sequences from the N terminus of the kinase domain, shares some similarities with pleckstrin homology (PH) domains (Figure 1).

In the present review we will discuss the functional properties of ILK, which are governed by ILK's interaction partners and kinase activity. The first part of this review summarizes biochemical and cell biological studies of ILK and the second part deals with *in vivo* experiments from invertebrates and mice.

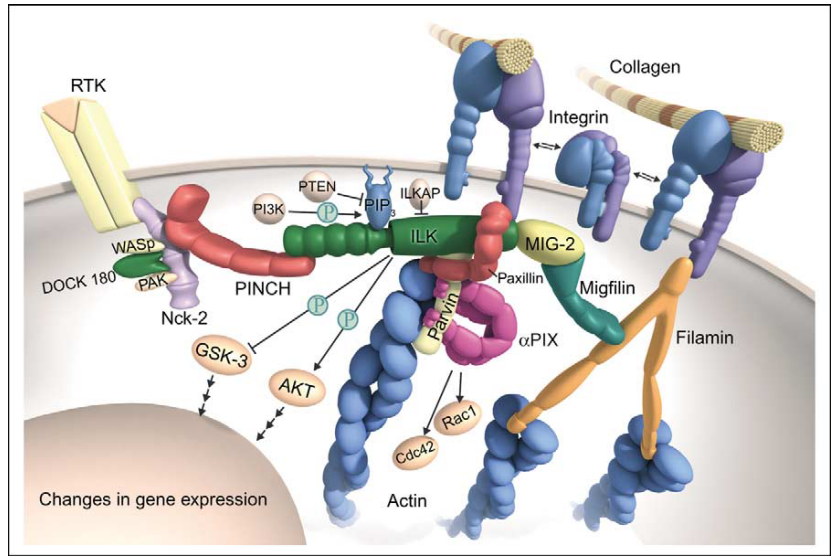
Cell biology and biochemistry of ILK

Overexpression of ILK as well as loss or reduction of ILK expression in cells profoundly affects their morphology and function. The most striking changes are impaired cell spreading, abnormal cell adhesion to and assembly of ECM proteins, delayed formation of FAs and altered cell proliferation [3–6,7••]. How can these defects be explained? Important hints have come from the identification of ILK binding partners (Table 1), from their mode of interaction with ILK and from the identification of substrates for the ILK kinase domain (Table 2).

ILK — a platform for actin regulatory proteins

Almost all adaptor proteins that bind either directly or indirectly to ILK regulate the actin cytoskeleton and

Figure 1



ILK binds Pinch and parvin and this ternary complex subsequently locates to the plasma membrane through the interaction with the cytoplasmic domain of activated β1 and β3 integrin subunits as well as unknown FAs component(s). Binding to phospholipids results in the activation of the kinase function of ILK, which in turn leads to the phosphorylation of GSK3β and PKB/Akt. Finally, ILK can recruit several adaptor proteins, which are able to regulate actin dynamics or actin attachment to FAs. The molecules presented in Figure 1 are not drawn to scale. AKT, protein kinase B/Akt; RTK, receptor tyrosine kinase; WASP, Wiskott-Aldrich syndrome protein.

hence could be responsible for the shape change and FA dysfunction associated with altered ILK expression (Figure 1). Pinch ('particularly interesting new cysteine-histidine-rich protein') was the first interactor to be identified [8]. Pinch-2, a Pinch homologue, was subsequently identified in mice and humans [9,10]. They are both composed of five LIM domains and a nuclear localization signal (NLS) at the C terminus. The first LIM domain binds the first ankyrin repeat of ILK. The interaction has been well-characterized using structural [11], biochemical and cell biological approaches [8,9]. The fourth LIM domain of Pinch-1 was shown to bind with very low affinity to the SH2/SH3 adaptor protein Nck2, which in turn interacts with growth factor receptors and recruits a large number of proteins, including actin modulators such as Dock180 (180-kDa protein downstream of CRK) and the p21-activated serine/threonine kinase (PAK) [8,12,13]. Whether Pinch-1 interacts with Nck2 *in vivo* is not clear. Since mice and cells lacking Nck2 are normal [14] but mice lacking Pinch-1 die during implantation (F Stanchi and R Fässler, unpublished) this interaction does not seem to be crucial for Pinch-1 function. It has been shown that Pinch-2 can translocate into the nucleus [9]. Its role there, however, is unclear.

Table 1

ILK interacting proteins, the location of their binding site on ILK and the method(s) used to confirm their interaction.

Interactor	Domain	Detection	Reference
β1 integrin	C terminus	Y2H/IP	[3]
β3 integrin	C terminus	IP	[3,61]
ILKAP	N terminus	Y2H/IP	[62]
Mig-2/Kindlin-2	C terminus	Y2H	[21••]
α-parvin	C terminus	Y2H/IP	[18]
β-parvin	C terminus	Y2H/IP	[19]
paxillin	C terminus	IP	[15]
Pinch-1	N terminus	Y2H/IP/CC	[8,11]
Pinch-2	N terminus	IP	[9]
PIP3	PH	—	[26]

CC, co-crystallization; IP, co-immunopreciptiation; Y2H, yeast-two-hybrid assay

Table 2

Putative targets of the ILK kinase activity and the amino acid residue(s) phosphorylated by ILK.

Target	Phosphorylation site	Reference
ILK	(Ser343)	[35,40]
β1 integrin	(Ser785)	[3]
β3 integrin	—	[61]
β-parvin	—	[19]
GSK-3β	(Ser9)	[26,62]
PKB/Akt	(Ser473)	[26]
MLC-20	(Thr18/Ser19)	[42]
MYPT-1	(Thr695, Thr495/Thr709)	[43,44]
CPI-17	(Thr38)	[45]
PHI-1	(Thr57)	[45]

MYPT1, myosin phosphatase target subunit isoform 1.

A search for paxillin binding proteins showed that the kinase domain of ILK contains sequences resembling a paxillin binding subdomain (PBS) motif, which firmly binds paxillin [15]. The ILK–paxillin interaction is necessary but not sufficient to recruit ILK into FAs, where the complex may modulate the function of other paxillin binding proteins such as vinculin, α-actinin, talin and FAK.

Several laboratories have simultaneously shown that parvins, a new family of F-actin binding proteins, bind the kinase domain of ILK [16–19]. The parvins comprise three members (α-parvin or actopaxin or CH-ILK binding protein; β-parvin or affixin; and γ-parvin) and are composed of two calponin homology (CH) domains that bind ILK, paxillin and F-actin. β-parvin was shown to interact with the guanine nucleotide exchange factor α-PIX (PAK-interactive exchange factor-α), which may activate Rac1 and Cdc42 [20]. Parvins are found in FAs and apparently do not colocalize to stress fibers [16,17]. An important future task will be to map the binding sites of ILK, paxillin and F-actin on the CH domains and to test whether their binding occurs simultaneously or is mutually exclusive.

A recent paper identified an additional ILK binding partner in *Caenorhabditis elegans*, termed UNC-112 [21••]. UNC-112 contains a FERM domain [22] and is important for the recruitment of the ILK orthologue, Pat-4, to muscle attachment sites. The mammalian orthologue of UNC-112, Mig-2/Kindlin-2, was shown to bind the LIM-domain-containing adaptor protein migfilin, which in turn binds filamin [23•]. It will be interesting to see whether Mig-2/Kindlin-2 also binds ILK in mammalian cells and whether this interaction modulates the function of filamin, which is mutated in a variety of human diseases.

ILK, Pinch and parvin — a ternary complex required for stability and focal adhesion localization

The association of ILK, Pinch and parvin into a ternary protein complex happens before their recruitment into FAs [24•] and serves at least two purposes: it stabilizes the individual proteins and targets the individual components into FAs [24•,25•]. Loss of ILK expression in cells leads to the degradation of Pinch and parvin and, conversely, loss of Pinch expression diminishes ILK and parvin levels [25•]. The degradation can be prevented either by inhibiting the proteasome [25•] or by expressing short N-terminal fragments of ILK (the ankyrin repeats) in ILK-deficient cells (C Grashoff, R Fässler, unpublished data) or Pinch (the first LIM domain) in Pinch-deficient cells (F Stanchi, R Fässler, unpublished data). Their recruitment into FAs, however, cannot be rescued with these fragments. These results support the notion that ILK and Pinch must have binding partner(s) that facilitate FA targeting. Possible candidates for ILK targeting partners are integrins, paxillin and Mig-2/Kindlin-2. It has been shown that nematodes lacking β integrin fail to localize ILK to cell attachment sites [21••]. Mammalian cells may have a similar requirement for β integrin to localize ILK, but this has not been shown yet with cell lines lacking either β1 or β3 or both integrin subunits. Paxillin binds ILK via its N-terminal leucine-rich motifs and targets to FAs via the C-terminal LIM domains. Mutation in the paxillin binding site of ILK prevents ILK/Pinch/parvin recruitment to FAs [15]. Mig-2/Kindlin-2 could also play a role since the worm orthologue UNC-112 is essential for localization of Pat-4/ILK to integrin-containing attachment sites [21••]. No candidate binding partners are currently known that could promote recruitment of Pinch into FAs.

The dependence of ILK, Pinch and parvin stability on the formation of a ternary complex has implications for the interpretation of overexpression experiments. Accumulation of ILK in the cytoplasm of ILK-overexpressing cells may cause a partial depletion of Pinch and parvin from FAs, resulting in an impaired FA function. This could explain why cells either lacking [7••] or overexpressing ILK [3] have similar phenotypes: they both show a rounded morphology and have decreased adhesive properties.

The kinase activity of ILK

Despite the sequence differences between the ILK kinase domain and other protein kinases (important residues in the activation loop of the kinase are not conserved) the similarity was immediately recognized and investigated [3]. Initial studies showed that GST-tagged ILK purified from bacteria or mammalian cells could phosphorylate serine and threonine residues in peptides representing the β1 integrin tail, and model substrates such as myelin basic protein [3].

ILK kinase activity took center stage when it was suggested to be directly associated with cell proliferation, tumor growth and metastasis [4,26–29]. On the one hand, overexpression of ILK in cells results in anchorage-independent cell cycle progression [5] and epithelial-to-mesenchymal transition (EMT) of non-tumorigenic as well as tumorigenic epithelial cells [4,29]. Inhibition of ILK kinase activity, on the other hand, suppresses cell growth in culture as well as growth of human colon carcinoma cells in SCID mice [30]. Several lines of experimental evidence suggest that these phenotypes are largely attributed to enhanced ILK kinase activity and phosphorylation of GSK3β and PKB/Akt [26], two key enzymes involved in a diverse array of cell functions including cell proliferation, survival and insulin responses [31,32]. ILK-dependent phosphorylation of GSK3β in epithelial cells downregulates GSK3β kinase activity [26]. This in turn is associated with reduced E-cadherin expression, enhanced AP1 activity and increased β-catenin–Lef/Tcf activity [4,33], which induces the expression

of cell-cycle-promoting genes such as cyclins and c-myc [5,34]. The reduced E-cadherin expression could be due to a direct effect of the β-catenin-Lef/Tcf complex on E-cadherin gene expression [4]. Alternatively, ILK can reduce E-cadherin levels indirectly by triggering snail expression, which in turn represses E-cadherin gene expression [30].

Full activation of PKB/Akt requires PIP3-dependent phosphorylation of two residues: Thr308 and Ser473 [32]. Whereas PDK-1 (3-phosphoinositide-dependent kinase 1) phosphorylates Thr308, ILK has been identified as 'PDK-2', which phosphorylates Ser473 via a direct interaction at the plasma membrane [26,35]. Besides possessing a kinase activity, ILK fulfils other requirements of a PDK2, including PIP3 binding and regulation of its activity by PI3K (phosphatidylinositol-3-kinase) or PTEN (protein tyrosine phosphatase and tensin homolog) [26,27]. However, some doubts about ILK's kinase activity arose when it was reported that it has no Ser473 phosphorylation activity [36,37•]. These doubts were reinforced by genetic studies in invertebrates and mice that demonstrated normal Ser473 phosphorylation in the absence of ILK [7••,21••,38]. Loss-of-function mutations of ILK in worms and flies show no defects that can be explained by impaired PKB/Akt activity, but develop severe muscle defects that are fully rescued when different kinase-dead versions of ILK are expressed [21••,38]. Similarly, fibroblasts with or without the ILK gene phosphorylate Ser473 to a similar extent following insulin or PDGF stimulation [7••], and neither chondrocytes nor keratinoyctes change their steady-state Ser473 phosphorylation after ILK gene ablation *in vivo* [39] (T Sakai and R Fässler, unpublished). These findings convincingly demonstrate that ILK — even if it has Ser473 phosphorylation activity — is not the only PDK2. These findings, however, do not exclude the possibility that ILK mediates the phosphorylation of PKB/Akt and other target proteins in an indirect manner, for example by recruiting a kinase or inhibiting a phosphatase [37•,40]. Support for such a notion also comes from gene ablation experiments. Monocytes lacking ILK expression show reduced Ser473 phosphorylation [41•]. Similarly, ILK-null fibroblasts, which respond normally to insulin treatment, fail to maintain Ser473 phosphorylation levels to the same extent as normal cells upon PDGF treatment [7••]. Furthermore, they display a slightly reduced steady state level of Ser473 phosphorylation under normal culture conditions (T Sakai and R Fässler, unpublished).

Other targets of the ILK kinase activity (Table 2) are β-parvin [19], the regulatory myosin light chain (MLC) [42], and MLC phosphatase [43,44] and its regulators CPI-17 (protein-kinase-C-dependent phosphatase inhibitor of 17 kDa) and PHI-1 (phosphatase holoenzyme inhibitor 1) [45]. The significance of their phosphorylation, however, is not clear.

Since ILK regulates so many essential cellular functions it is important to settle the debate on ILK's kinase activity. Solving the structure of the ILK kinase domain will be very informative, as will the analysis of mice carrying 'kinase-dead' versions of the ILK gene and the identification of PDK2(s). In addition to these new experimental approaches, new reagents to probe ILK's function will be useful. The E359K mutation in ILK, for example, was originally found to lack kinase activity and was therefore used in many studies as a 'kinase-dead' version of ILK. It turns out, however, that the mutation does not affect kinase activity but rather impairs paxillin binding and FA targeting [46•]. Furthermore, a polyclonal anti-ILK antiserum that recognizes a 59 kDa band of unknown origin instead of the 52 kDa sized ILK has been used in a large number of studies and could potentially have given misleading results [3,6,47].

Studies of ILK/Pinch/parvin in invertebrates and mice

The attachment sites of the body wall muscle to the hypodermis of *C. elegans* are called dense bodies and resemble FA-like structures. They contain β-*pat*-3 integrin (the only β integrin subunit in *C. elegans*), pat-4/ILK, UNC-97/Pinch, pat-6/parvin and UNC-112/Mig-2 and loss-of-function alleles of these proteins lead to severe adhesion defects manifesting as muscle detachment and embryonic lethality [21••,22,48,49•]. The loss-of-function studies also reveal that β-pat-3 integrin is required to recruit ILK to the plasma membrane [21••] and that integrins are partially mislocalized in the absence of pat-4/ILK [21••] or UNC-112/Mig-2/Kindlin-2 [22]. A recent report showed that the Zn^{2+}-finger-containing transcription factor UNC-98 can bind UNC-97/Pinch and is also required for muscle attachment to the body wall [50•]. UNC-98 shuttles between dense bodies and the nucleus where it binds DNA and probably regulates gene transcription. So far an ortholog of the UNC-98 gene has not been identified in flies or mammals.

Drosophila melanogaster has a similar requirement for βPS integrins, ILK and Pinch in muscle cell attachment [38,51••]. Interestingly, loss of βPS integrin function in flies leads to detachment of ECM from the cell membrane, while loss of ILK function leads to detachment of F-actin from the plasma membrane, indicating an important role for ILK in actin stabilization at integrin attachment sites [38]. The severe muscle defect in worms or flies lacking ILK can be fully rescued by the expression of different kinase-dead ILK transgenes, supporting the idea that ILK functions as an important adaptor protein, independent of its kinase activity [21••,38].

The loss of ILK expression in mice leads to peri-implantation lethality similar to what is seen upon loss of β1 integrin expression [7••,52]. The cause of the developmental arrest was studied in embryoid bodies (EBs)

[7••,53,54]; these studies showed that β1-integrin-mutant EBs are unable to deposit a basement membrane (BM), while ILK-null EBs produce a BM but fail to polarize the epiblast (a primitive tissue that will give rise to all three germ layers). Since addition of laminin to β1-integrin-null EBs rescues the BM assembly phenotype and allows epiblast development it is likely that β1 integrin and ILK function independently during the peri-implantation period [54].

Conditional loss of ILK in chondrocytes leads to skeletal growth retardations characterized by abnormal chondrocyte shape and decreased proliferation *in vivo* [39,55], and diminished chondrocyte spreading on ECM and reduced stress fiber formation *in vitro* [39]. Similar, albeit more severe, defects are also observed in mice with a chondrocyte-specific deletion of the β1 integrin gene [56], indicating that β1 integrins and ILK are both required for normal chondrocyte function. The mechanism leading to reduced chondrocyte proliferation in the absence of ILK expression is not understood; altered phosphorylation of PKB/Akt or GSK-3β was excluded [39]. A conditional deletion or reduction of ILK gene expression in macrophages, on the other hand, results in a strong inhibition of the PKB/Akt-Ser473 phosphorylation associated with apoptosis [41•], indicating that ILK kinase activity might differ depending on the cell type.

Overexpression of ILK in mammary glands of transgenic mice leads to tumor formation [29]. Similarly, pharmacological inhibition of ILK in prostate carcinoma cells causes them to proliferate much less rapidly *in vivo* [57••]. These findings can principally be explained by the oncogenic activities of ILK (activation of PKB/Akt, inhibition of GSK-3β, and stimulation of AP-1, NF-κB and β-catenin–Lef/Tcf transcription factors) and its ability to promote tumor angiogenesis. ILK promotes blood vessel invasion into tumors in two ways: ILK induces HIF1α-dependent VEGF expression in tumor cells, which in turn regulates endothelial cell migration and proliferation in an ILK kinase-dependent manner [57••]. The importance of ILK for tumor pathology is underscored by the fact that a large number of malignant tumors display increased ILK levels and kinase activity [58], and in some tumor types ILK levels correlate with tumor grade [59,60].

Outlook

ILK has many interesting functional facets and work in both invertebrates and mice has revealed an essential role for ILK in development. There is a general consensus that ILK plays a central role in the reorganization of the F-actin cytoskeleton and its attachment to FAs. The role of ILK as a kinase is more controversial. Since a large number of ILK functions rely on kinase activity, including EMT, proliferation and VEGF expression, this controversy should urgently be settled. This can be assisted by solving the structure of the ILK kinase domain, using continued genetic approaches or the well-defined antibodies that have become available over the past few years. As has already been done in flies and worms, it should be tested in mice whether point mutations in the kinase domain of ILK impair the function of the molecule.

An important future task will be to identify the signals that trigger assembly of the ILK/Pinch/parvin complex, to identify the proteins that recruit the core complex into FAs, and to establish how the core complex modulates integrin functions and regulates actin dynamics. The availability of cell lines and mice that lack ILK and the progress in proteomics and live cell imaging should together help to dissect these mechanisms and to clarify ILK's role in integrin-mediated cell adhesion.

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Publication 2

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Integrin-linked kinase is required for epidermal and hair follicle morphogenesis

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Integrin-linked kinase is required for epidermal and hair follicle morphogenesis

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Integrin-linked kinase (ILK) links integrins to the actin cytoskeleton and is believed to phosphorylate several target proteins. We report that a keratinocyte-restricted deletion of the ILK gene leads to epidermal defects and hair loss. ILK-deficient epidermal keratinocytes exhibited a pronounced integrin-mediated adhesion defect leading to epidermal detachment and blister formation, disruption of the epidermal–dermal basement membrane, and the translocation of proliferating, integrin-expressing keratinocytes to suprabasal epidermal cell layers.

The mutant hair follicles were capable of producing hair shaft and inner root sheath cells and contained stem cells and generated proliferating progenitor cells, which were impaired in their downward migration and hence accumulated in the outer root sheath and failed to replenish the hair matrix. In vitro studies with primary ILK-deficient keratinocytes attributed the migration defect to a reduced migration velocity and an impaired stabilization of the leading-edge lamellipodia, which compromised directional and persistent migration. We conclude that ILK plays important roles for epidermis and hair follicle morphogenesis by modulating integrin-mediated adhesion, actin reorganization, and plasma membrane dynamics in keratinocytes.

Introduction

The skin is composed of an epithelial (epidermis and hair follicle [HF]) and a mesenchymal compartment (dermis, subcutis, and dermal papilla [DP]) joined and maintained together by a basement membrane (BM). The interfollicular epidermis contains multiple layers of keratinocytes at different stages of differentiation, from a basal layer of undifferentiated, proliferating keratinocytes attached to the BM, to terminally differentiated, cornified cells (Fuchs and Raghavan, 2002). The HF is an epidermal appendage, which arises as an epithelial cone from the fetal epidermis after a series of epithelial–mesenchymal cues. The mature HF epithelium consists of a central hair shaft (HS), surrounded by an inner and an outer root sheath (IRS and ORS, respectively). HS and IRS differentiation from the hair matrix (HM) is induced by mesenchymal cues from the connective tissue sheath and the DP. The mature HF has the ability to involute and regenerate, with cyclically alternating periods of rapid growth (anagen), apoptosis-driven regression (catagen), and relative quiescence (telogen). During each growth period, the progeny (transient amplifying [TA] cells) of epithelial stem cells located in the bulge region of the ORS extends into the mesenchymal compartment and generates a new HM. Here, epithelial cells change migration direction and terminally differentiate into IRS or HS (Paus and Cotsarelis, 1999).

Basal keratinocytes express several integrins, including $\alpha2\beta1$, $\alpha3\beta1$, $\alpha9\beta1$, $\alpha v\beta5$, and $\alpha6\beta4$ integrins (Watt, 2002). The $\alpha6\beta4$ integrin is the core component of hemidesmosomes anchoring keratin filaments to the BM, whereas $\alpha3\beta1$ and $\alpha9\beta1$ integrins link the actin cytoskeleton to the BM. The $\alpha2\beta1$ is found around the entire basal keratinocytes, where it is thought to mediate cell–cell interactions. ORS cells express $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta4$ integrins at different levels according to the region of the HF (Commo and Bernard, 1997). In vitro studies with keratinocytes and genetic manipulations in mice revealed that $\beta1$ integrins regulate adhesion and differentiation of epidermal cells and play an essential role for hair germ invagination, ORS cell migration, and sustained HM proliferation during HF

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Abbreviations used in this paper: BM, basement membrane; DEJ, dermal–epidermal junction; DP, dermal papilla; FA, focal adhesion; FC, focal complex; GSK, glycogen synthase kinase; HF, hair follicle; HM, hair matrix; HS, hair shaft; ILK, integrin-linked kinase; IRS, inner root sheath; ORS, outer root sheath; P, postnatal day; PKB, protein kinase B; TA, transient amplifying.

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morphogenesis (Brakebusch et al., 2000; Raghavan et al., 2000; Watt, 2002; Grose et al., 2002). An important and still largely unanswered question is how integrins mediate these functions in skin and HFs. Because integrin cytoplasmic domains lack actin binding sites and enzymatic activity, signaling is implemented through accessory molecules such as talin, α-actinin, and integrin-linked kinase (ILK; Brakebusch and Fässler, 2003). ILK is composed of N-terminal ankyrin repeats, a pleckstrin homology–like domain and a putative, C-terminal kinase domain (Hannigan et al., 1996; Grashoff et al., 2004, Legate et al., 2006). ILK was given its name based on the enzymatic activity of its kinase domain (Delcommenne et al., 1998; Novak et al., 1998; Persad et al., 2000), which was shown to phosphorylate several target proteins, including protein kinase B (PKB)/Akt and glycogen synthase kinase (GSK) 3β. The significance of the ILK activity, however, is controversial as in vitro and in vivo results in flies, worms, and mice point toward an adaptor rather than an enzymatic function of ILK (Lynch et al., 1999; Zervas et al., 2001; Mackinnon et al., 2002; Hill et al., 2002; Grashoff et al., 2003; Sakai et al., 2003). A recent report proposed that the ILK activity is biologically relevant for transformed epithelial cells but not normal cells (Troussard et al., 2006). Whether the controversy may indeed be ascribed to the different biological systems used in the past to investigate ILK function awaits further studies. Another important function of ILK is its ability to link integrins to the actin cytoskeleton and to modulate actin reorganization (Zervas et al., 2001; Mackinnon et al., 2002; Grashoff et al., 2003; Sakai et al., 2003). Almost all proteins that bind ILK bind and/or regulate actin dynamics. They include PINCH1 and PINCH2, which bind actin modulators and connect ILK to growth factor receptors, the parvin family of F-actin binding proteins, and paxillin, which recruits actin binding and regulatory proteins, including vinculin, talin, α-actinin, and FAK (for reviews see Grashoff et al., 2004; Legate et al., 2006). HF development and cycling is crucially dependent on the inactivation of GSK-3β in HM cells (Fuchs et al., 2001; Huelsken et al., 2001). Active, nonphosphorylated GSK-3β can phosphorylate β-catenin bound to a protein complex, collectively called the β-catenin degradation complex. Phosphorylation of GSK-3β inactivates the kinase and leads to stabilization and translocation of β-catenin to the nucleus, where it associates with the Lef1/Tcf family of DNA binding proteins to activate the transcription of target genes, such as cyclin D1, c-myc, homeobox containing transcription factors, Lef1, and hair-specific keratins (Zhou et al., 1995; for review see Logan and Nusse, 2004). ILK can modulate the stability of β-catenin either through phosphorylating GSK-3β (Delcommenne et al., 1998; Novak et al., 1998) or through inhibiting the β-catenin degradation complex (Oloumi et al., 2006) and could therefore play a central role for HF morphogenesis.

To test the function of ILK during epidermis and HF development, we deleted the ILK gene in keratinocytes. We found that loss of ILK compromises epidermal keratinocyte adhesion and disrupts HF formation, leading to progressive hair loss. The HF defect was not due to an abnormal β-catenin stability, HM differentiation, or stem cell maintenance. Instead, the accumulation of proliferating ORS cells points to an impaired HF downward growth in vivo.

Results

Deletion of ILK in keratinocytes leads to progressive hair loss

To delete the ILK gene in keratinocytes, floxed ILK mice were intercrossed with animals carrying the keratin 5 (K5)–Cre transgene (ILK-K5 mice). Littermates carrying heterozygous floxed

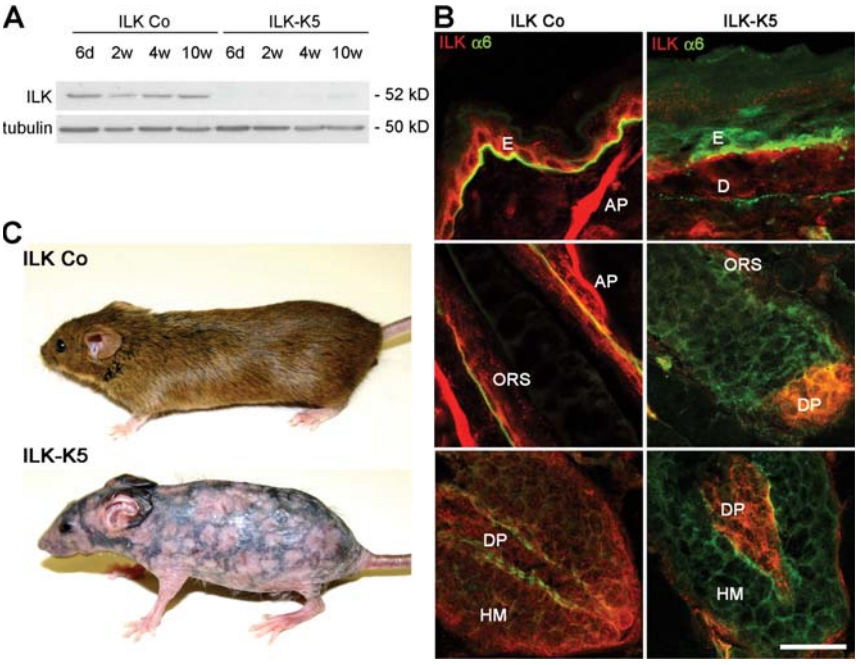

Figure 1. **Keratinocyte-restricted deletion of ILK causes progressive hair loss.** (A) ILK protein level in epidermal lysates of ILK Co and ILK-K5 mice. (B) Back skin of 2-wk-old ILK Co and ILK-K5 animals stained for ILK and α6 integrin. ILK is expressed in basal keratinocytes of the epidermis (E), ORS, HM, DP, arrector pili muscle (AP), and dermis (D). ILK-K5 skin retains ILK expression in DP and dermis but lacks ILK expression in epidermis, HM, and ORS. Bar, 25 μm. (C) Control and ILK-K5 animals at 8 wk of age.

ILK gene and the K5-Cre transgene served as controls (ILK Co). K5-mediated Cre expression deleted the ILK gene in back skin at around embryonic day 15, decreased ILK levels in newborn skin, and led to the loss of the ILK protein thereafter (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1). Western blot analysis in back skin epidermis of 6-d-, 2-wk-, 4-wk-, and 10-wk-old mice confirmed the sustained absence of ILK (Fig. 1 A). Immunostained sections of 2-wk-old control mice revealed ILK in basal epidermal keratinocytes, ORS, HM, DP, and the arrector pili muscle (Fig. 1 B). ILK was absent from epidermis and HF epithelium of ILK-K5 skin but still present in DP (Fig. 1 B).

ILK-K5 animals were indistinguishable from control littermates at birth. At 1–2 wk, when control animals developed their hair coat, ILK-K5 animals had scattered hair with partial alopecia. This appearance endured until around 4 wk of age and was followed by progressive hair loss, leading to persistent alopecia by 6–8 wk (Fig. 1 C). A reticular pigmentation pattern developed on the back skin of 8-wk-old ILK-K5 mice (Fig. 1 C), whereas hair coat and hair cycle–dependent skin color changes occurred normally in control mice.

Loss of ILK causes severe epidermal and HF abnormalities

The epidermis of ILK-K5 mice was morphologically normal at birth and postnatal day (P) 2 but became progressively hyperplastic (at P7–9, four to five cell layers, and at P28, six to seven cell layers; Fig. 2, A and B). Although basal keratinocytes were polarized and tightly attached to the BM in control skin, they appeared flattened in the mutant epidermis and were often detached along the dermal–epidermal junction (DEJ; Fig. 2, A and B, asterisks). The detachment became more severe with age (at P7, 5–10% of total epidermal length; at P14, 30–50%; and at P70, up to 70%) but did not result macroscopically in visible skin blisters.

The most striking phenotype was a severe impairment of HF development in ILK-K5 mice characterized by a progressive growth retardation, which was first visible at around P2 (Fig. 2, A and D). By P14, control mice had completed HF morphogenesis, with all hair bulbs residing deep in the subcutis. In contrast, ILK-K5 HFs diverged into two subpopulations. (1) Approximately 33% of the mutant HFs reached the final stages of HF morphogenesis but were shortened and profoundly distorted. They displayed substantial hyperplasia of the ORS with up to six cell layers and condensed DPs (Fig. 2, A, C, and D, ▲). (2) Approximately 66% of the mutant HFs were arrested in their development. They failed to reach down deeper than the reticular dermis and showed defective morphogenesis with distorted or absent HS formation and misshapen HM and DP (Fig. 2, A, C, and D, ■). A plausible explanation for the varying HF populations is the combination of an asynchronous HF morphogenesis (Paus et al., 1999) and the perinatal loss of ILK protein

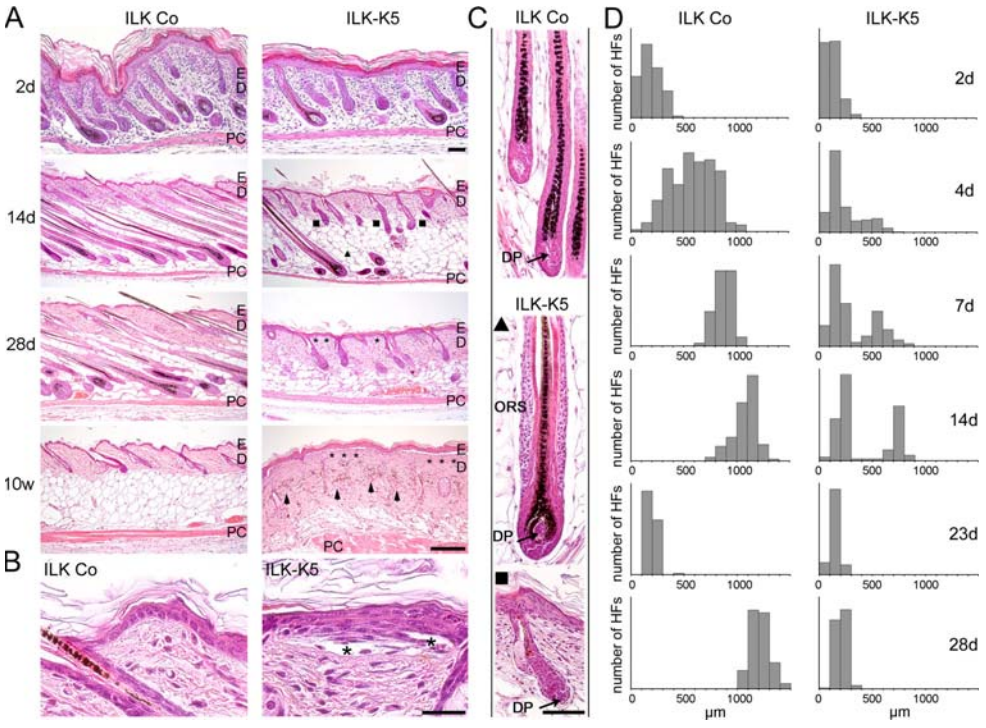

Figure 2. **Keratinocyte-restricted deletion of ILK leads to epidermal hyperplasia and epidermolysis and perturbs HF development and growth.** (A) Hematoxylin-eosin staining of sections derived from back skin of control and ILK-K5 mice. ILK-K5 mice display stunted HF morphogenesis leading to two HF types (▲, fully developed; ■, shortened and prematurely arrested), progressive epidermal detachment (asterisks), and dermal pigment deposition (arrowheads). Bar, 100 µm. (B) Epidermis from 2-wk-old ILK-K5 mice is hyperplastic and detached from the underlying dermis (asterisks). ILK-K5 keratinocytes show a flattened morphology. Bar, 25 µm. (C) High magnification of hematoxylin-eosin–stained HFs from 9-d-old back skin. ILK-K5 HFs have multilayered ORS (▲) or show premature growth arrest with loosely attached, malformed DP (arrow; ■). Bar, 50 µm. (D) ILK-K5 HF growth is perturbed during morphogenesis and cycling. HF lengths of a minimum of 100 HFs per time point are presented as histograms. PC, panniculus carnosum; E, epidermis; D, dermis.

expression: fully developed HFs (Fig. 2, ▲) lost ILK late in morphogenesis, whereas arrested HFs (■) lost ILK early in morphogenesis. At P28, none of the ILK-K5 HFs was able to initiate anagen characterized by HF downgrowth into the subcutis (Fig. 2, A and D). By 10 wk of age, the ILK-K5 HFs were resorbed (Fig. 2 A) and melanin condensates within the dermis gave rise to a reticular skin pigmentation (Fig. 1 C).

Loss of ILK compromises keratinocyte adhesion and BM maintenance

Cell detachment in ILK-K5 skin points to a compromised integrin function that could be caused by altered expression, activity, localization, or weaker linkage to the actin cytoskeleton. Integrin function was tested with adhesion assays using fibronectin (FN), collagen I (Col I), collagen IV (Col IV), and laminin 332 (LM332) as substrates. Although interaction with poly-L-lysine was similar between ILK-K5 and control keratinocytes, adhesion to the ECM substrates was significantly diminished in ILK-K5 keratinocytes (Fig. 3 A).

Integrin expression determined by FACS revealed strong β1 integrin expression and a comparable Mn^{2+}-triggered activation of β1 integrins on freshly isolated control and ILK-K5 keratinocytes. However, a subpopulation of ILK-K5 cells expressed lower levels of β1 integrin (Fig. S1 B). The expression levels of the α6, β4, and αv integrin subunits were not changed on ILK-K5 keratinocytes, whereas the α3 and α2 integrin chains were slightly up-regulated (Fig. S1 B). In situ immunostaining revealed differences in integrin localization at the cellular level. In control skin, β1 integrin was expressed around the entire surface of basal keratinocytes (Fig. 3 B) and β4 and α6 integrins along the DEJ (Fig. 3 B and Fig. 4). In ILK-K5 skin, the β1 integrin subunit was present on basal keratinocytes but also on many suprabasal cells (Fig. 3 B), which maintained K14 expression (Fig. 3 B, middle). The localization of α6 and β4 integrins on ILK-K5 basal keratinocytes was comparable to control skin, with the exception of a few areas lacking detectable α6 and β4 integrin and some suprabasal cells showing a strong staining for α6 and β4 integrin (Fig. 3 B). The latter cell population likely expressed similar levels of α6β4 integrins as basal keratinocytes, as FACS analysis of freshly isolated keratinocytes did not distinguish two populations of α6β4-expressing keratinocytes (Fig. S1 B).

The decreased keratinocyte adhesion was associated with severe BM defects. Although control skin showed a linear staining of LM332 along the DEJ and around HFs, ILK-K5 skin displayed irregular deposits of LM332 at the DEJ with areas of massive LM332 (Fig. 3 B, asterisks) diffusion into the dermis and dotlike deposits adjacent to integrin-positive suprabasal keratinocytes (Fig. 3 B, right, arrowhead). EM of a control skin revealed a regular BM structure at the DEJ, whereas mutant skin showed an abnormal BM with discontinuities in the lamina densa between hemidesmosomes (Fig. 3 C). The number of hemidesmosomes was normal except in areas with detached epidermis, where the number was reduced. Collectively, these data demonstrate that loss of ILK weakens integrin-mediated adhesion of basal keratinocytes to the BM and abrogates BM integrity.

ILK regulates proliferation and differentiation of epidermal keratinocytes

ILK-deficient epidermis was hyperplastic (Fig. 2 A). Ki67 immunostaining revealed that P4 epidermis from control as well as

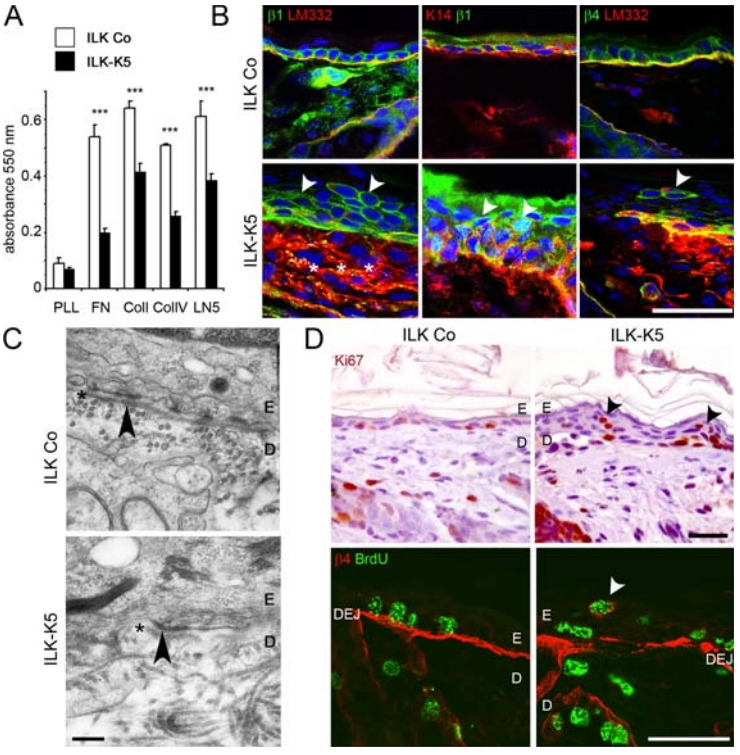

Figure 3. **ILK ablation impairs keratinocyte adhesion, integrin expression, and BM integrity and alters proliferation in vivo.** (A) Cell adhesion of ILK-K5 keratinocytes from 4-d-old mice on FN, Col I, Col IV, and LM332 is significantly reduced compared with control keratinocytes. Adhesion to poly-L-lysine (PLL) is not different (mean + SD of three independent experiments; ***, $P < 0.001$). (B) Integrin expression in epidermis from 2-wk-old mice. In control skin, β1 and β4 integrins are expressed in basal keratinocytes, whereas in ILK-K5 skin both integrins are also found on suprabasal keratinocytes (arrowheads). In ILK-K5 mice, β4 integrin shows discontinuous staining on basal keratinocytes, LM332 diffuses into the upper dermis (asterisks), and β1 integrin–expressing suprabasal cells retain K14 expression. Bars, 25 μm. (C) Electron micrographs of back skin sections of 2-wk-old control and ILK-K5 mice. Control skin exhibits a continuous lamina densa (asterisks) and hemidesmosomes (arrowhead), whereas ILK-K5 skin shows a discontinuous lamina densa, which is preserved at hemidesmosomes (arrowhead) but largely absent in between. Bar, 0.25 μm. (D) Ki67 staining revealed the presence of proliferating cells in ILK-K5 suprabasal layers. Suprabasal BrdU+ cells express β4 integrin. Bars, 25 μm.

ILK-K5 skin contained comparable numbers of proliferating cells almost exclusively in the basal layer. At P7, however, ILK-K5 skin contained normal numbers of proliferating cells in the basal layers and, in addition, a significant number of proliferating cells in the suprabasal layers (Fig. S2, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1). The ectopic keratinocyte proliferation was also observed in Ki67-immunostained skin (Fig. 3 D). It occurred in areas with aberrant and normal BM and was associated with β1 and β4 integrin expression (Fig. 3 D).

To test whether loss of ILK expression also affected proliferation of primary keratinocytes in vitro, we performed BrdU incorporation assays. In three independent experiments, we found an increased incorporation of BrdU in ILK-K5 keratinocytes when compared with control cells (Fig. S2 C). Surprisingly, however, the phosphorylation of known ILK targets involved in cell cycle control, such as Ser9 of GSK-3β and Ser473 of PKB/Akt, and the expression of D-type cyclins were not changed in ILK-K5 epidermal lysates (Fig. S2 D).

The defective keratinocyte adhesion could trigger a chronic wound healing response with infiltrating inflammatory cells, which in turn may induce the ectopic proliferation of suprabasal keratinocytes in vivo. To test this, we searched skin sections from control and ILK-K5 mice for the presence of granulocytes and macrophages. As expected, granulocyte and macrophage infiltrates were absent from P7 as well as P14 control skin (Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1). ILK-K5 skin also lacked granulocyte and macrophage infiltration at P7 (Fig. S3, A and B), when abundant proliferation of suprabasal keratinocyte was already evident (Fig. S2 B). At P14, however, macrophages accumulated around ILK-K5 HFs and granulocytes beneath the epidermis (Fig. S3, A and B).

The presence of proliferating, integrin-positive keratinocytes in suprabasal layers points to an aberrant differentiation and/or mislocalization of undifferentiated ILK-K5 keratinocyte. To investigate differentiation, we analyzed the expression of epidermal keratins. K14 was expressed in basal cells and weakly extended into the first suprabasal layer of control epidermis (Fig. 4 A). In ILK-K5 skin, K14 was expressed suprabasally in up to five cell layers (Fig. 4 A). Normal suprabasal cells switched off K14 and K5 expression and instead expressed K10 (Fig. 4 A). In ILK-K5 epidermis, K10 was absent from basal cells but strongly expressed in the four to five suprabasal cell layers. In addition, there were often patches of cells lacking K10 but expressing integrins (Fig. 4 A, asterisks) and high levels of K14 (Fig. 4 A and Fig. 3 B). Furthermore, although loricrin was confined to the stratum granulosum and appeared as a thin linear signal in control epidermis, in ILK-K5 epidermis, loricrin was found in two to three cell layers, which contained large and round keratinocytes with prominent nuclei (Fig. 4 A). These data suggest that loss of ILK sustains proliferation and expression of basal layer markers in suprabasal cell layers and delays keratinocyte differentiation.

ILK maintains polarity of epidermal keratinocytes

ILK-deficient keratinocytes have a flattened shape (Fig. 2 B), suggesting that their polarity was impaired. To investigate keratinocyte polarity in vivo, we compared F-actin and the distribution of cell–cell adhesion molecules between control and ILK-K5 epidermis. In control epidermis, F-actin distributed to the apical and lateral plasma membranes of basal keratinocytes, whereas in ILK-K5 epidermis, the F-actin was also present at the basal plasma membrane zone facing the BM, where it frequently colocalized with nidogen (Fig. 4 B). Similar F-actin defects were also seen in mutant HFs (Fig. 4 C).

In normal skin, E-cadherin and its junctional adaptor protein β-catenin were found at the lateral and apical plasma membrane of basal keratinocytes (Fig. 4 B and not depicted).

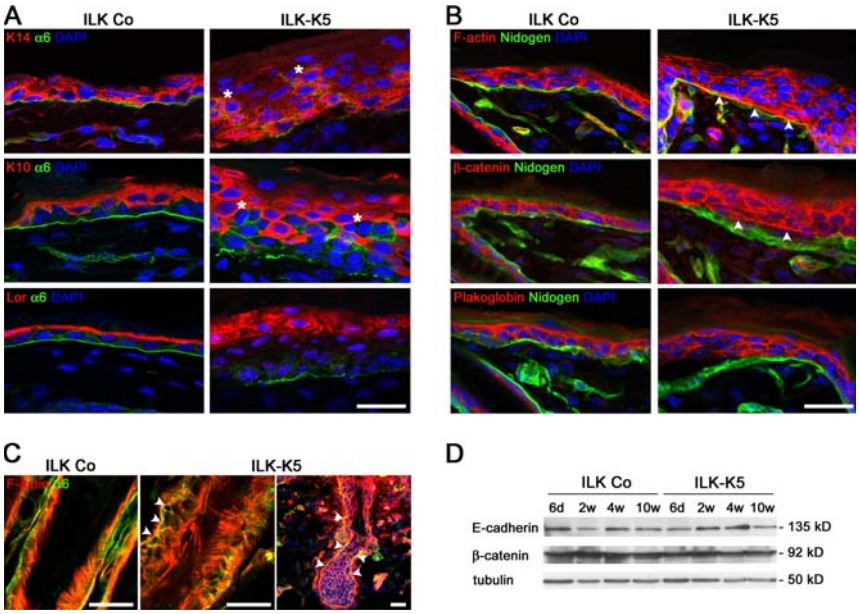

Figure 4. **Loss of ILK retards differentiation and disturbs polarity of epidermal keratinocytes.** (A) Double immunostaining for K14, K10, or loricrin (Lor) and α6 integrin on back skin of 2-wk-old control and ILK-K5 animals. ILK-K5 epidermis shows several cell layers expressing K14 and loricrin, respectively. Integrin α6 expression is discontinuous in ILK-K5 skin and present on suprabasal cells (asterisks). Bar 25 μm. (B) Immunostaining for F-actin, β-catenin, and plakoglobin in 2-wk-old mouse skin. In control epidermis, F-actin and β-catenin are absent from the basal side of basal keratinocytes. In ILK-K5 epidermis, F-actin and β-catenin are found basally adjacent to nidogen (arrowheads). Plakoglobin localizes to the lateral–apical sides of basal keratinocytes of both control and ILK-K5 mice. Bar, 25 μm. (C) F-actin overlaps with α6 integrin in the mutant HF (arrowheads). Bars, 50 μm. (D) Western blot analysis reveals similar expression levels of E-cadherin and β-catenin in control and ILK-K5 epidermal lysates.

In ILK-K5 skin, the E-cadherin and β-catenin staining was normally distributed in areas where the epidermis was attached to the dermis. In areas where the epidermis was detached from the BM, both E-cadherin and β-catenin were redistributed to the basal side of basal keratinocytes (Fig. 4 B and not depicted). In epidermal lysates, E-cadherin and β-catenin protein levels were indistinguishable between control and ILK-K5 samples (Fig. 4 D). The expression and localization of desmosomal components such as plakoglobin and desmoplakin (Fig. 4 B and not depicted), as well as the ultrastructure of desmosomes (Fig. S3 C), were unaffected in all areas of the ILK-K5 epidermis. We conclude that ILK controls cell polarity by maintaining the integrity of the actin cytoskeleton and BM and not by regulating E-cadherin expression or the formation of cell–cell junctions.

Loss of ILK permits normal β-catenin–Lef1 signaling and HF differentiation

A possible role of ILK for hair epithelium differentiation stems from the observation that ILK controls β-catenin–Lef1–mediated gene transcription either by phosphorylating and inactivating GSK-3β (Delcommenne et al., 1998) or by stabilizing β-catenin (Oloumi et al., 2006). To test whether GSK-3β and the downstream β-catenin–Lef1 complex were affected by the loss of ILK, we performed a series of different experiments. Immunoblotting of lysates from freshly isolated keratinocytes revealed that the total levels of GSK-3β and the extent of phosphorylation of Ser9 did not differ between control and ILK-K5 samples (Fig. S2 D). Immunostaining revealed that Lef1 and nuclear β-catenin were present in the precortical HM and HS cortex of control as well as fully developed ILK-K5 HFs (Fig. 5, A and B, ▲). Moreover, both proteins could clearly be detected in the Ki67-positive HM cells of prematurely growth-arrested ILK-K5 HFs (Fig. 5, A and B, ■). To determine the activity of the nuclear β-catenin–Lef1 transcription factor complex, ILK-K5 mice were intercrossed with reporter mice, in which β-galactosidase expression is controlled by nuclear β-catenin–Lef1 (Maretto et al., 2003). The expression of β-galactosidase was clearly visible in the HS of control and fully developed ILK-K5 HFs (Fig. 5 C, ▲) and in cells of growth-retarded ILK-K5 HFs (Fig. 5 C, ■). Normal activity of the β-catenin–Lef1 complex was further confirmed by determining the β-catenin–Lef1–dependent expression of IRS-specific keratins. The IRS keratin K6irs1 (Fig. 5 D) and K6irs2-4 (not depicted) were normally expressed in ILK-K5 HFs. Similarly, the expression of ORS keratins and several HS-specific markers (e.g., hHa1) was also normal in both populations of ILK-K5 HF (even though the localization of the K6irs1-positive cells in the shortened mutant HF was abnormal; Fig. 5 D). Altogether, these findings demonstrate that ILK regulates neither the phosphorylation of GSK-3β and the stability and activity of β-catenin in HFs nor the differentiation of HM into the IRS or HS.

ILK loss leads to accumulation and premature proliferation of ORS cells

Loss of β1 integrin expression leads to reduced proliferation of epidermal keratinocytes and HF matrix cells (Brakebusch et al., 2000; Raghavan et al., 2000). To assess whether altered proliferation of the ILK-K5 HM accounts for the abnormal hair

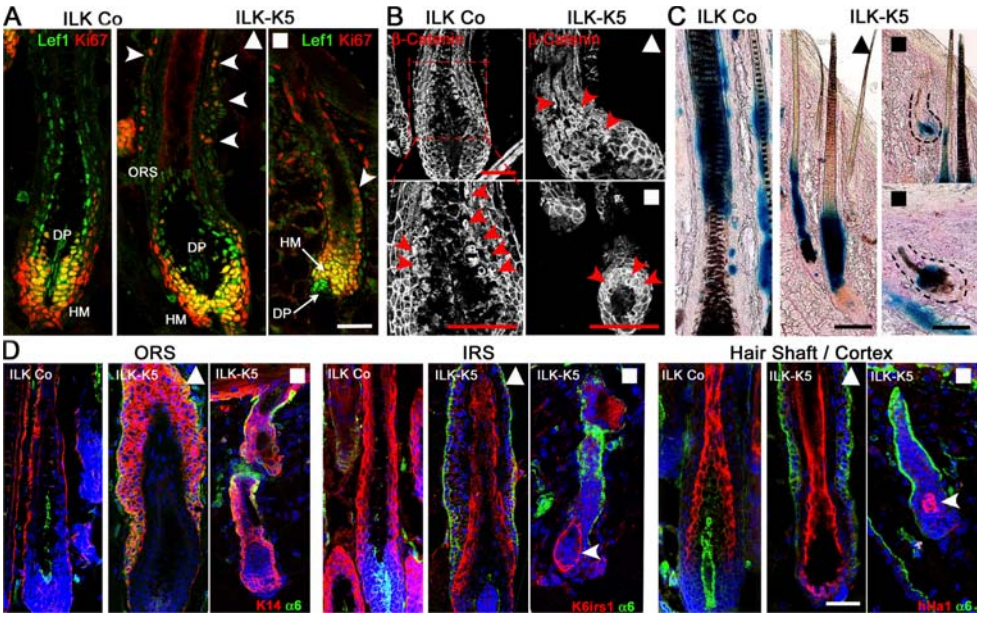

Figure 5. **ILK-K5 HFs show normal β-catenin stability and hair-specific differentiation.** (A) Control and mutant 2-wk skin sections stained for Ki67 and Lef1 show an increased number of Ki67+ cells in the ORS (arrowheads), yet retained Lef1 expression in the HM and DP of ILK-K5 HFs. (B) Control and mutant 2-wk skin sections stained for β-catenin revealed nuclear β-catenin (arrowheads) in precortical HM and proximal HS cortex in both control and long (▲) and short (■) ILK-K5 HFs. (C) BatGal reporter mice were intercrossed with ILK-K5 and control animals. LacZ activity is present in precortical HM and HS cortex of both control and ILK-K5 HFs. (D) Immunostaining of K14 for the ORS, of keratin K6irs1 for the IRS, and of keratin hHa1 for HS cortex and α6 integrin. ILK-K5 HFs revealed the presence of a multilayered K14+ ORS. K6irs1 and hHa1 were expressed but mislocalized in short ILK-K5 HFs (arrowheads). Bars, 50 μm.

development, we performed BrdU incorporation assays and determined Ki67 expression. At P7, both fully developed ILK-K5 HFs (Fig. 6 A, ▲) as well as growth-retarded HFs (Fig. 6 A, ■) showed an elevated number of proliferating cells in the ORS. To quantify the number of proliferating cells, we counted their numbers on fully developed ILK-K5 HFs (Fig. 6 A, ▲), thereby ensuring comparison of identical HF developmental stages. Counting of proliferating cells in P7 ILK-K5 HFs revealed that the increased number of proliferating ORS cells was associated with a slight but not significantly lower amount of proliferating cells in the HM (Fig. 6, B–D). At P14, however, the number of proliferating cells significantly diminished in the HM and further increased in the ORS (Fig. 6, B–D), suggesting that ILK-deficient, rapidly proliferating TA cells are capable of proliferating but accumulate in the ORS. Moreover, neither TUNEL assays nor immunostaining for activated caspase-3 revealed an elevation in apoptotic cell numbers, indicating that cell survival was unaffected in the ILK-K5 HFs (unpublished data).

The ORS cells originate from the CD34-positive stem cell population that is located in the hair bulge (Blanpain and Fuchs, 2006). To determine whether ILK loss led to the elimination of CD34-positive cells, we immunostained P24 skin sections. Both control and ILK-K5 HFs contained CD34-positive cells in their hair bulges (Fig. 6 E). The formation of secondary hair germs is driven by the proliferation of hair bulge-derived TA cells triggered by the inductive activity of the DP. At P24, normal HFs are at the onset of anagen, and Ki67+ TA cells appeared adjacent to the DP (Fig. 6 F, left). Ki67 staining of ILK-K5 skin revealed the presence of two types of HFs: ∼65% contained proliferating cells, suggesting that ILK-K5 HFs were principally capable of entering early stages of anagen (Fig. 6 F, middle). The remaining ILK-K5 HFs lacked proliferating cells (Fig. 6 F, right), likely because they were detached from the DP (Fig. 6 E, right) or connected to a malformed DP (Fig. 2 C) and, hence, did not receive the inductive signals. Collectively, these data suggest that ILK-K5 HFs contain CD34-positive stem cells that give rise to TA cells, which require ILK to migrate down to the HM or to trigger the downward growth of hair germs.

ILK is required for directional migration of keratinocytes

In ILK-K5 HFs, rapidly proliferating TA cells accumulate in the ORS, suggesting that ILK regulates their migration along the LM332-containing BM lining the HF. To test this assumption, we isolated keratinocytes from control and ILK-K5 mice and compared their migration behavior using different assays.

We first performed transwell migration assays and observed that migration of primary ILK-K5 keratinocytes on LM332, as well as their invasion through laminin-rich matrigel, was significantly impaired (Fig. 7 A). Next, we scratched monolayers of primary keratinocytes and observed the closure of the scratch over 12 h using time-lapse video microscopy.

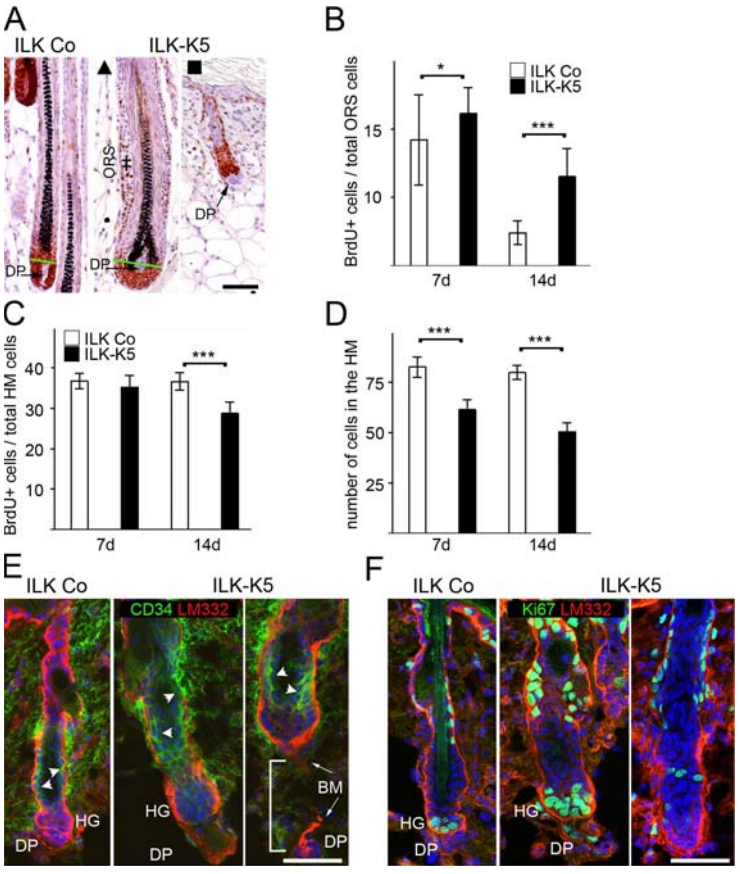

Figure 6. **ILK-deficient HFs accumulate proliferating cells in the ORS.** (A) Fully developed ILK-K5 HFs display a hyperplastic ORS (++). Both types of ILK-K5 HFs (▲, ■) show an elevated number of Ki67-positive ORS cells. Auber's line (green line; Auber, 1952) demarks the border between the proliferative and nonproliferative zones of the HM. Bar, 50 μm. (B) Quantification of BrdU+ cells in ILK Co and ILK-K5 HFs as a percentage of total cells in the ORS. The number of proliferating cells in the ORS is significantly increased in mutant HFs. (C) The percentage of proliferating cells in the HM is normal in 7-d HFs but significantly reduced in 14-d ILK-K5 HFs. (D) The overall number of cells is significantly reduced in the HM of 7- and 14-d ILK-K5 HFs. A minimum of 25 HFs were evaluated for B, C, and D at each time point (error bars indicate 95% confidence interval of mean values; *, $P < 0.05$; ***, $P < 0.001$). (E) Double immunostaining of CD34 and LM332 on skin sections of 24-d-old animals reveals the presence of a CD34+ bulge region (arrowheads) in ILK Co as well as ILK-K5 HFs. Note that ILK-K5 HFs display a severely abnormal morphology at this stage sometimes with detached DP (bracket). Bar, 25 μm. (F) Double immunostaining of Ki67 and LM332 on skin sections of 24-d-old animals. Proliferating hair germ is formed in HFs with DP and absent in ILK-K5 HFs without DP. HG, hair germ. Bar, 25 μm.

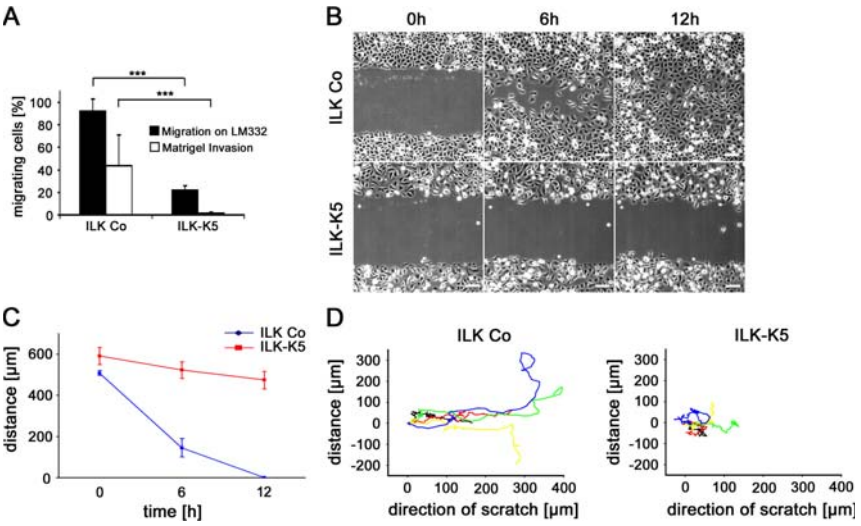

Figure 7. **Loss of ILK impairs migration.** (A) Freshly isolated keratinocytes were subjected to migration on LM332 and invasion through Matrigel. ILK-K5 keratinocytes show impaired migration and invasion (mean + SD of three independent experiments; ***, $P < 0.001$). (B) Time-lapse microscopy of a scratch assay. ILK-K5 keratinocyte exhibit delayed wound closure. Bar, 100 µm. (C) Quantification of the wound closure in the scratch assay. ILK-K5 wound closure is retarded (error bars indicate 95% confidence interval of mean values). (D) Reduced directionality of single ILK-K5 keratinocytes in the leading front of keratinocytes after scratch induction (five representative cells selected out of 40 analyzed for each genotype).

After scratching, control keratinocytes displayed directional migration and invaded the denuded area (Fig. 7, B and D) with a mean wound closure speed of 42.3 µm/h, leading to the closure of the scratch within 12 h (Fig. 7 C). In contrast, ILK-K5 keratinocytes often stopped and migrated back- and sideward (Fig. 7 D), with a reduced wound closure speed of 9.7 µm/h (Fig. 7, B and C). Furthermore, single-cell tracking at the migration front revealed a migration velocity of 0.7 ± 012 µm/min by ILK-K5 keratinocytes versus 0.9 ± 016 µm/min by control cells ($P < 0.01$).

To more closely evaluate the migration defect, we performed time-lapse microscopy of single keratinocytes. Control keratinocytes formed broad, usually single and stable leading edge lamella with a mean persistence of 985 ± 339 s that allowed single cells to directionally migrate (Fig. 8, A and B; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1). In sharp contrast, ILK-K5 lamellae were instable and collapsed within 618 ± 332 s (Fig. 8 B). Furthermore, the mutant cells constantly extended new lamellae toward different directions simultaneously, which gave rise to frequent changes of the migration direction and consequently prohibited directional movement (Fig. 8 A and Video 2).

To precisely characterize lamellipodia behavior, we monitored and quantified the plasma membrane extension rates of migrating cells using kymography (Hinz et al., 1999) over a period of 20 min. The lamellipodia of ILK-K5 keratinocytes persisted for a significantly shorter time (Fig. 8 C) and protruded more frequently than those of control keratinocytes (Fig. 8 D). Collectively, these data indicate that ILK is important for the stability and dynamics of the lamellae/lamellipodia and hence for directional migration of keratinocytes.

Loss of ILK leads to reduced spreading, focal adhesion (FA) formation, and FAK activation

The reduced adhesion of ILK-K5 keratinocytes to the ECM (Fig. 2 B and Fig. 3 A) can diminish the fixation of plasma membrane protrusions to the ECM, impair cytoskeletal reorganizations, and compromise integrin-triggered signaling, which in turn can cause the abnormal formation of leading-edge lamellipodia and impaired directional migration.

To test whether ILK is critical for the formation of integrin adhesion sites and integrin signaling, we isolated control and ILK-K5 keratinocytes. Both cultured cell types had comparable integrin profiles, β1 integrin activity, and α6β4-containing migration track patterns at the rear of the cell (Fig. S4, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1). The size of ILK-K5 cells was smaller, reaching a threefold smaller spreading area 40 h after plating on a mixture of Col I and FN (Fig. S4 C). Talin staining of adherent cells revealed that ILK-K5 keratinocytes formed fewer

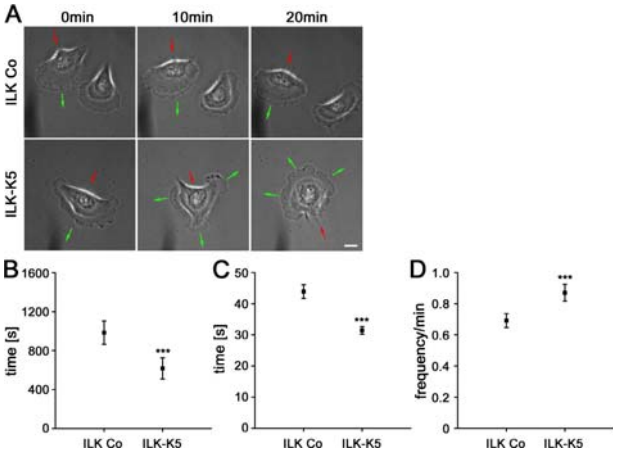

Figure 8. **ILK-K5 keratinocytes exhibit reduced lamellipodia stability.** (A) Time-lapse microscopy of single control and ILK-K5 keratinocytes. ILK-K5 keratinocytes formed instable lamellipodia, leading to a constant change of direction (green arrows indicate the protrusion and red arrows the retraction of the cell). Single frames chosen from Videos 1 and 2 (available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1). Bar, 10 µm. (B) Quantification of lamella stability. ILK-K5 keratinocytes exhibit significantly reduced lamella stability. (C and D) Quantification of lamellipodia persistence (C) and lamellipodia frequency (D). Compared with control keratinocytes, ILK-K5 lamellipodia protrusions are significantly less stable and occur more frequently. Error bars in indicate 95% confidence interval of mean values. ***, $P < 0.001$.

focal complexes (FCs) in the leading-edge lamellipodia (Fig. 9 A). Additional immunostaining for paxillin and FAK showed that only 30% of the cells contained mature FAs (Fig. 9, B and D) whose number per cell and size were significantly reduced (Fig. 9 C). The number of FAs in relation to the cell contact area, however, was not altered between ILK Co and ILK-K5 keratinocytes. In line with the severe spreading defect, ILK-K5 keratinocytes contained fewer stress fibers than control cells (Fig. 9, A, B, and D).

ILK can associate with several FA components, which in turn can modulate the activity of adaptor and signaling proteins, including FAK and Rac1 (Legate et al., 2006). Therefore, we tested whether their function is affected in ILK-K5 cells. Although total FAK levels were normal in ILK-K5 keratinocytes, the auto-activated form of FAK (pY397-FAK), as well as other tyrosine residues, such as Y861, were reduced (Fig. 9 E).

To test whether Rac-1 can be activated upon cell adhesion, we determined the levels of GTP-loaded Rac1 before and after cell seeding on LM322. Both ILK-K5 and control keratinocytes activated Rac1 to a similar extent (Fig. 9 F), indicating that the absence of ILK does not impair Rac1 activation in keratinocytes. Moreover, growth factor–induced activation of Rac1 became similarly increased in control and ILK-K5 keratinocytes (unpublished data).

Discussion

In the present paper, we report that a keratinocyte-restricted deletion of the ILK gene in mice leads to abnormal HF morphogenesis and epidermal defects with blisters, ectopic keratinocyte proliferation in suprabasal cell layers, and abnormal keratinocyte differentiation. Mutant HFs produced proliferating progenitor cells, which accumulated in the ORS and failed to replenish the HM. In vitro experiments revealed that ILK-deficient keratinocytes were unable to firmly stabilize lamellipodia, leading to impaired directional migration and providing a potential explanation for the accumulation of progenitor cells in the ORS.

Epidermal morphogenesis

The most prominent defects of the ILK-deficient epidermis were detachment from the dermal–epidermal BM and hyperthickening. The hyperthickened epidermis contained a normal number of proliferating keratinocytes in the basal layer and, surprisingly, also proliferating keratinocytes ectopically in the suprabasal layers. The cycling cells in the suprabasal layers expressed markers of basal keratinocytes, including K5 and K14; β1, α6, and β4 integrins; and LM332, and were unevenly distributed. They were detected in areas where the epidermis was firmly attached to the BM but also in epidermal stretches above microblisters. A similar hyperplastic epidermis was previously observed in transgenic mice ectopically expressing β1 integrin in the stratum granulosum (Carroll et al., 1995). The β1 transgenic keratinocytes were hyperproliferative, which was thought to be triggered by an excessive cytokine release from infiltrating inflammatory cells. Because we did not observe a chronic wounding response with an obvious inflammatory infiltrate (likely because the blistering was mild) at P7, when suprabasal proliferation was already evident, the proliferation and hyperplasia of the ILK-K5 epidermis must be triggered by a different

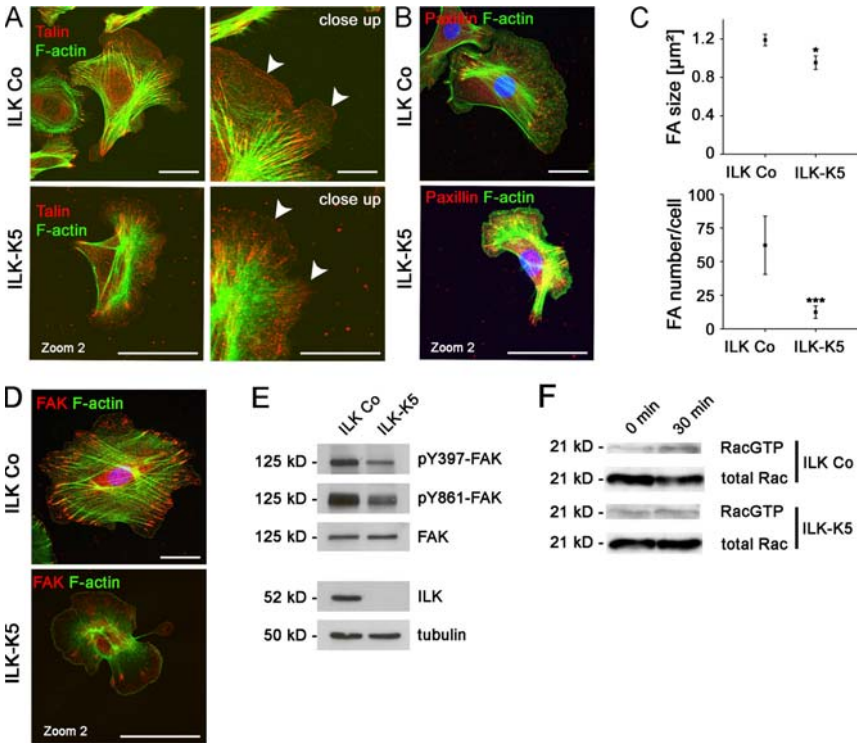

Figure 9. **Impaired formation of FAs but normal Rac1 activation in ILK-K5 keratinocytes.** (A, left) Immunostaining of primary control and ILK-K5 keratinocytes for talin and F-actin. Control and ILK-K5 keratinocytes contain talin in FAs. ILK-K5 cells are enlarged twofold compared with control cells. Bars, 10 µm. (right) A close up demonstrates talin in FCs at the leading edge of control cells (arrowheads). ILK-K5 keratinocytes have fewer talin-positive FCs at the leading edge (arrowheads). Bars, 2.5 µm. (B) Immunostaining of control and ILK-K5 keratinocytes for paxillin, F-actin, and DAPI. Bars, 10 µm. (C) Quantification of paxillin-containing FAs. ILK-K5 keratinocytes exhibit reduced size and amount of paxillin containing FAs compared with control keratinocytes. Error bars indicate 95% confidence interval of mean values. *, $P < 0.05$; ***, $P < 0.001$. (D) Immunostaining of primary control and ILK-K5 keratinocytes for FAK, F-actin, and DAPI. Note that mutant keratinocytes form fewer FAs that are poorly linked to thin and disorganized actin fibers. Bars, 10 µm. (E) Western blot analysis of protein lysates from primary keratinocytes showing reduced FAK phosphorylation levels in the absence of ILK. (F) Western blot analysis of a GTPase pull-down assay showing normal activation of Rac1 in primary ILK-K5 keratinocytes after 30 min of adhesion on a LM322-rich matrix.

mechanism. A possible explanation is that the proliferating basal keratinocytes detach because of an impaired adhesion strength, which leads to their ectopic location and marked thickening of the epidermis. It could also be that an accelerated proliferation rate contributes to the ectopic distribution of proliferating keratinocytes. Such a notion is supported by the elevated proliferation rate of primary keratinocytes in vitro. However, it is currently unclear why ILK-K5 keratinocytes would proliferate better than their normal control counterparts. Finally, delayed terminal differentiation of suprabasal keratinocytes may additionally contribute to the epidermal hyperplasia. In ILK-K5 epidermis, the K5- and K14-positive keratinocyte zone extended into several layers of the K10-positive stratum granulosum. Also the loricrin-positive cell compartment was increased. Interestingly, epidermal thickening and delay in keratinocyte differentiation was also observed in the β1 integrin–deficient epidermis (Brakebusch et al., 2000). In contrast to the ILK-K5 skin, however, the hyperthickened, β1 integrin–null epidermis contained fewer proliferating basal keratinocytes (Brakebusch et al., 2000; Raghavan et al., 2000), suggesting that β1 integrins accomplish keratinocyte differentiation through ILK and keratinocyte proliferation through an ILK-independent mechanism.

The diminished integrin-mediated attachment of keratinocytes to the BM resulted in blister formation, deterioration of the BM, and abnormal distribution of E-cadherin and β-catenin above blisters. In attached epidermis, E-cadherin and β-catenin were normally distributed, suggesting that ILK affects E-cadherin–based cell–cell adhesion structures rather indirectly. This is in contrast to previous reports showing that ILK regulates E-cadherin expression (Tan et al., 2001) and assembly of E-cadherin–based cell–cell adhesions (Vespa et al., 2005).

HF development and cycling

The most impressive phenotype of ILK-K5 mice is their progressive hair loss, which is completed at the age of 6–8 wk. Upon completion of morphogenesis, ILK-K5 skin revealed two types of abnormal HFs: long HFs with multilayered ORS and short, immature HFs that were stuck in the dermis. The existence of two types of HFs is most easily explained by the asynchronous development of HFs over a period of several days. The depletion of the ILK protein around birth is consequently hitting HFs later (long HFs; Figs. 2, 5, and 6, ▲) or earlier (short HFs; Figs. 2, 5, and 6, ■) in their development. In both types of HFs, although much more pronounced in long HFs, we observed an accumulation of proliferating cells in the hyperthickened ORS. The concomitant reduction of proliferating cells in the HM and the presence of CD34-postive stem cells in the hair bulge suggest that TA cells are generated but fail to migrate down to and replenish the HM, arresting HF development and maintenance. It is conceivable that hyperproliferation, like in the epidermis, may additionally contribute to the hyperthickening of the ORS.

We also observed abnormal localization of DPs during HF morphogenesis and detachment of the DP from ∼35% ILK-K5 HFs in P24 mice. Because the DP is releasing signals that are required for HF development and maintenance (Panteleyev et al., 1998), such abnormalities are likely to contribute to the hair loss. Interestingly, ILK-K5 HFs still connected to the DP could respond to the inductive signals and trigger sustained proliferation. In spite of the successful induction of anaphase, however, downward migration of the mutant HF epithelium was never observed.

What could be the underlying mechanism for the defective migration? Our analysis of primary keratinocytes revealed that loss of ILK alters the formation of mature FAs and prevents persistent, directional migration. Single-cell imaging demonstrated that ILK-K5 keratinocytes are perfectly able to form membrane protrusions but are unable to stabilize them over a prolonged period of time. As a consequence, lamellipodia are short-lived and frequently collapse. Interestingly, ILK-K5 keratinocytes swiftly respond with the formation of new lamellipodia, often simultaneously at multiple sites of the cell. In vivo such a high turnover rate of lamellipodia would force migrating ILK-K5 ORS cells to continuously change the direction of movement, which, along with the reduced migration velocity, could explain their accumulation in the ORS and their handicap to arrive in the HM.

Molecularly, we found several defects that could account for the impaired directional persistence and migration speed. First, ILK-K5 keratinocytes showed weakened integrin adhesion, which could compromise the fixation of lamellipodia. Second, the defective formation of integrin adhesion sites could lessen integrin-signaling pathways crucial for cell migration, such as the activation of FAK. Third, diminished integrin signaling could, in turn, lead to an impaired spatiotemporal activation of small Rho-like GTPases. The stabilization of lamellipodia and directional migration of keratinocytes critically depends on the optimal activation of the small GTPase Rac1 (Nobes and Hall, 1999; Ridley et al., 2003). In vitro studies with human keratinocytes revealed that high Rac1 activity can lead to inefficient migration with low lamellipodia persistence (Borm et al., 2005). Likewise, reduced Rac1 activity in α3β1 integrin–deficient keratinocytes can also result in directional migration defects and short-lived leading-edge lamellipodia (Choma et al., 2004). The ILK-K5 keratinocytes show a normal Rac1 activation after seeding on a LM322-enriched ECM, indicating that either ILK is not required for modulating Rac1 activity in keratinocytes or we were unable to detect small but critical differences in Rac1 activation between control and ILK-K5 keratinocytes. Thus, we anticipate that loss of ILK is sufficient to compromise the dynamics of lamellipodia and FAs and, consequently, results in altered cell migration.

An abnormal proliferation rate of HM cells could also potentially contribute to hair loss in ILK-K5 mice. Loss of β1 integrins impairs ORS cell migration and proliferation of HM cells (Brakebusch et al., 2000; Raghavan et al., 2000). In sharp contrast, we found robust proliferation in the HM of short ILK-K5 HFs. In fully developed ILK-K5 HFs, the number of proliferating HM cells diminished with the accumulation of proliferating cells in the ORS. These findings, along with the increased proliferation rate of primary ILK-K5 keratinocytes in vitro, suggest that HM cell proliferation can be sustained in the absence of ILK.

ILK does not regulate GSK-3β activity in HFs

The inactivation of GSK-3β and the subsequent stability and nuclear translocation of β-catenin and formation of a Lef1–Tcf–β-catenin complex plays a fundamental role for the differentiation of the HM cells into the precortical HM and HS (DasGupta and Fuchs, 1999; Huelsken et al., 2001). The inactivation of GSK-3β and stabilization of β-catenin is achieved by Wnt signals (Logan and Nusse, 2004) or by ILK-dependent phosphorylation of GSK-3β (Delcommenne et al., 1998) and/or inhibition of the β-catenin destruction complex (Oloumi et al., 2006). Despite the high expression of ILK throughout the entire HM, we found no evidence for reduced GSK-3β phosphorylation in ILK-K5 keratinocytes, decreased β-catenin levels, diminished Lef1–Tcf–β-catenin activity (both in prematurely arrested as well as fully developed HFs), or impaired differentiation of HM keratinocytes into trichocytes. These findings indicate that, contrary to what has been reported for intestinal and mammary epithelial cells (Novak et al., 1998) and for HEK293 and L2 cells, ILK is not required to stabilize β-catenin in the HF epithelium to induce expression of HS keratins. These findings, along with recent observations in mice with other organ-specific ILK deletions (Grashoff et al., 2003; Niewmierzycka et al., 2005), suggest that the ILK activity, at least toward GSK-3β and PKB/Akt, may not be required under physiological conditions in vivo.

Materials and methods

Mouse strains

To obtain mice with a keratinocyte-restricted deletion of the ILK gene, transgenic mice expressing *Cre* under the control of the keratin-5 promoter (Brakebusch et al., 2000) were crossed with floxed ILK mice (Grashoff et al., 2003; Sakai et al., 2003). Offspring were genotyped as described previously (Grashoff et al., 2003). BatGal transgenic mice carry Lef1/Tcf binding sites in front of a minimal promoter and the *lacZ* gene (Maretto et al., 2003) and were intercrossed with the ILK mutant mice.

Keratinocyte, epidermal lysate, and GTPase pull-down assay

Primary keratinocytes were cultured in keratinocyte growth medium containing 8% FCS and low Ca^{2+} (45 μM) on cell culture dishes coated with a mixture of Col I (Cohesion) and FN (Invitrogen) to subconfluence as described previously (Romero et al., 1999). Protein lysates from keratinocytes or epidermis were separated by SDS gel electrophoresis, blotted, and incubated with the indicated antibodies.

For GTPase pull-down assays, keratinocytes were cultivated to 70% confluence. Cells were then serum starved overnight and detached by Trypsin/EDTA treatment (Invitrogen). Detached cells were resuspended in serum-free keratinocyte growth medium and kept for 30 min in suspension. For adhesion-induced GTPase activation, cells were plated on a LM332-rich matrix produced by Rac-11P/SD squamous cell carcinoma cells for 30 min (Sonnenberg et al., 1993). Cells were washed twice with PBS and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM $MgCl_2$, 1 mM NaF, and 1 mM Na_3VO_4; all from Sigma-Aldrich) supplemented with protease inhibitor cocktail tablets (Complete Mini, EDTA-free; Roche) and containing biotinylated PAK-CRIB peptide (a gift from J. Collard, Netherlands Cancer Institute, Amsterdam, Netherlands). Lysates were centrifuged at 20,000 *g* for 10 min at 4°C, and the supernatant was subsequently incubated for 45 min at 4°C. Next, lysates were incubated with streptavidin-conjugated agarose beads (GE Healthcare) for 30 min at 4°C. Beads were washed three times with lysis buffer, resuspended in 2× SDS sample buffer, and boiled for 5 min at 95°C. The supernatant was subjected to SDS gel electrophoresis, Western blotting, and immunodetection by the indicated antibodies. The following antibodies were used for Western blot analysis: mouse mAb against ILK (clone 3; BD Biosciences); rat mAb against α-tubulin (Kilmartin et al., 1982); rabbit pAb against PKB/Akt and phospho-PKB/Akt (Ser473; Cell Signaling Technology); mouse mAb against GSK-3β (BD Biosciences); rabbit pAb against phospho–GSK-3β (Ser9; Biosource International); mouse mAb against cyclin D1/2 (Upstate Biotechnology); rabbit pAb against cyclin A (Santa Cruz Biotechnology, Inc.); rabbit pAb against p42/44 MAPK (Cell Signaling Technology); mouse mAb against phospho-p42/44 MAPK Thr202/204 (New England Biolabs, Inc.); rat mAb against E-cadherin (Zymed Laboratories); rabbit pAb against β-catenin (Sigma-Aldrich); rabbit pAbs against FAK (Upstate Biotechnology) and pFAK (Tyr397and Tyr861; Biosource International); mouse mAb against Rac1 (BD Biosciences); and goat anti–rat HRP, goat anti–mouse HRP, and goat anti–rabbit HRP (Bio-Rad Laboratories).

Histology and immunohistochemistry

Skin samples were fixed in 4% PFA in PBS, pH 7.2, overnight, dehydrated in a graded alcohol series, and embedded in paraffin (Paraplast X-tra; Sigma-Aldrich) or frozen unfixed in OCT (Thermo Shandon). Immunohistochemistry of skin sections was performed as described previously (Brakebusch et al., 2000). For cellular immunostainings, keratinocytes were seeded on chamber slides (Nunc) coated with 5 μg/ml of purified LM332 or 30 μg/ml Col I and 10 μg/ml FN and allowed to spread for 40 h. Cells were washed in PBS, fixed in 4% PFA, and incubated with the indicated antibodies. To determine BrdU incorporation, mice were injected with BrdU (100 μg/g body weight) 2 h before killing. Assessment of proliferation of cultured keratinocytes was performed with the Cell Proliferation ELISA according to the manufacturer's protocol (Roche). The following antibodies were used for immunohistology: rabbit pAb against ILK (Cell Signaling Technology); FITC-conjugated mAb against integrin α6 (BD Biosciences); rat mAb against β1 integrin (Chemicon); rat mAb against β4 integrin (BD Biosciences); rabbit pAb against laminin-5 (Brakebusch et al., 2000); rabbit pAbs against keratins 6, 10, and 14 and loricrin (Covance); rat mAb against E-cadherin; rabbit pAb against β-catenin; rabbit pAb β-catenin (Huelsken et al., 2000); rat mAb against nidogen (Chemicon); rabbit pAb against desmoplakin (Research Diagnostics); rabbit pAb against plakoglobin (Santa Cruz Biotechnology, Inc.); rabbit pAb against Lef1 (obtained from R. Grosschedl, Max Planck Institute of Immunobiology, Freiburg, Germany); rat Ki67 (Dianova); guinea pig pAbs against HF keratins (K6hf, K6irs1, K6irs2, K6irs3, K6irs4, hHa4, hHa5, hHb2, hHb5, CK5, and CK14; made by L. Langbein, German Cancer Research Center, Heidelberg, Germany); rat mAb against CD34 (clone RAM34; eBioscience); FITC-conjugated mouse mAb and POD-conjugated mAb against BrdU (Roche); rabbit pAb against cleaved caspase-3 (Asp175; Cell Signaling Technology); mouse mAb against paxillin (BD Biosciences); rabbit pAbs against FAK (Upstate Biotechnology) and phospho-FAK (Tyr397 and Tyr861; Biosource International); mouse mAb against Talin (Sigma-Aldrich); phalloidin Alexa488 (Invitrogen); goat anti–mouse Cy3, goat anti–rat Cy3, goat anti–rabbit FITC, and donkey anti–rabbit Cy3 (Jackson ImmunoResearch Laboratories); goat anti–rabbit Alexa488 (Sigma-Aldrich); and goat anti–rat Alexa488 (Invitrogen). Images were collected at room temperature by confocal microscopy (DMIRE2; Leica) using the Leica Confocal Software (version 2.5 Build 1227) with 63× NA 1.4 or 100× NA 1.4 oil objectives or by bright field microscopy (Axioskop; Carl Zeiss MicroImaging, Inc.) with 10× NA 0.3, 20× NA 0.5, or 40× NA 0.75 objectives, a camera (DC500; Leica), and IM50 software.

FACS analysis

Flow cytometry was performed as described by Brakebusch et al. (2000). Antibodies used for FACS analysis are as follows: FITC-conjugated hamster mAb against integrin β1; rat mAb against integrin β1 9EG7; FITC-conjugated rat mAb against integrin α6; biotinylated rat mAb against integrin αV; rat mAb against integrin β4; FITC-conjugated hamster mAb against integrin α2; biotinylated rat mAb against integrin α5 (all obtained from BD Biosciences); mouse mAb against integrin α3 (BD Biosciences); Streptavidin-Cy5 (BD Biosciences); mouse mAb anti–rat FITC (BD Biosciences); and goat anti–mouse FITC (Jackson ImmunoResearch Laboratories).

Adhesion and transwell assays

Adhesion of epidermal keratinocytes to ECM proteins (poly-L-lysine [Sigma-Aldrich], Col I, Col IV [a gift from R. Timpl, Max Planck Institute of Biochemistry, Martinsried, Germany], FN, and LM332) was measured as described previously (Fässler et al., 1995). Transwell migration and matrigel invasion assays of primary keratinocytes were performed as described by Thomas et al. (2001).

Cell-wounding assay

Monolayers were treated with 4 μg/ml Mitomycin C (Sigma-Aldrich) for 4 h before scratching with a 200-μl plastic micropipette to obtain wound

widths of 500–600 μm. Live-cell recordings were performed immediately after wounding for 12 h at 37°C and 5% CO_2 using a microscope (Axiovert; Carl Zeiss MicroImaging, Inc.) equipped with 10× NA 0.3, 20× NA 0.4, 40× NA 0.6, and 100× NA 1.3 objectives, motorized scanning table (Märzhäuser) and a stage incubator (EMBL Precision Engineering). Images were captured every 10 min with a cooled charge-coupled device camera (MicroMAX; Roper Scientific) using the MetaMorph software (Universal Imaging Corp.) for microscope control and data acquisition. Wound closure was quantified by measuring the distance between both leading edges moving toward the wound in 20 randomly chosen regions. At least four independent scratch-wound experiments were used for calculations. Migration velocity was determined by calculating the slope of a linear regression line. Single-cell tracking of cells within the leading edge was performed using MetaMorph software, choosing 15 cells each in at least three independent experiments.

Cell spreading

Cells were seeded on Col I/FN-coated dishes (MatTek Corporation) and allowed to spread for the indicated time. Four images were taken by the live-cell recording unit for each time point, and cell area was assessed using MetaMorph software.

Kymograph analysis

Lamellipodia dynamics and lamella stability was analyzed using kymography (Hinz et al., 1999). We monitored at least 10 migrating cells over a period of 20 min with a frame rate of 4 s using the live-cell imaging unit (100× NA 1.3 objective). Subsequently, eight areas of interest across the cell lamella with a 1-pixel width were defined. The 1-pixel-wide images were pasted side-by-side to generate a composite image of membrane dynamic at a single point along the cell lamella. As described by Hinz et al. (1999), slopes of these lines were used to calculate the velocities, and projections of these lines along the x axis (time) were used to calculate the persistence of protrusions.

Transmission EM

Transmission EM was performed as described previously (Grose et al., 2002).

Statistical analysis

Statistical evaluation was performed with SPSS software (SPSS, Inc). Statistical significance between data groups was determined by Whitney U test and subdivided into three groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Online supplemental material

Fig. S1 shows the ILK expression on newborn and 2-d-old skin sections and the integrin-expression pattern on freshly isolated control and ILK-K5 keratinocytes. Fig. S2 shows the numbers of proliferating cells in basal and suprabasal layers of control and ILK-K5 epidermis, in vitro proliferation of primary control and ILK-K5 keratinocytes, and the phosphorylation levels of GSK-3β and PKB/Akt. Fig. S3 shows immunostaining for Mac1 and Gr1 on skin sections of 7-d- and 2-wk-old mice and transmission EM of desmosomal contacts in the epidermis. Fig. S4 shows the integrin-expression pattern and immunostaining for integrins on cultured primary control and ILK-K5 keratinocytes and spreading kinetics of freshly isolated keratinocyte. Video 1 shows time-lapse video microscopy of control keratinocytes. Video 2 shows time-lapse video microscopy of ILK-K5 keratinocytes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608125/DC1.

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Publication 3

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PINCH-1 works in an ILK-dependent as well as independent manner in keratinocytes

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Manuscript in preparation

PINCH1 works in an ILK-dependent as well as independent manner in keratinocytes

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Running title: PINCH1-deficient epidermis and hair follicles

Key words: integrin, PINCH, IPP complex, adhesion, migration, hair follicle, epidermis

Abstract

PINCH1 is a LIM-only domain protein that forms a ternary complex together with integrin-linked kinase (ILK) and parvin (IPP-complex) and is subsequently recruited to integrin adhesion sites. We report here that the keratinocyte-restricted deletion of the PINCH1 gene results in epidermal detachment from the epidermal-dermal basement membrane, epidermal hyperthickening and progressive hair loss in mice. The hyperthickened epidermis was characterized by the presence of cycling, integrin-positive keratinocytes in the suprabasal layers. This was likely due to impaired adhesion to and displacement from the BM rather than an aberrant orientation of mitotic spindles and thereby abnormal positioning of cycling cells. Interestingly, PINCH1-deficient keratinocytes displayed severe cell-cell adhesion defects, which were neither observed in ILK- nor β1 integrin-null epidermis. The mutant hair follicles were highly distorted and shortened. They failed to initiate epithelial downgrowth during cycling resulting in their elimination by inflammatory macrophages. Impaired hair downgrowth was associated with severe defects in focal adhesion formation, actin cytoskeleton assembly and migration of keratinocytes lacking PINCH1 *in vitro*. Our data show that PINCH1 is important for epidermis and hair follicle biology and that it acts together with as well as independent of β1 integrin and ILK.

Abbreviations

BM, basement membrane; Col1: collagen type 1; d: days; DEJ: dermal-epidermal junction; DP, dermal papilla; DSP: desmoplakin; F-actin: filamentous actin; FA: focal adhesion; FC: focal complex; FN: fibronectin; HF, hair follicle; HS: hair shaft; IF: immunofluorescence; IFE: interfollicular epidermis; ILK, integrin-linked kinase; IRS: inner root sheath; IPP: ILK-PINCH-Parvin; K: keratin; KGM: keratinocyte growth medium; LM332: laminin332, laminin-5; Lor: loricrin; ORS: outer root sheath; P: postnatal day; PFA: para-formaldehyde; PG: plakoglobin; TA: transit-amplifying; w: weeks

Introduction

The skin is the largest organ in mammals and provides multiple critical functions required for survival. It forms a tight barrier which prevents loss of body fluids from the inside and protects against infectious agents, temperature changes, trauma or substance uptake from the outside. The skin is composed of an epithelial compartment called epidermis, a mesenchymal compartment called dermis, a basement membrane (BM) which separates them, and the adipose subcutis. The epidermis is a stratified squamous epithelium composed of several layers. The basal cell layer is in direct contact with the underlying BM and contains a pool of stem and/or progenitor cells. Upon differentiation keratinocytes withdraw from cell cycle, undergo a distinct differentiation program and move upwards through spinous, granular layers and finally the stratum corneum before they slough off (Fuchs and Raghavan, 2002). Two distinct processes have been described to ensure epidermal stratification from the basal cell layer: (i) down-modulation of the BM adhesion and suprabasal translocation of committed cells or (ii) asymmetric cell division of basal cells leading to the suprabasal positioning of one daughter cell (Lechler and Fuchs, 2005; Vaezi et al., 2002). It was recently reported, however, that the spindle orientation in dividing basal keratinocytes of adult mouse skin occurs parallel to the BM, thus favoring BM detachment as mechanism leading to epidermal stratification (Clayton et al., 2007).

Hair follicles (HFs) arise from the epidermis during embryogenesis in response to a tightly controlled exchange of signaling molecules with the underlying mesenchyme. HF morphogenesis lasts until postpartum and is characterized by epithelial-mesenchymal interactions, localized downgrowth of the epithelium into the dermis and keratinocyte differentiation. The mature HF forms a unit between the epithelial cone and a specialized mesenchymal compartment, the dermal papilla (DP) located at the distal tip of the cone. The HF epithelium contains concentric layers such as the outer root sheath (ORS), inner root sheath (IRS), the central hair shaft (HS), and at its base the proliferating hair matrix cells that is in contact with the DP. The HF stem cells reside in the bulge, a specific region

within the proximal ORS. From there, transit-amplifying (TA) cells constantly migrate downwards along the ORS to replenish the proliferative cell pool of the hair matrix. After completion of morphogenesis HFs undergo life-long cycles of regression (catagen), quiescence (telogen) and re-growth (anagen) (Paus and Cotsarelis, 1999).

Both cell-matrix and cell-cell adhesions play fundamental roles in maintaining the protective and barrier functions of the epidermis. Attachment to the BM is mediated by α6β4 integrins in hemidesmosomes and several members of the β1 integrin subfamily. Intercellular adhesion within the epidermis in turn occurs via adherens junctions, desmosomes and tight junctions (Fuchs and Raghavan, 2002). The tight interplay of the different adherens structures is crucial for the functional integrity of the epithelium. Disruptions of any of these components affects epidermal homeostasis, function and differentiation as well as HF formation/maintenance (Jamora and Fuchs, 2002).

Integrins play a fundamental role in mediating cell-matrix adhesion. They are heterodimeric transmembrane receptors composed of an α and a β subunit that mediate binding to extracellular matrix components. Integrin engagement anchors the cytoskeleton to the cell membrane and regulates intracellular signaling cascades. Various integrins are found in the epidermis. Under normal conditions α2β1, α3β1, α6β4 and αvβ5 are expressed by basal and αvβ8 in suprabasal keratinocytes. Under pathological conditions such as response to injury or *in vitro* culture keratinocytes upregulate α5β1, αvβ6 and α9β1 (Watt, 2002). α6β4 integrin is the core component of the hemidesmosomes anchoring basal keratinocytes to the underlying BM. It binds laminin-5 (LM332) and recruits intermediate filaments to hemidesmosomes. α3β1 integrin also binds LM332 and was suggested to be involved in hemidesmosome nucleation (Litjens et al., 2006). The role of α2β1 integrin is still unclear. It is abundantly expressed, localizes both to the baso-lateral and apical surfaces and is required for keratinocyte adhesion to type 1 collagen *in vitro* (Zhang et al., 2006). A requirement for α2β1 integrin in keratinocyte migration is debated (Grenache et

al., 2007; Zweers et al., 2007). The ORS keratinocytes express α2β1, α3β1 and α6β4 integrins (Commo and Bernard, 1997).

The role of the β1 integrin family in keratinocytes affects epidermal adhesion, proliferation and differentiation, BM maintenance, HF formation and wound healing (Brakebusch et al., 2000; Grose et al., 2002; Raghavan et al., 2000).

As integrin cytoplasmic tails lack enzymatic activity as well as binding sites for cytoplasmic proteins, their functions depend on the recruitment of accessory proteins. One member of the “integrin adhesome” (Zaidel-Bar et al., 2007) is the integrin-linked kinase (ILK) that directly binds the β1 integrin cytoplasmic tail. ILK is composed of N-terminal ankyrin repeats and a C-terminal putative Ser/Thr protein kinase domain interspersed with a pleckstrin-homology-like domain. ILK binds C-teminally to the actin-binding parvin family consisting of α-, β- and γ-parvin and via the first N-terminal ankyrin repeat to PINCH (Tu et al., 1999). The formation of the heterotrimeric ILK-PINCH-Parvin (IPP)-complex occurs in the cytosol and precedes its translocation to the integrin adhesion site. Interestingly, protein stability of each of the IPP-complex members depends on complex formation as depletion of one member leads to proteasomal degradation of the others (Fukuda et al., 2003; Grashoff et al., 2004; Legate et al., 2006).

The PINCH family in vertebrates consists of two members: PINCH1 (LIMS1) and PINCH2 (LIMS2) whereas invertebrates such as *Caenorhabditis elegans* or *Drosophila melanogaster* possess only one PINCH ortholog. All PINCH proteins are composed of five LIM domains composed of two cysteine-rich zinc-fingers that mediate protein-protein interactions, and C-terminal nuclear localization sequences (Braun et al., 2003a; Rearden, 1994). Binding to ILK occurs through the first LIM domain (Tu et al., 1999). Genetic studies revealed that Unc-97/PINCH is required for the integrity of integrin attachment sites and thus for normal development of *C. elegans* (Hobert et al., 1999; Norman et al., 2007) and *D. melanogaster* (Clark et al., 2003). In mice, PINCH1 and PINCH2 are expressed in a large number of tissues (Braun et al., 2003a). PINCH2 deficient mice do not show any overt phenotype, but upregulation of PINCH1 in tissues with prominent PINCH2 expression suggests functional compensation of the two

isoforms *in vivo* (Stanchi et al., 2005). Genetic ablation of PINCH1 in mice leads to early embryonic lethality during implantation characterized by impaired endodermal and epiblast adhesion to an abnormal BM, abnormal cell-cell adhesion and increased apoptosis (Li et al., 2005; Liang et al., 2005). *In vitro* studies in Hela cells showed that PINCH1 – together with ILK - is required for actin-dependent processes such as spreading and migration, and for cell survival. The loss of both PINCH1 and ILK triggered apoptosis via impairing Akt phosphorylation. Interestingly, loss of PINCH1 reduced Akt phophorylation on both the Ser473 and Thr 308, whereas loss of ILK selectively affected Ser473 phosphorylation (Fukuda et al., 2003).

The fourth LIM domain of PINCH can weakly bind Nck2, a SH3- and SH2-containing adaptor protein. Nck2 in turn interacts with key components of growth factor receptor and GTPase signaling (Tu et al., 1998). Nck2 regulates actin dynamics through several pathways: via the WASP family members and the Arp2/3 complex, small GTPases and PAK or DOCK180 (Buday et al., 2002). Recent findings highlight the importance for the PINCH-Nck2 interaction for the regulation of cytoskeletal dynamics. Mutant PINCH defective for Nck2 binding is unable to localize to integrin adhesion sites. Furthermore, this mutant fails to rescue the spreading defects of PINCH1 and PINCH2 double null cells (Vaynberg et al., 2005; Velyvis et al., 2003). The use of deletion mutants of PINCH suggests that PINCH binding to different partners, i.e. Nck2 or ILK, might be crucial the for coupling integrin functions to downstream effectors (Norman et al., 2007; Xu et al., 2005). However, it is currently unclear whether the PINCH-Nck2 interaction is of any relevance *in vivo*. Mice with a genetic deletion of either Nck1 or Nck2 are phenotypically normal due to functional redundancy although PINCH binds Nck2 only (Bladt et al., 2003; Tu et al., 1998).

One additional link between the IPP-complex and the actin cytoskeleton is the interaction of PINCH and ILK with thymosin β4, a small, G-actin monomer sequestering peptide (Bock-Marquette et al., 2004). Furthermore, PINCH1 has been shown to interact with Ras-suppressor protein RSU-1 in both vertebrates (Dougherty et al., 2005) and *D. melanogaster* (Kadrmas et al., 2004) which

negatively regulates the JNK activity. Several studies *in vivo* and *in vitro* have also revealed nuclear localization of PINCH (Hobert et al., 1999; Li et al., 2005; Zhang et al., 2002). Although other LIM domain proteins also shuttle between adhesion sites and the nucleus (Hervy et al., 2006), no nuclear function has so far been attributed to PINCH. Despite the characterization of PINCH-specific interactors, the interdependence of protein stability within the IPP complex has hampered the identification of IPP-independent functions of PINCH or any other IPP-complex member. Only very recent findings that implicate PINCH1 in cell-cell adhesion or ILK in centrosome assembly assign separate functions, however, without addressing the molecular mechanisms (Fielding et al., 2008; Li et al., 2005).

To analyze PINCH1 function *in vivo* we conditionally ablated the PINCH1 gene in keratinocytes using the keratin5 (K5)-Cre transgene. By comparing the offsprings with the keratinocyte-specific depletions of ILK (Lorenz et al., 2007) we addressed the question whether ILK and PINCH1 function exclusively in concert with each other or whether IPP-independent functions play a role in keratinocytes. Our data demonstrate that PINCH1 is important for integrin-mediated cell adhesion and cytoskeletal dynamics downstream of integrin and ILK. *In vitro* analysis of PINCH1-deficient keratinocytes revealed defective actin cytoskeleton formation and dynamics during adhesion, migration and spreading, reminiscent of but more pronounced than in the absence of ILK. Importantly, defects in intercellular adhesion were a consequence specific to PINCH1-deficiency. These results suggest that PINCH1 functions downstream of integrins in the regulation of cell-matrix and cell-cell adhesion, partly in cooperation with ILK but partly via IPP-independent pathways.

Materials and Methods

Mouse Lines

To obtain mice with a keratinocyte-restricted deletion of the PINCH1 gene, a transgenic line expressing *Cre* under the control of the K5 promoter (Ramirez et al., 2004) was crossed with floxed PINCH1 animals (Li et al., 2005). Offspring were genotyped by PCR as described (Li et al., 2005).

Keratinocyte cell culture and epidermal lysates

Primary keratinocytes were isolated and cultured in keratinocyte growth medium (KGM) based on MEM (Spinner Modification; Sigma) containing 8% FCS, low Ca^{2+} (45 µM) and supplemented with growth factors on a mixture of Col1 (Cohesion) and FN (Merck) (10µg/ml) as described (Montanez et al., 2007). *In vitro* differentiation of primary keratinocytes was induced by adding 1.2 mM $CaCl_2$ to KGM for 24 hours.

Epidermal lysates were prepared from freshly isolated epidermal keratinocytes lysed in lysis buffer (1% Triton X-100; 1% Na-deoxycholate; 0.1% SDS; 50 mM HEPES, pH7.4; 150 mM NaCl; 10% glycerol; 100 mM NaF; 10 mM $Na_4P_2O_7$; 1.5 mM $MgCl_2$; 1 mM EGTA; 1mM Na-orthovanadate; all from Sigma) supplemented with protease inhibitor cocktail tablets (Complete Mini, EDTA-free; Roche). Protein lysates were separated by SDS gelelectrophoresis, blotted and incubated with the indicated antibodies.

The following antibodies were used for western blot analysis: mouse mAb against ILK (clone 3, BD Biosciences); mouse mAb against PINCH (BD Biosciences); rat mAb against tubulin (clone YL1/2, Millipore); goat anti rat-HRP, goat anti mouse-HRP (Biorad).

Cell wounding assay and single cell analysis

Cell wounding assays were performed with monolayers of primary keratinocytes treated with 4 µg/ml Mitomycin C (Sigma) for 4h prior to scratching with a 200µl plastic micropipette to obtain wound widths of 500–600 µm. Wound closure was

monitored for 12h with a frame rate of 10 min and quantified by measuring the distance between both leading edges moving towards the wound in 20 randomly chosen regions per time point. Migration velocity was determined by calculating the slope of a linear regression line. For single cell analysis, cells were monitored over a period of 20 min with a frame rate of 4s. All live cell recordings were performed at 37°C and 5% CO_2 using a Zeiss Axiovert microscope equipped with a 10x NA0.3, 20x NA0.4, 40x NA0.6, 100x NA1.3 objectives, motorized scanning table (Märzhäuser) and a stage incubator (EMBL Precision Engineering). Images were captured with a cooled CCD camera (Roper Scientific MicroMAX) using the Metamorph software (Universal Imaging Corporation) for microscope control and data acquisition.

FACS analysis

Flow cytometry was carried out as previously described (Brakebusch et al., 2000). Antibodies used for FACS analysis: FITC-conjugated hamster mAb against integrin β1; rat mAb against integrin β1 9EG7; FITC-conjugated rat mAb against integrin α6; biotinylated rat mAb against integrin αV; rat mAb against integrin β4; FITC-conjugated hamster mAb against integrin α2; biotinylated rat mAb against integrin α5 (all BD Biosciences); Streptavidin-Cy5 (BD Biosciences); mouse mAb anti rat-FITC (BD Biosciences); goat anti mouse-FITC (Jackson Immunoresearch).

Adhesion assay

Adhesion of epidermal keratinocytes to extracellular matrix proteins (Poly-L-Lysine (Sigma), Col1 (Cohesion), FN (Merck), LM332 (M. Aumailley)) was measured as previously described (Fässler et al., 1995).

Transmission Electron Microscopy

TEM was performed as described in (Grose et al. 2000).

Histology and immunohistochemistry

Skin samples were fixed in 4% PFA in PBS, pH 7.2, overnight, dehydrated in a graded alcohol series, and embedded in paraffin (Paraplast X-tra; Sigma) or frozen unfixed in OCT (Thermo Shandon). Histology and immunohistochemistry of skin sections was performed as described (Brakebusch et al., 2000; Montanez et al., 2007). Cultured keratinocytes were seeded on Col1 (Cohesion) and FN (Merck) (10µg/ml)-coated glass cover slips and allowed to spread for 40h prior to optional differentiation and fixation in 4% fresh PFA. Cells were incubated with the indicated antibodies as described in (Montanez et al., 2007). To determine BrdU incorporation, mice were injected with BrdU (100 µg/g body weight) 4h before sacrificing.

The following antibodies were used for immunohistology: FITC-conjugated mouse mAb and POD-conjugated mAb against BrdU (Roche); rat mAb against E-cadherin (Zymed); rabbit pAb against α-catenin; rabbit pAb against β-catenin (Sigma); rabbit pAb β-catenin (Huelsken et al., 2000); rat mAb against CD34 (clone RAM34, eBioscience); rabbit pAb against desmoplakin (Research Diagnostics); rat mAb against Gr1 (BD Biosciences); mouse mAb against ILK (Upstate); FITC-conjugated mAb against integrin α6 (BD Pharmingen, CA, USA); rat mAb against β1 integrin (Chemicon); rat mAb against β4 integrin (BD Pharmingen); rabbit pAbs against keratin 6, 10 and 14, Loricrin (Covance); rat mAb against Ki67 (Dianova); rabbit pAb against LM332 (obtained from M. Aumailley, Cologne, Germany); rat mAb against Mac1 (BD Biosciences); rat mAb against nidogen (Chemicon); rabbit pAb against Paxillin (Santa Cruz); mouse mAb against PINCH (BD Biosciences); rabbit pAb against plakoglobin (Santa Cruz); mouse mAb against Vinculin (Sigma); Phalloidin Alexa488 (Invitrogen); goat anti mouse-Cy3, goat anti rat-Cy3, goat anti rabbit-FITC and donkey anti rabbit-Cy3 (Jackson Immunoresearch); goat anti rabbit-Alexa488 (Sigma); goat anti rat-Alexa488 (Invitrogen). Images were collected at room temperature by confocal microscopy (DMIRE2; Leica) using the Leica Confocal Software (version 2.5 Build 1227) with 63x NA1.4 or 100x NA1.4 oil objectives, or by bright field microscopy (Axioskop; Zeiss) with 20x NA0.5 or 40x NA0.75 objectives,

Leica DC500 camera and the IM50 Software. For quantification of HF lengths mosaic images of entire skin sections were taken using the Zeiss Axio Imager.Z1 with 10x NA0.25 objective. Mosaics were stitched and analyzed with the Axiovision software.

Analysis of epidermal cell division

Wholemounts of tail epidermis were prepared and stained as described by (Braun et al., 2003b). The following antibodies were used: rabbit pAb against phospho-histone H3 (Ser10) (Millipore); goat anti rabbit-Alexa488 (Sigma). Confocal stacks with a step size of 0.2 µm were collected at room temperature with the 63x NA1.4 oil objective using the DMIRE2 microscope (Leica) and Leica Confocal Software (version 2.5 Build 1227). Confocal stacks were 3D reconstructed and further analyzed by AMIRA® software (Visage Imaging).

Results

Linkage of PINCH1 locus to the Cre-transgene insertion site

In order to specifically delete PINCH1 from the keratinocytes in skin we intercrossed PINCH1$^{loxP/loxP}$ female animals (Li et al., 2005) with heterozygous PINCH1$^{loxP/lwt}$ males expressing the Cre recombinase under the control of the K5 promoter (Ramirez et al., 2004). The number of newborn PINCH1$^{loxP/loxP}$/K5-Cre (**P1-K5**) animals was significantly less than the expected Mendelian distribution of 25% (Table 1). Since the number of PINCH1$^{loxP/wt}$ pups without the K5-Cre transgene was comparably reduced and the other genotypes were evenly distributed with a frequency of 44 % (Table 1), we suspected a genetic linkage of the PINCH1 locus with the Cre-transgene, leading to co-segregation of the wild-type PINCH1 allele and the K5Cre-transgene. We tested this hypothesis by breeding the heterozygous PINCH1$^{loxP/lwt}$/K5-Cre males to wild type C57BL/6 mice. From this mating in turn we obtained a strongly reduced number of PINCH1$^{loxP/lwt}$/K5-Cre mice, supporting the assumption of genetic linkage between the loci. The resulting PINCH1$^{loxP/lwt}$/K5-Cre males were subsequently used for intercrosses with PINCH1$^{loxP/loxP}$ female animals. The distribution of genotypes among the offspring followed the expected ratio with a “yield” of more than 40% of P1-K5 animals (Table 1). PINCH1$^{loxP/lwt}$ animals without Cre transgene were used as controls for all experiments (**P1 Co**). PINCH1 homo- or heterozygous floxed animals did not display abnormalities (Li et al., 2005).

Deletion of PINCH1 in skin leads to progressive hair loss

The efficient deletion of PINCH1 from the skin epithelium was confirmed by Western blotting of epidermal lysates from P1 Co and P1-K5 animals. Whereas the PINCH1 protein was efficiently ablated two days after birth, PINCH1 expression was recovering with time resulting in a considerable amount of PINCH1 re-expression by the age of ten weeks (10w; Figure 1A). Important to note is that ILK levels are reduced upon deletion of PINCH1 but to a smaller extent than PINCH1 itself, suggesting that the residual ILK is stabilized even in

the absence of PINCH1. PINCH2 is not expressed under normal conditions in the epidermis or HFs and we did also not detect a compensatory upregulation in P1-K5 epidermal lysates as the antibody used recognizes both PINCH isoforms.

P1-K5 animals appeared normal at birth. During early postnatal development when control animals developed a dark skin color due to HF morphogenesis, P1-K5 animals developed patchy skin pigmentation. At postnatal day 14 (P14) control mice had developed their normal hair coat. The hair coat of P1-K5 animals by P14 was scattered with partial alopecia. By 8w after birth, when the first postnatal hair cycle was completed in controls, P1-K5 animals were almost without hairs (Figure 1B). Boldness and patchy skin pigmentation persisted during their entire life span, which was normal.

Epidermal homeostasis and HF development depend on PINCH1

Postnatal skin morphology was analyzed by hematoxylin and eosin staining on backskin sections. First signs of abnormal HF development in P1-K5 animals could be detected as early as P2 (*arrow* in Figure 2), while the interfollicular epidermis (IFE) appeared morphologically normal. At 2w of age clearly the IFE displayed several defects including hyperthickening, blistering at the dermal-epidermal junction (DEJ) (*asterisks* in Figure 2), flattened shape and increased intercellular spacing of keratinocytes (Figure 2, 3A). At 10w of age epidermal aberrations became reverted in large areas of the P1-K5 skin with an almost complete absence of blistering and reduction of the hyperthickening, while only in a few areas the defects persisted or became even more severe (Figure 3A). Interestingly, melanin deposits were frequently observed in the P1-K5 IFE, which was never observed in the controls (*arrow heads*, Figure 3A).

HF malformations in P1-K5 animals gradually aggravated with the age. During early postnatal development HFs normally asynchronously pass through the distinct stages of morphogenesis and steadily increase in length (Figure 2, 3C). By P14 control mice completed HF morphogenesis with all HFs residing deep in the subcutis and containing pigmented hair shafts (Figure 2, 3B, 3C). At P2 the HF defects became clearly visible in P1-K5 mice and ranged from severe

distortions to slight ORS hyperthickening, abnormal melanin deposition in the HF epithelium and condensed DPs. By P14 two subtypes of P1-K5 HFs could be observed: fully developed HFs that reached into the subcutis and developed a hyperthicknened ORS and condensed DP (▲; Figure 2, 3B); and short HFs that were residing in the dermis and were severely distorted with a misshapened or often absent DP (■; Figure 2, 3B). At 23d when control mice were in telogen, P1-K5 HFs had a similar length as control HFs and morphologically resembled telogen HFs indicating that they responded to cycling cues. However, the HF outgrowth during the following anagen as seen in controls by 4w was absent in P1-K5 skin (Figure 2, 3C). By 10w HFs were absent from the P1-K5 skin and prominent melanin condensates were visible in the upper dermis mainly causing the reticular pigmentation (*arrow heads* in Figure 2). These data indicate that PINCH1 is required to maintain epidermal and HF homeostasis.

Loss of PINCH1 impairs keratinocyte adhesion and integrin expression

The epidermal blistering could be caused by impaired integrin expression, distribution and/or function, which would result in diminished keratinocyte adhesion. To test these possibilities we determined integrin levels on freshly isolated keratinocytes and found that their levels were only slightly altered. Small subpopulations of cells contained reduced levels of β1, β4 and α6 integrins (Figure 4A). Interestingly this population was lost from the P1-K5 populations in culture (Supplementary Figure 1). Immunostaining of control and P1-K5 skin sections revealed a similar distribution of β1 around the entire basal keratinocyte, and of β4 and α6 at the basal side adjacent to the BM. However, in P1-K5 skin β1 and β4 integrin were also frequently reduced in basal cells or found in clusters of suprabasal cells (*arrow heads*, Figure 4B). Important to note is also the accumulation of strong β1 integrin expressing cells underneath the epidermis when the BM is detached, indicating substantial alterations of the underlying mesenchyme (*asterisks*, Figure 4B). Adhesion assays with primary keratinocytes expanded in culture for 4-5 days revealed that loss of PINCH1 severely

abrogated adhesion to LM332, fibronectin (FN) and collagen type 1 (Col1) and was significantly more pronounced than in the absence of ILK (Figure 4C).

Immunostaining for LM332 revealed that the BM was continuous and tightly attached to the basal keratinocytes in control skin. In contrast, P1-K5 skin often showed BM detachment from the basal keratinocytes and diffusion of LM332 into the dermis (Figure 4B). TEM of skin samples from 2w- and 4w-old animals also revealed a defective BM organization of P1-K5 skin with discontinous lamina densa. Hemidesmosomes were only present in areas with intact BM (*arrowhead*, Figure 4D). Interestingly, P1-K5 epidermis developed prominent cell-cell adhesion defects with large intercellular spaces with filamentous membrane protrusions connecting adjacent cells to each other (Figure 4D, right panels).

These findings demonstrate that loss of PINCH1 impairs integrin-mediated adhesion of keratinocytes to the BM by affecting both integrin function and integrin expression and that PINCH1 is required for the maintenance of stable intercellular adhesion.

PINCH1 ablation alters epidermal differentiation and proliferation

The presence of integrin expressing cells in suprabasal layers pointed to an abnormal differentiation of P1-K5 epidermis. Therefore, we analyzed differentiation of IFE by immunstaining for characteristic keratins for basal (K14), spinous (K10) and granular layers (loricrin). In control skin K14 was restricted to the basal keratinocytes while P1-K5 epidermis frequently contained several layers of K14 expressing cells. Likewise, the number of K10 expressing suprabasal layers cells was also increased in P1-K5 skin (Figure 5A). Loricrin was confined to a very thin granular layer in the normal epidermis, while in P1-K5 epidermis loricrin expression was observed in several layers of cells containing large, prominent nuclei. K6 known to be strongly expressed in injured skin was strongly upregulated in P1-K5 epidermis (Figure 5D).

Thickening of the epidermis is often indicative for hyperproliferative epithelia. Quantitative analysis of Ki67-positive keratinocytes revealed ectopic proliferation in the suprabasal layers of P1-K5 hyperthickened epidermis (Figure 5B, 5C)

whereas proliferation was restricted to the basal layer in control epidermis. The suprabasal proliferating keratinocytes expressed β1 and β4 integrins (Figure 5D). Next we excluded an abnormal orientation of the division plane as cause for the presence of proliferating cells in the suprabasal layers of P1-K5 epidermis by determining the mitotic spindle orientation in wholemount tail skin preparations from control and P1-K5 animals (Figure 5E).
These data suggest that the loss of PINCH1 alters epidermal differentiation and proliferation, but not mitotic spindle orientation in epidermal keratinocytes.

Inflammatory responses in the P1-K5 skin

Increased proliferation, epidermal hyperplasia and abnormal differentiation could also be caused by inflammation. As the skin is an immuno-competent organ, a small number of resident macrophages was present in the dermis of control animals at all stages analyzed (Figure 6A). At P2 the number of macrophages was scarce in P1-K5 skin. At P4 and P7, however, abnormal numbers of macrophages accumulated throughout the dermis and subcutis and around HFs (Figure 6A). Gr-1 expressing granulocytes were virtually absent from control skin while they were found around malformed HFs both at P4 and P7 (Figure 6B).

Polarity and cell-cell adhesion defects P1-K5 keratinocytes

Cell-cell adhesion, cell-ECM adhesion and cytoskeletal organization are essential for inducing and maintaining epithelial polarity. Filamentous (F)-actin and cell adherens junctions components, such as E-cadherin and β-catenin, were found laterally and apically in basal keratinocytes and around the entire cell surface of suprabasal cells. Basal keratinoyctes in the skin of P1-K5 lost their polarized F-actin distribution and contained F-actin at the basal cell surface (Figure 7A). Similarly, cell adherens junction components such as E-cadherin or β-catenin were also found at the basal side of basal keratinocytes (Figure 7A). Furthermore, suprabasal layers developed intercellular spaces, most prominently in non-recovered areas of 10w-old skin (*asterisks*, Figure 7A, B). Plakoglobin (PG) is incorporated into both adherens junctions and desomosomes throughout

the IFE and thus expressed in a polarized, lateral-apical manner in control epidermis. In P1-K5 epidermis, however, PG was also often found adjacent to the DEJ where it colocalized with α6 integrin (Figure 7B). Interestingly, we observed patches of suprabasal cells and corneocytes lacking PG (*arrowheads*, magnified insert, Figure 7B). Defects in PG localization raised the question whether desmosomes were affected by P1-K5 skin. Desmoplakin marks desmosomal junctions (Figure 7B) and was clearly mislocalized to the basal side of basal P1-K5 keratinoyctes (Figure 7B).

These data implicate PINCH1 in maintaining polarity and intercellular adhesion of keratinocytes by regulating the localization of components of cell-adherens junctions and desmosomes.

P1-K5 HFs maintain stem cells, but their progeny fails to move along the ORS

The HF as a micro-organ is maintained by stem cells in the bulge from which TA cells move along the ORS to replenish the proliferating hair matrix. To asses whether loss of PINCH1 affects HF proliferation, we stained for Ki67 and performed *in vivo* BrdU incorporation assays. To ensure the comparison of identical developmental stages we compared long, fully developed P1-K5 HFs (▲) with control HFs with completed morphogenesis. HFs from P1-K5 animals showed a higher proliferation rate in the ORS at P7 and P14 (Figure 8A, 8B) and a reduced HM size (Figure 8C). The proportion of proliferating cells in the HM was not altered in PINCH1 deficient HFs (Figure 8D). Shortened and developmentally arrested P1-K5 HFs (■) also showed a sustained proliferation in the epithelium proximal to the DP (Figure 8A).

The HF stem cell compartment was visualized by immunostaining CD34 on P24 skin (Figure 8E). By P24 HFs are at the onset of anagen. Signals from the DP induce proliferation of HF keratinocytes triggering the anew outgrowth of the epithelium. Ki67 was strongly induced in the newly formed hair germs in control skin at P24. Proliferation was also induced in P1-K5 HFs, while HF downgrowth did not occur (Figure 8F, 3C). To further address the presence and functionality

of the DP we stained P14 and P26 skin sections for versican, which is expressed in the active DP and the dermal sheet of anagen HFs. Versican expression was also strong in P1-K5 DPs and dermals sheets and extended into the upper dermis adjacent to the DEJ (Supplementary Figure 2).

Together these data demonstrate that keratinocytes in the P1-K5 HFs are able proliferate and respond to signals from active DPs. However, during morphogenesis proliferating cells accumulate within the ORS and at the beginning of the first HF cycle the outgrowth of the follicular epithelium fails to occur.

P1-K5 keratinocytes display a spreading and migration defect

Reduced cell adhesion implies a weakened interaction of the cell membrane with the ECM. This in turn can lead to destabilization of membrane protrusions, defects in cytoskeletal reorganizations and integrin signaling, thereby affecting spreading and migration. We therefore analyzed PINCH1 keratinocytes *in vitro*.

Primary keratinocytes were isolated and cultured for 4-5 days prior to the experiments. P1-K5 keratinocytes tended to grow in tight colonies of round, poorly spread cells as compared to control keratinocytes (Figure 9A).

When cells were allowed to spread on FN/Col1, P1-K5 keratinocytes poorly increased their size and reached a 4.3-fold smaller spreading area than control cells (Figure 9B).

Control keratinocytes were polarized with a single, broad lamella at the front and retracting fibers at the rear and were randomly migrating under normal culture conditions (*arrows*, Figure 9C). In contrast, P1-K5 keratinocytes were hardly spread and had small, unstable protrusions with the entire cell body remaining round. Frequently, P1-K5 cells had several protrusions at the same time (*arrows*, Figure 9C). Collapses of membrane protrusions were often leading to almost complete detachment of the cell body. Interestingly, a high retrograde flow of actin could be detected in P1-K5 keratinocytes. In line with the lack of stable lamellae formation the PINCH1 null cells were more stationary than their control counterparts. The migratory potential of P1-K5 cells was tested in a scratch

wounding assay. Control keratinocytes developed a leading edge and migrated towards the scratch with a speed of 22.9 µm/h. In contrast, P1-K5 keratinocytes failed to from a leading edge and hardly moved towards the scratch (Figure D, E). In transwell assays and invasion through laminin-rich Matrigel P1-K5 cells were also drastically impaired (data not shown).

These data indicate that PINCH1 is crucial for keratinocyte spreading and migration and the formation and anchoring of plasma membrane protrusions to the underlying substratum.

Loss of PINCH1 affects focal adhesions (FAs) and F-actin distribution

Defects in spreading and migration and the high rate of retrograde actin flow of PINCH1 deficient keratinocytes could be the cause of an impaired formation of integrin adhesion sites, inefficient anchoring of actin filaments to the plasma membrane or altered actin dynamics. We therefore cultured primary keratinocytes on glass cover slips coated with FN/Col1 and analyzed FA formation.

Control keratinocytes were spread and formed stress fibers anchored to abundant FAs that contained PINCH1 and Paxillin (Figure 10A). The clusters of small P1-K5 cells were frequently surrounded by contaminating non-deleted cells, but could be easily distinguished by their morphology and the loss of PINCH1 expression (Figure 10A). Interestingly, Paxillin and Vinculin could not be detected in any discrete “FA”-like structure of P1-K5 cells (Figure 10A, 10B). The only more intensely stained cellular structures were thick, collapsed lamellar protrusions (*arrowheads*, Figure 10A). FAs mature from initial adhesions, called focal complexes (FCs) and are found at the leading edge of lamellipodia in normal keratinocytes (*arrowheads*, Figure 10B). In P1-K5 cells also “FC”-like structures were absent.

In control keratinocytes PINCH1 colocalized with ILK in FAs at the tip of actin stress fibers. In the P1-K5 cells ILK was detected evenly in the cytoplasm (Figure 10C). Contaminating melanocytes in the P1-K5 primary cultures expressed high amounts of ILK (*asterisks*, Figure 10C). The actin cytoskeleton was also severely

affected by loss of PINCH1. Stress fiber formation was observed in controls but virtually absent in P1-K5 cells. Furthermore, the overall staining intensity for F-actin was lower than in control cells (Figure 10A, 10B, 10C). However, the defects in FA formation did not result from an impaired integrin expression, as the integrin expression of cultured keratinocytes was virtually normal, with the exception of an upregulation of α5 integrin (Supplementary Figure 1).

Defects in LM332 assembly into an intact BM was observed in the skin of P1-K5 animals. Abnormal secretion of LM332, one of the major BM molecules that keratinocytes bind to, could also compromise keratinocyte adhesion in cell culture. LM332 is deposited on the culture surface by control keratinocytes and left behind, when they are randomly migrating and secreted LM332 can also be observed around the P1-K5 keratinocytes (Figure 10D).

Taken together these data suggest that PINCH1 is crucial for stress fiber formation and the assembly of FAs.

In the absence of PINCH1 Vinculin is not recruited into cell-cell contacts

In addition to impaired cell-matrix interactions, cell-cell adhesion was abnormal in P1-K5 skin. To further investigate the cause for the cell-cell adhesion abnormalities *in vivo*, we differentiated cultured primary keratinocytes *in vitro* by Ca^{2+} induction and analyzed the molecular composition of the cell-cell adhesion plaque. β-catenin and E-cadherin are the core components of cell-adherens junctions, which were found in the cytoplasm of undifferentiated keratinocytes and were recruited into cell-cell adhesions upon Ca^{2+}-induced differentiation in control cells (Figure 11A). P1-K5 keratinocytes were able to assemble cell-adherens junctions containing both β-catenin and E-cadherin (Figure 11A). However, P1-K5 cells were frequently detached and only connected via filamentous membrane protrusions (Figure 11A, *arrowheads*). It is further important to note that the architecture of the differentiated epithelial sheets was different between controls and P1-K5 cells. In contrast to control cells, cell shape

of P1-K5 cells remained roundish leading to an increased thickness of the differentiated epithelial clusters (left stripes, Figure 11A).

We next addressed the question whether PINCH1 itself would localize to cell-cell adherens junctions. Therefore, keratinocytes were co-stained for β-catenin and PINCH1. No junctional localization of PINCH1 was observed (Figure 11A). Intriguingly, however, β-catenin and E-cadherin were found to be colocalized with PINCH1 outside of cell-cell adhesion in peripheral FA-resembling structures at the tips of actin fibers when the cell-cell contacts were not fully established (*arrowheads*, Figure 11B). Further analysis of the cell-adherens junction composition revealed that α-catenin was recruited to cell-cell adhesions in both control and P1-K5 keratinocytes but vinculin was absent from cell-cell contacts of PINCH1 null cells (Figure 11C, 11D).

Desmosomal proteins such as Plakoglobin and Desmoplakin were found at the sites of cell-cell adhesions in control and PINCH1-deficient cells and no colocalization of PINCH1 with these proteins was observed (Figure 11D, 11E).

Together these data indicate that PINCH1, although not localizing to cell-cell junctions, regulates the molecular composition and/or stabilization of cell adherens junctions as well as the cell shape in differentiated keratinocytes.

Discussion

In this manuscript we present the keratinocyte-specific deletion of the IPP-complex member PINCH1 *in vivo*. We report that PINCH1 was required for epidermal and HF integrity, similarly as its binding partner ILK. Genetic ablation of PINCH1 caused epidermal hyperplasia and detachment of the epidermis from the underlying dermis, abnormal differentiation, polarity and ectopic suprabasal proliferation in the epidermis. PINCH1 mutant HFs contained stem cells and showed a sustained proliferative capacity in combination with an impaired HF growth. This caused severe HF distortions and hair loss due to absent HF cycling. *In vitro* PINCH1 was required for cell adhesion and actin-dependent processes such as cell spreading and migration, FA and lamellipodia formation and/or stabilization. In sharp contrast to ILK, PINCH1 is required for the formation of stable cell-cell adhesions both *in vivo* and *in vitro*.

Conditional ablation of PINCH1 resulted in skin abnormalities that did not affect life span. We previously published that loss of β1 integrin and ILK with K5-Cre caused similar postnatal defects in the skin epithelium (Brakebusch et al., 2000; Lorenz et al., 2007). Studies from other groups using K14-driven gene deletions of both ILK and β1 integrin in the skin epithelium resulted in early postnatal lethality which likely resulted from differences in the K14-transgene expression (Nakrieko et al., 2008; Raghavan et al., 2000). In order to ensure the direct comparability of the effects of PINCH1 deletion with our previous findings on ILK and β1 integrin function in keratinocytes, we ablated the PINCH1 gene using the K5-Cre transgene.

Epidermal hyperthickening

The epidermis of P1-K5 developed normally until the mice where 4d old. Thereafter, similarly to integrin β1 and ILK-deficient mice, the epidermis became hyperthickened, showed abnormal differentiation and blisters at the DEJ. Epidermal hyperplasia is frequently associated with hyperproliferation as observed in human pathologies such as psoriasis (Galadari et al., 2005). The P1-

K5 hyperthickened epidermis frequently contained suprabasal proliferating cells. Interestingly, transgenic mice expressing suprabasal β1 integrin showed similar epidermal hyperthickening and hyperproliferation. In these mice the hyperproliferation was attributed to a dermal inflammatory response (Carroll et al., 1995). Abnormal differentiation, as observed in PINCH1 mutants, is also characteristic of psoriasis and was observed in integrin β1 transgenic mice. Inflammatory infiltrates of macrophages and granulocytes were present in the skin of PINCH1-deficient mice representing a potential explanation for the observed alterations. However, similar defects might also be triggered independently from inflammation. In integrin β1-deficient hyperthickenend epidermis the keratinocyte proliferation was reduced (Brakebusch et al., 2000) and in ILK-deficient epidermis the onset of epidermal hyperproliferation clearly preceded the inflammatory response (Lorenz et al., 2007). In addition, it was shown that the increased proliferation in the ILK-deficient keratinocytes was cell-autonomous as primary cultured keratinocytes also proliferated more.

Alternatively, the occurrence of suprabasal proliferating and integrin-expressing cells could result from misplacement of non-committed basal cells into suprabasal localization. In accordance with the latest findings of epidermal cell division, we observed mitotic spindles orientated parallel to the BM in normal IFE (Clayton et al., 2007) and this pattern remained unchanged upon PINCH1 loss. This finding was unexpected as PINCH1 clearly altered cell polarity with an abnormal actin cytoskeleton *in vivo*. Several lines of evidence point towards an involvement of IPP-complex members in cell polarity and mitotic spindle orientation. PINCH1 was identified as a binding partner of LKB1, a member of the PAR proteins involved in cell polarity (Brajenovic et al., 2004). ILK is found in centrosomes of HEK293 cells and has a functional role in mitotic spindle assembly and DNA segregation (Fielding et al., 2008). It remains, however, puzzling since loss of ILK decreased the mitotic index of these cells and we were at least not able to identify ILK in the centrosome in fibroblasts (A. Raducanu and R.Fässler, unpublished observations). Furthermore, it was shown previously that spindle axis orientation was controlled by integrin β1 in keratinocytes (Lechler

and Fuchs, 2005). These questions open an exciting field for further investigations if IPP complex members are indeed implicated in mitotic spindle orientation downstream of integrin in keratinocytes.

As there no evidence for altered mitotic spindle orientation, another possible explanation is that non-committed basal keratinocytes detach because of an impaired adhesion, which leads to their ectopic localization and the epidermal hyperplasia.

Epidermal cell-matrix and cell-cell adhesion

The most striking difference compared to the ILK and integrin β1 mutant epidermis was the defective cell-cell junction formation in PINCH1-deficient epidermis. The ultrastructural analysis revealed the presence of desmosomes but increased intracellular spaces between them pointing towards a decreased stability of intercellular adhesion likely due to impaired stability of cell-adherens junctions. Primary keratinocytes lacking PINCH1 could be differentiated in cultured and formed cell-cell adhesions containing both adherens junctions and desmosomal components. Nonetheless, cellular morphology and cell-cell contacts were abnormal in the PINCH1-deficient cells. ILK had been described to contribute to epithelial sheet formation in keratinocytes representing a potential mechanism for PINCH1 localization to cell-cell junctions (Vespa et al., 2005; Vespa et al., 2003). ILK involvement in cell-cell junctions was, however, not confirmed in keratinocyte-specific ILK knock-outs *in vivo* (Lorenz et al., 2007; Nakrieko et al., 2008). Likewise, PINCH1 was not detected in mature cell-cell adhesions in Ca^{2+}-differentiated keratinocytes in culture. Interestingly, however, PINCH1 colocalized at the tip of actin fibers with β catenin and E-cadherin in normal keratinocytes at the tip of membrane extensions probably involved in initial steps of cell-cell adhesions. β catenin was previously reported to localize together with dynein to filopodia-like extensions in developing contacts in an actin-dependent manner (Ligon et al., 2001). The analysis of the molecular composition of cell-cell junctions *in vitro* revealed an impaired recruitment of vinculin. Compromised *unc-97*/PINCH in *C.elegans* embryos was also leading to

an impaired spatial organization of integrin and vinculin during muscle body wall adhesion (Hobert et al., 1999). Vinculin localizes to both cell-matrix and cell-cell adhesions and binds in both locales to actin filaments. Although, this molecule might serve as an interesting link to the involvement of a FA protein in cell-cell adhesions as it localizes to both structures, the potential function of PINCH1 in cell-cell adhesion remains unclear to date.

Blister formation at the DEJ indicated impaired integrin function. Interestingly, the integrin-mediated adhesion was even stronger affected upon loss of PINCH1 than in the absence of ILK. Reduced adhesion to BM components resulted *in vivo* in impaired BM assembly leading to diffusion of BM molecules in the dermis and ultrastructurally abnormal deposits of BM components underneath the epithelium. The presence of hemidesmosomes in PINCH1-deficient basal keratinocytes strongly correlated with the quality of the BM. This is in line with current concepts that hemidesmosome formation is also driven from LM332 outside the cell (Litjens et al., 2006).

HF morphogenesis and homeostasis

The most striking phenotype of the conditional P1-K5 mice was the progressive hair loss reminiscent of the phenotypes of both ILK- and integrin β1-deficient mice (Brakebusch et al., 2000; Lorenz et al., 2007). In normal mice HF morphogenesis was completed by P14 whereas in P1-K5 animals two types of HFs were found: fully developed HFs with a hyperthickened ORS (▲) and shortened, developmentally arrested HFs (■) with a misshapen DP. Initiation of HF cycling appeared in a synchronous manner with the entry in telogen but subsequent HF outgrowth was completely blocked. Potential causes for HF growth defects were multiple, such as stem cell depletion, defective ORS cell migration, impaired hair matrix proliferation or impaired DP-keratinocyte cross-talk. Neither an impaired stem cell maintenance nor proliferation defects *per se* were observed. ORS proliferation, however, was increased. A subpopulation of P1-K5 HFs lacked a DP by P24. For those HFs impaired DP interactions and

signaling are the most likely explanation for growth arrest (Panteleyev et al., 1998).

In general PINCH1 mutant HF characterization revealed strongly overlapping defects with the ILK mutants (Lorenz et al., 2007). Impaired HF growth after ILK deletion in keratinocytes was explained by an impaired downward migration of TA cells from the bulge. This defect was caused by an impaired stabilization of lamellipodia extensions compromising directional and persistent migration causing the accumulation of proliferative cells along the ORS *in vivo*. Expecting comparable defects of P1-K5 keratinocytes *in vitro*, it was remarkable to detect an even stronger effect of PINCH1 ablation on actin-driven processes such as cell spreading and migration. The severe alterations of keratinocyte actin-dynamics represent a potential explanation for the high degree of HF malformations *in vivo* resulting in an earlier onset of dermal inflammation upon deletion of PINCH1 as compared to ILK.

PINCH1 in actin dynamics

Our analysis of primary keratinocytes revealed that P1-K5 primary cells formed only few, unstable membrane protrusions with the cell body remaining roundish, impairing stable cell adhesion, cell spreading and migration.

What could be the underlying mechanisms for such strong requirement of PINCH1 exceeding the ILK phenotype? First, weakened integrin adhesion could compromise the stable fixation of lamellipodia to the substratum impairing force generation required for lamellipodia extension. Second, it is also conceivable that impaired stress fiber formation or anchoring compromises FA formation (Burridge et al., 1997). Indeed, these notions are further supported by the observation that detectable FC and FA formation is entirely absent in PINCH1-deficient keratinocytes. In addition, P1-K5 keratinocytes display a poorly developed actin filaments in culture. Molecularly, PINCH1 and ILK were shown to bind Thymosin β4, a small G-actin sequestering protein (Bock-Marquette et al., 2004). Therefore, it will be interesting to investigate whether the ratio of monomeric G-actin to F-actin is changed in these keratinocytes. Interestingly, Thymosin β4 was

also implicated in HF growth via the regulation of stem cell progenitor migration from the bulge (Philp et al., 2004; Philp et al., 2007). Besides the pathways that are connecting PINCH1 via ILK to the regulation of the actin cytoskeleton via Parvins and Kindlins (Grashoff et al., 2004), PINCH1 also binds Nck2. Recent worked dissected the specific functions of PINCH1 subdomains and their interactors revealing that the LIM4-mediated interaction with Nck2 regulates cell morphology and migration independently from ILK (Xu et al., 2005).

In summary our analysis clearly shows that PINCH1 must have IPP-complex and integrin-independent functions likely through the recruitment of other interaction partners or though IPP-independent stabilization in new cellular compartments. PINCH1 is implicated in cell-cell adhesion in keratinocytes as thus far only shown in embryoid bodies (Li et al., 2005). PINCH1 is crucial for FA formation assembly, thereby impairing cellular key functions such as spreading and migration. The challenging task for the upcoming investigations will be the identification of PINCH1-specific pathways and potential new interacting molecules involved in those.

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Figure Legends

Figure 1*: Keratinocyte-restricted deletion of PINCH1 causes progressive hair loss in mice.*

(A) PINCH1 and ILK protein levels in epidermal lysates of P1 Co and P1-K5 mice. **(B)** Control and P1-K5 animals at 2 weeks (w) and 8w of age.

Figure 2: *Keratinocyte-restricted deletion of PINCH1 leads to progressive HF abnormalities and HF resorption, epidermal hyperthickening and epidermolysis.*

Hematoxylin-eosin staining of back skin sections of P1 Co and P1-K5 mice from 2 days (d) to 10w of age. First signs of abnormal HF morphogenesis in P1-K5 skin are detected starting from 2d (*arrows*) leading to two HF types (▲fully developed; ■ shortened and developmentally arrested) by 14d, followed by progressive HF resorption. With HF loss dermal pigment accumulation occurs (*arrowheads*). P1-K5 epidermis becomes hyperplastic and detaches from the underlying dermis (*asterisk*) (bars 100 µm). epidermis (E); dermis (D); panniculus carnosum (PC); subcutis (SC)

Figure 3: *Loss of PINCH1 causes epidermal detachment at the DEJ, increased intercellular spaces, HF deterioration and growth retardation.*

(**A**) Epidermis from 2-w-old P1-K5 mice is hyperplastic and detaches from the underlying dermis (*asterisk*). P1-K5 keratinocytes show increased intercellular spaces. Melanin in the IFE is indicated (*arrow heads,* bar 25 µm). (**B**) High magnification of hematoxylin-eosin stained HFs from 14d-old back skin. P1-K5 HFs have multilayered ORS and a condensed DP (▲) or show premature growth arrest with a malformed or absent DP (■) (bar 50µm). (**C**) P1-K5 HF growth is perturbed during morphogenesis and cycling. HF lengths of a minimum of 150

HFs per time point from each two animals are presented as histograms. HF length was measured from hematoxylin-eosin stained back skin sections. epidermis (E); dermis (D); panniculus carnosum (PC); subcutis (SC)

Figure 4: ***PINCH1 loss severely impairs keratinocyte adhesion, integrin expression and BM integrity***

(**A**) Cell adhesion of primary P1-K5 keratinocytes is significantly reduced compared to control and ILK-K5 keratinocytes on FN, Col1 and LM332 (mean+SD of three independent experiments). (**B**) Immunofluorescence (IF) of β1 and β4 integrin and LM332 on sections of back skin from 2-w-old mice. In P1 Co skin both integrins are expressed in basal keratinocytes, while in P1-K5 skin integrins are devoid from basal cells in some areas and are also found on suprabasal keratinocytes (*arrowheads*). LM332 mainly diffuses from the DEJ (*dashed line*) into the upper dermis, as a sheet (BM, first panel) or in patches (second panel). Note the high β1 integrin expression in the cells of the upper dermis (*asterisks*) (bar 25 µm). (**C**) Cell surface expression of integrins on freshly isolated keratinocytes by FACS analysis (black histogram: P1 Co; red: P1-K5; dashed histograms: negative controls). The antibody β1 Ha2/5 recognizes total β1 integrin and β1 9EG7 a ligand-induced binding site and hence the active β1 integrin. (**D**) EM of back skin sections of 2-w-old control and P1-K5 mice. Control skin exhibits a continuous lamina densa and hemidesmosomes (*arrowheads*). P1-K5 skin shows a discontinuous lamina densa (BM). Hemidesmosomes (*arrowhead*) are found when lamina densa is preserved. Desmosomal contacts (*asterisks*) are formed in both control and P1-K5 keratinocytes, while intercellular spaces are increased in P1-K5 epidermis (bar left 0.1 µm and right 0.2 µm). basement membrane (BM); dermal-epidermal junction (DEJ)

Figure 5: ***PINCH1 ablation alters keratinocyte differentiation and proliferation***

(**A**) Double-IF for K14, K10, loricrin (Lor), K6 and α6 integrin on back skin of 2-w-old mice. P1-K5 epidermis shows increased layers of K14, K10 and Lor expression. Integrin α6 expression is discontinuous in P1-K5 skin (bar 25 µm). K6 is upregulated in P1-K5 epidermis. (**B**) Ki67 staining on back skin of 7-d-old mice revealed the presence of suprabasal proliferating cells in P1-K5 epidermis (bar 50 µm). (**C**) Quantification of Ki67-positive cells in the IFE of 7-d-old mice. a) basal; b) suprabasal (error bar represent mean+SD) (**D**) Double-IF for integrin β4 and BrdU showing suprabasal, integrin-expressing cells in P1-K5 7d skin sections (bar 25 µm). (**E**) Slices in the direction of mitotic spindles taken from 3D reconstructions of tail epidermis wholemounts from 7-d-old mice stained with DAPI and quantification of the angles between mitotic axis and BM.

Figure 6: ***Inflammatory cells are progressively recruited to the dermis of P1-K5 skin***

(**A**) IF for Mac-1 and LM332 on skin sections of 2-, 4- and 7-d-old mice. No macrophages are detected in the skin of 2-d-old control and P1-K5 mice, while skin from 4- and 7-d-old P1-K5 mice shows macrophage infiltration predominantly at the distorted HFs and underneath the epidermis (*arrowhead*, bar 100 µm). (**B**) IF for Gr1 and LM332 on skin sections of 2-, 4- and 7-d-old mice. At 2d and 4d Gr1-positive granulocytes accumulate in the dermis of P1-K5 skin (bar 100 µm).

Figure 7: ***PINCH1 loss disturbs polarity of epidermal keratinocytes***

(**A**) IF for F-actin and E-cadherin on sections of 2-w-old skin. In control epidermis F-actin and E-cadherin are absent from the basal side of basal kerationcytes. In P1-K5 epidermis, F-actin and E-cadherin are found basally (*arrowheads*). Intercellular gaps are frequently observed by E-cadherin staining in 10-w-old P1-

K5 (*asterisks*, bar 50µm). (**B**) IF for desmoplakin (DSP) and plakoglobin (PG) on sections of 2-w-old skin. Desmosomal components localize to the lateral-apical sides of basal keratinocytes of control epidermis, whereas they colocalize with α6 integrin at the basal side of basal keratinocytes in P1-K5 epidermis. Intercellular spaces (*asterisks*) and PG negative corneocytes (*arrow heads*) were detected by PG staining in 10-w-old P1-K5 (bar 50µm; insert 25 µm).

Figure 8: ***P1-K5 HFs display a hyperproliferative ORS and respond to DP derived signals***

(**A**) P1-K5 HFs display a hyperplastic ORS with an elevated number of Ki67+ positive cells (▲,■ (bar 50 µm)). Auber's line (green line; Auber, 1952) demarks the border between the proliferative and non-proliferative zones of the HM. (**B**) The proportion of proliferating cells in the ORS is increased in 7-d and 14-d mutant HFs. (**C**) The total number of HM cells is significantly reduced in 7-d and 14-d P1-K5 HFs. (**D**) The percentage of proliferating cells in the HM is unchanged in mutant HFs compared to the controls. A minimum of 20 HFs was evaluated for (B, C, D) per genotype (error bars indicate mean + SD of two mice, *=$p<0.05$). (**E**) Double-IF of CD34 and LM332 on skin sections of 24-d-old animals reveals the presence of a CD34+ bulge region (*arrowheads*) in control and P1-K5 HFs (bar 50 µm). Note that P1-K5 HFs display a severely abnormal morphology at this stage sometimes with detached DP (*bracket*). (**F**) Double-IF of Ki67 and LM332 on skin sections of 24-d-old animals. Proliferation is induced in control and mutant HFs with DP and absent in P1-K5 HFs without DP (bar 50 µm).

Figure 9: ***P1-K5 keratinocytes display impaired cell spreading and migration***

(**A**) Primary keratinocytes were cultured for 6 days. P1-K5 keratinocytes form clusters of small, round cells. (**B**) P1 Co and P1-K5 keratinocytes were seeded

on Col1 and FN. The spreading was monitored at the indicated time points and the cell area of more than 100 cells was quantified per time point. P1-K5 keratinocytes show reduced spreading (error bars indicate the 95% CI of mean values). (**C**) Time lapse microscopy of single control and P1-K5 keratinocytes. Control cells form stable lamellae, whereas P1-K5 keratinocytes are poorly spread, form small lamellar protrusions that frequently collapse and retract and show frequent collapses of membrane protrusions (green arrows indicate protrusion and red arrows retraction of the cell). (**D**) Time lapse microscopy of a scratch assay. P1-K5 keratinocytes exhibit impaired wound closure (bar 100µm). (**E**) Quantification of the wound closure in the scratch assay. P1-K5 wound closure is significantly retarded (error bars indicate 95% CI of mean values).

Figure 10: ***No FA formation in P1-K5 keratinocytes***

IFs of primary control and P1-K5 keratinocytes. (**A**) In control keratinocytes PINCH1 localizes to Paxillin (Pax)-containing FAs at the tip of F-actin stress fibers. No FA formation is observed in P1-K5 keratinocyte clusters. Weak actin staining reveals the absence of stress fiber formation. Note that thickened cell protrusions appear Paxillin-rich (*arrowheads*). Cultures of PINCH-1 deficient cells are often contaminated with non-deleted cells (ND) (bar 25 µm). (**B**) Vinculin is recruited to FCs (*arrowheads*) and mature FAs in control keratinocytes. Both structures are missing in P1-K5 cell clusters (bar 25µm). (**C**) ILK is distributed diffusively in the cytoplasm of P1-K5 cells. High ILK expressing cells are melanocytes (*asterisks*) (bar 25 µm). (**D**) LM332 is normally deposited by P1-K5 keratinocytes (bar 25 µm).

Figure 11: ***Aberrant Ca^{2+}-induced cell-cell adhesion in P1-K5 keratinocytes***

IFs of primary control and P1-K5 keratinocytes differentiated with 1.2mM $CaCl_2$. (**A**) E-cadherin (E-Cad) and β-catenin (β-Cat) localize to cell-cell junctions in differentiated control and P1-K5 keratinocytes. Filamentous membrane

protrusions are formed between distant cells (*arrowheads*). Left panels show perpendicular view through a confocal Z-stack 3D-reconstruction which reveals the increased thickness of differentiated P1-K5 sheets. PINCH does not localize to cell-cell junctions (bars 50 µm). (**B**) β-catenin colocalizes with PINCH in peripheral FAs during the process of differentiation in P1 Co keratinocytes. E-cadherin is found at the tip of actin stress fibers (*arrowheads*) (bars 50 µm, right panels bars 25µm). (**C**) α-catenin (α-Cat) is recruited to cell-cell adhesions of P1-K5 keratinocytes (bars 50 µm). (**D**) Vinculin is recruited to cell-cell junctions in P1 Co keratinocytes but is absent from cell-cell adhesions in mutant cells (bars 50 µm). (**E**,**F**) Desmosomal components Plakoglobin (PG) and Desmoplakin (DSP) are found in cell-cell junctions of control and mutant keratinocytes (bars 50 µm).

***Supplementary Figure 1*: Integrin profile of cultured, primary keratinocytes**

Cell surface expression of integrins on primary keratinocytes after 4-5 days in culture by FACS analysis (black histogram: P1 Co; red: P1-K5; dashed histograms: negative controls).

***Supplementary Figure 2*: IF for versican on skin sections**

Double IF for versican and integrin α6 on skin sections of 14-d- and 26-d-old animals. Note the presence of active DPs and dermal sheet in control and mutant HFs as well as the strong upregulation of versican underneath the epidermis of mutants. Inserts show IFE from P1 Co skin sections. (bars 50 µm; inserts 25 µm).

Tables

	PINCH1$^{loxP/loxP}$ K5-Cre +	PINCH1$^{loxP/wt}$ K5-Cre +	PINCH1$^{loxP/loxP}$ K5-Cre -	PINCH1$^{loxP/wt}$ K5-Cre -
Offsprings before recombination [%] (n=241)	5.81	43.98	43.98	6.22
Offsprings after recombination [%] (n=71)	40.85	5.63	9.86	43.66

Table 1: Genotype distributions from PINCH1 and K5-Cre intercrosses

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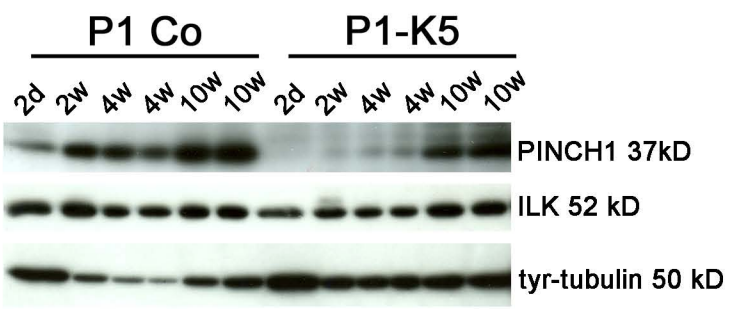

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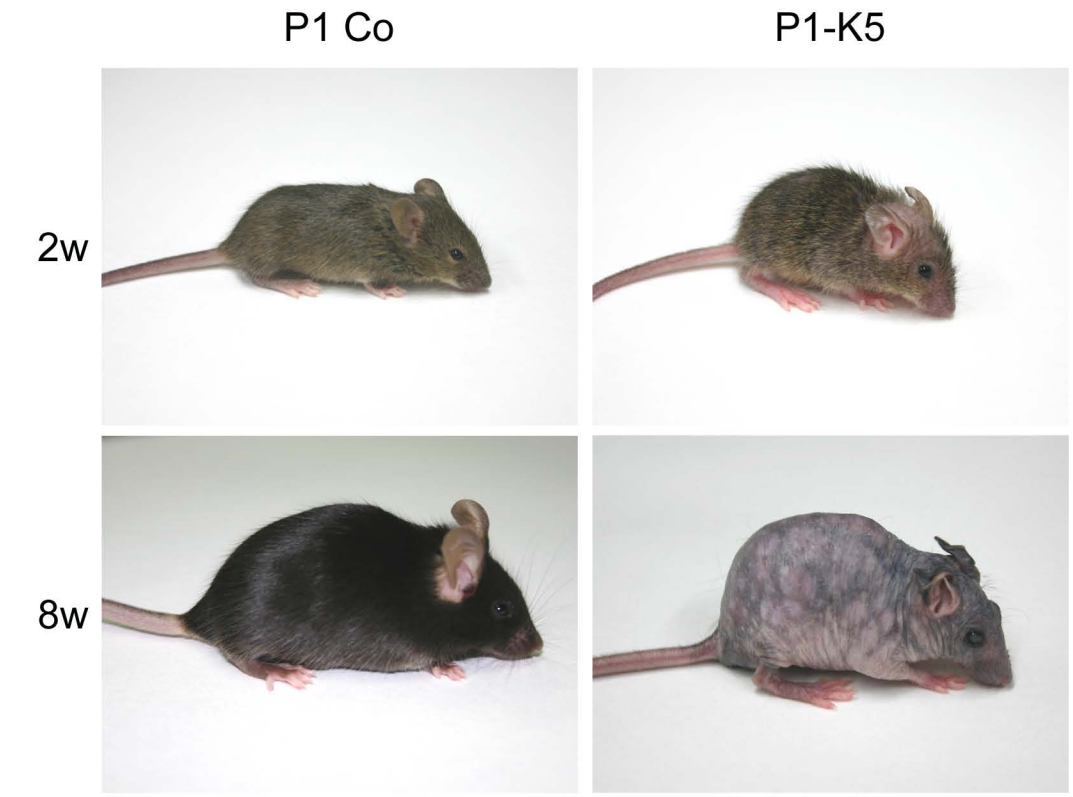

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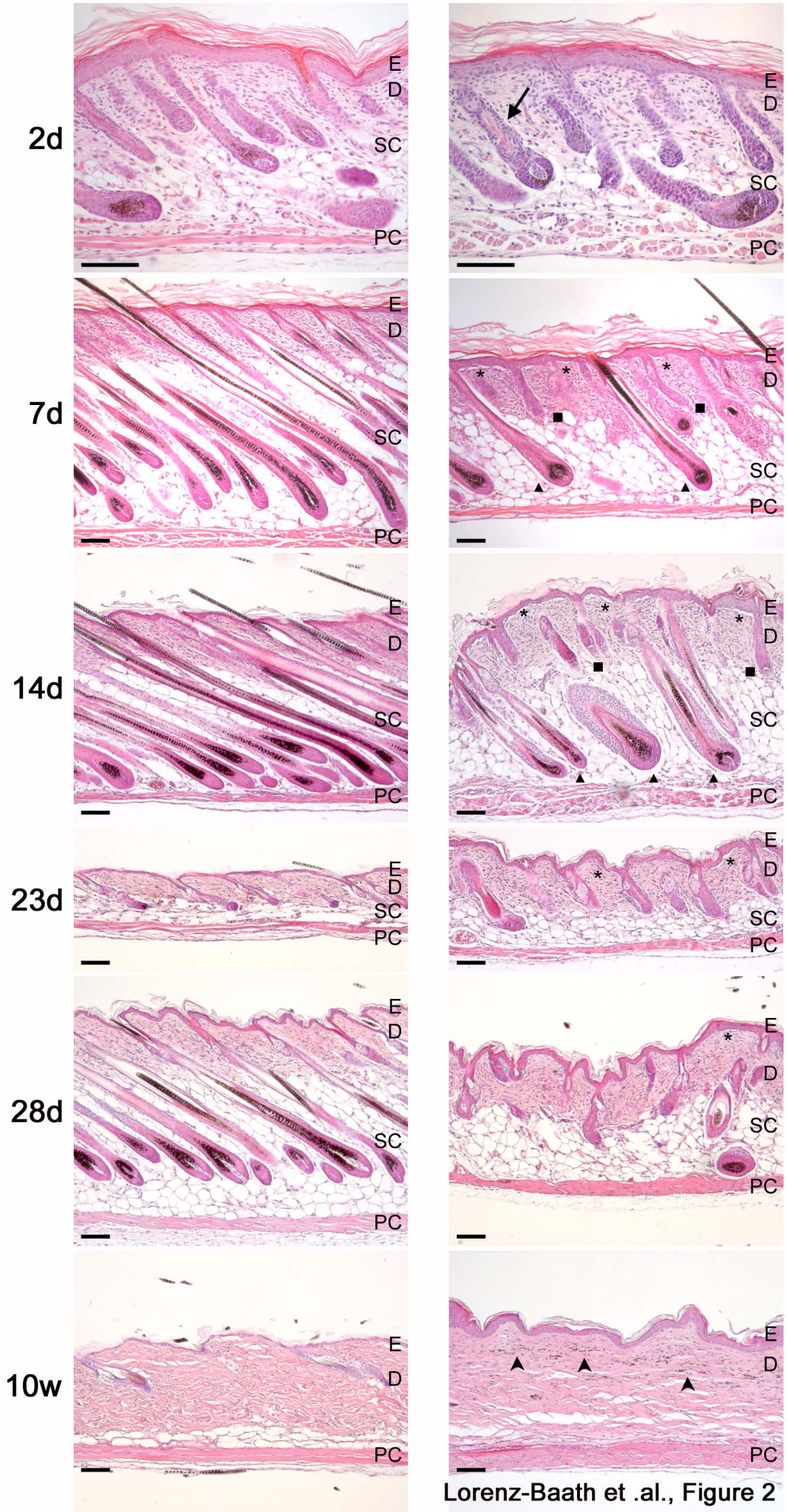

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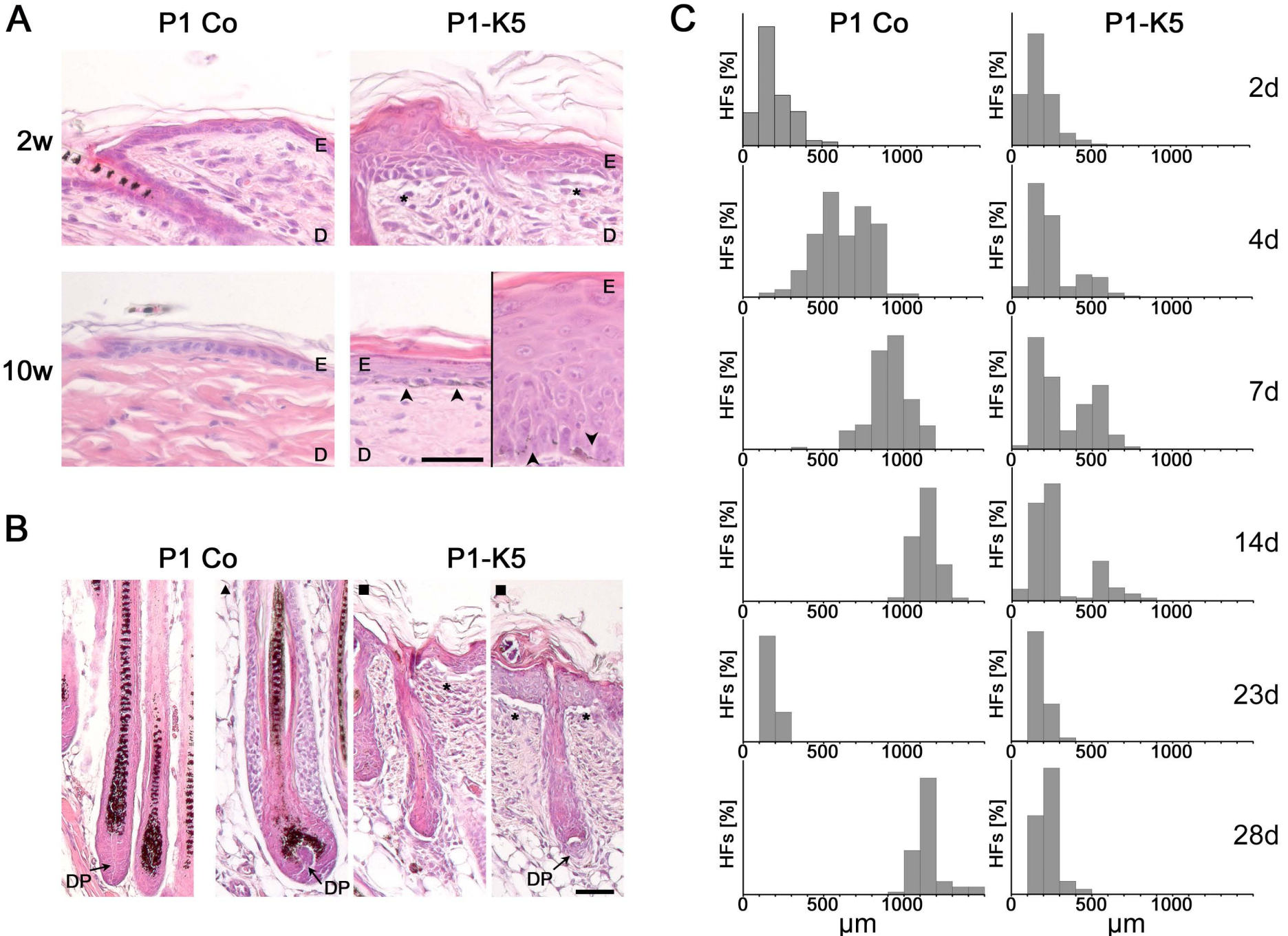

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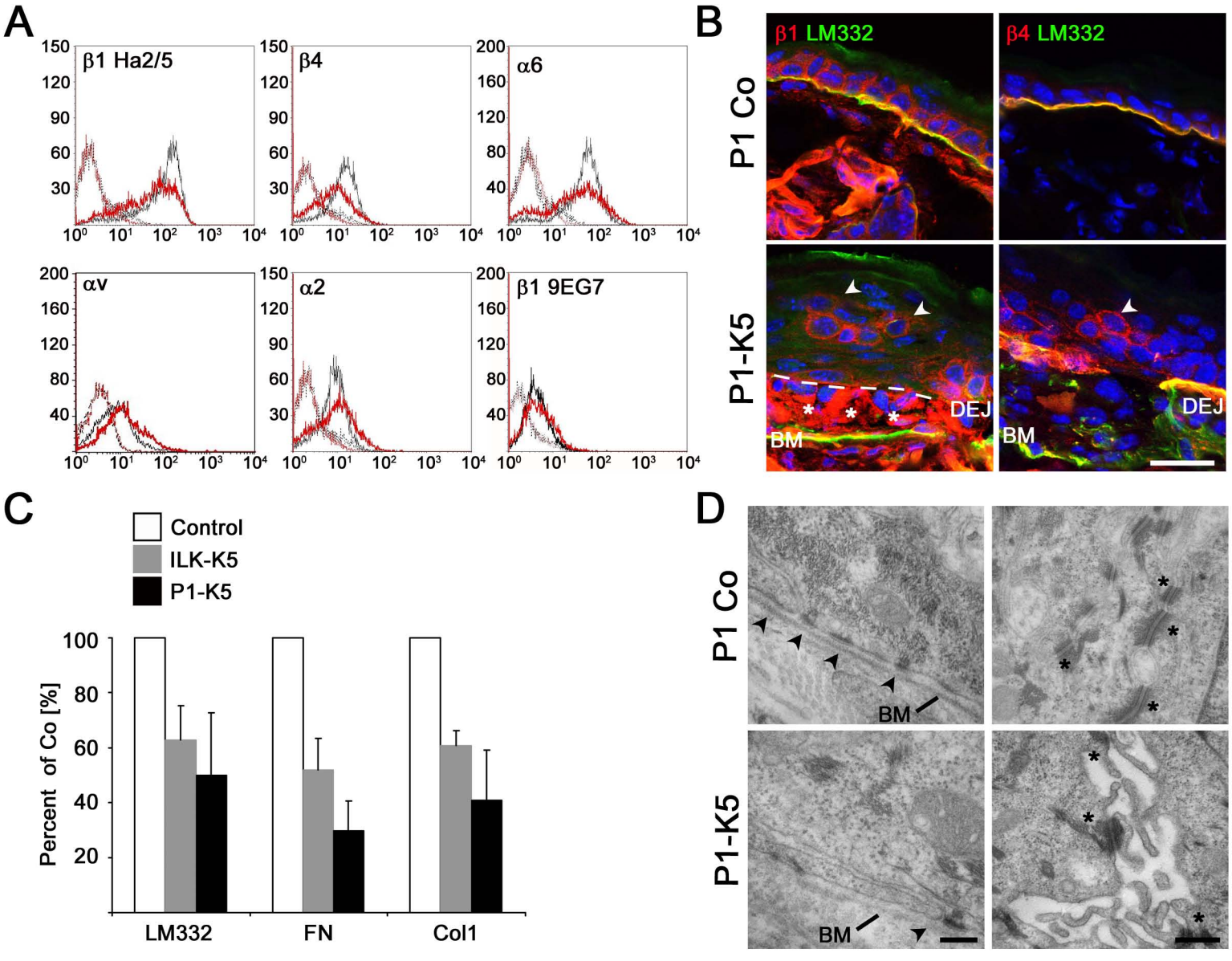

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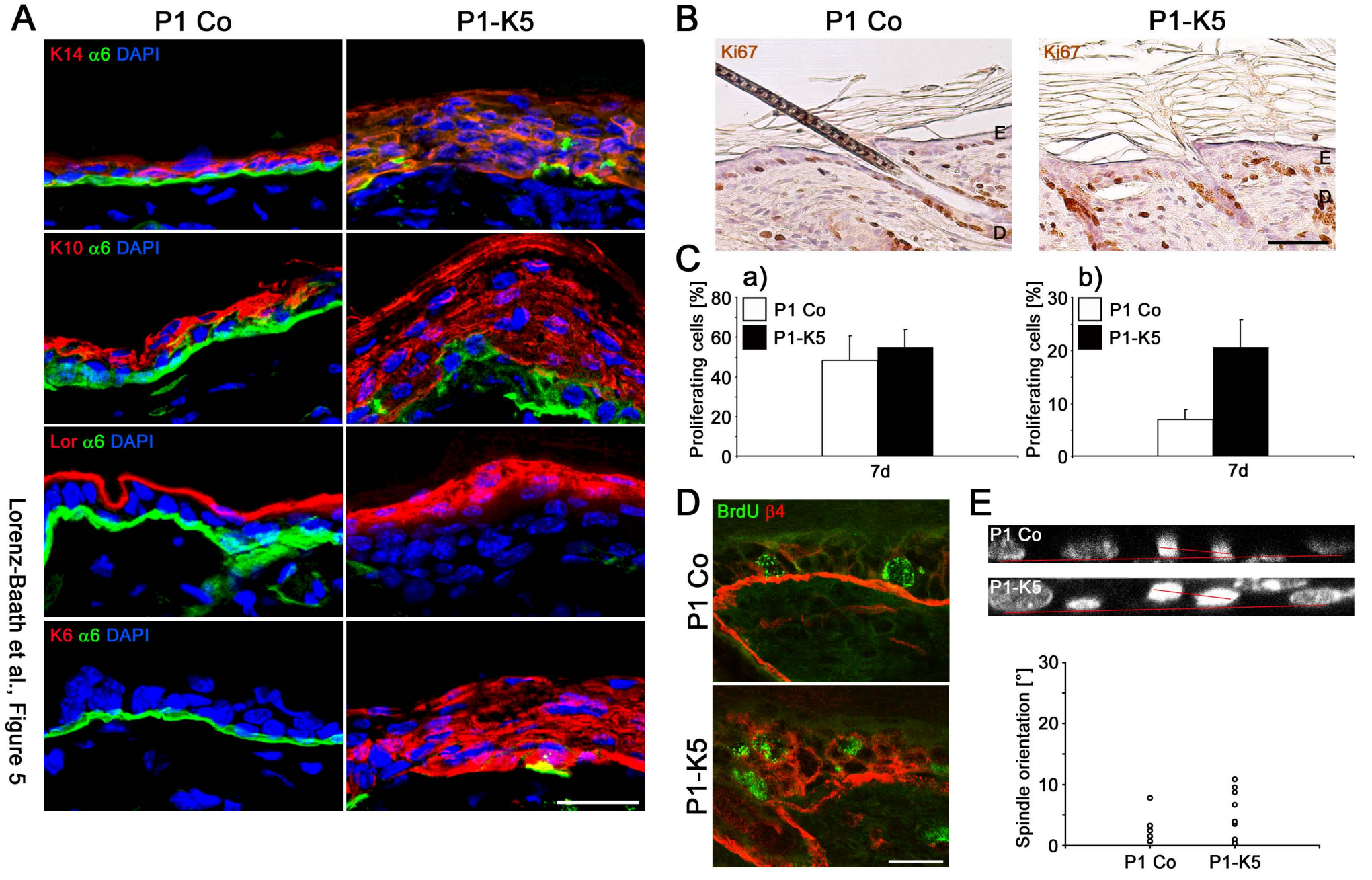

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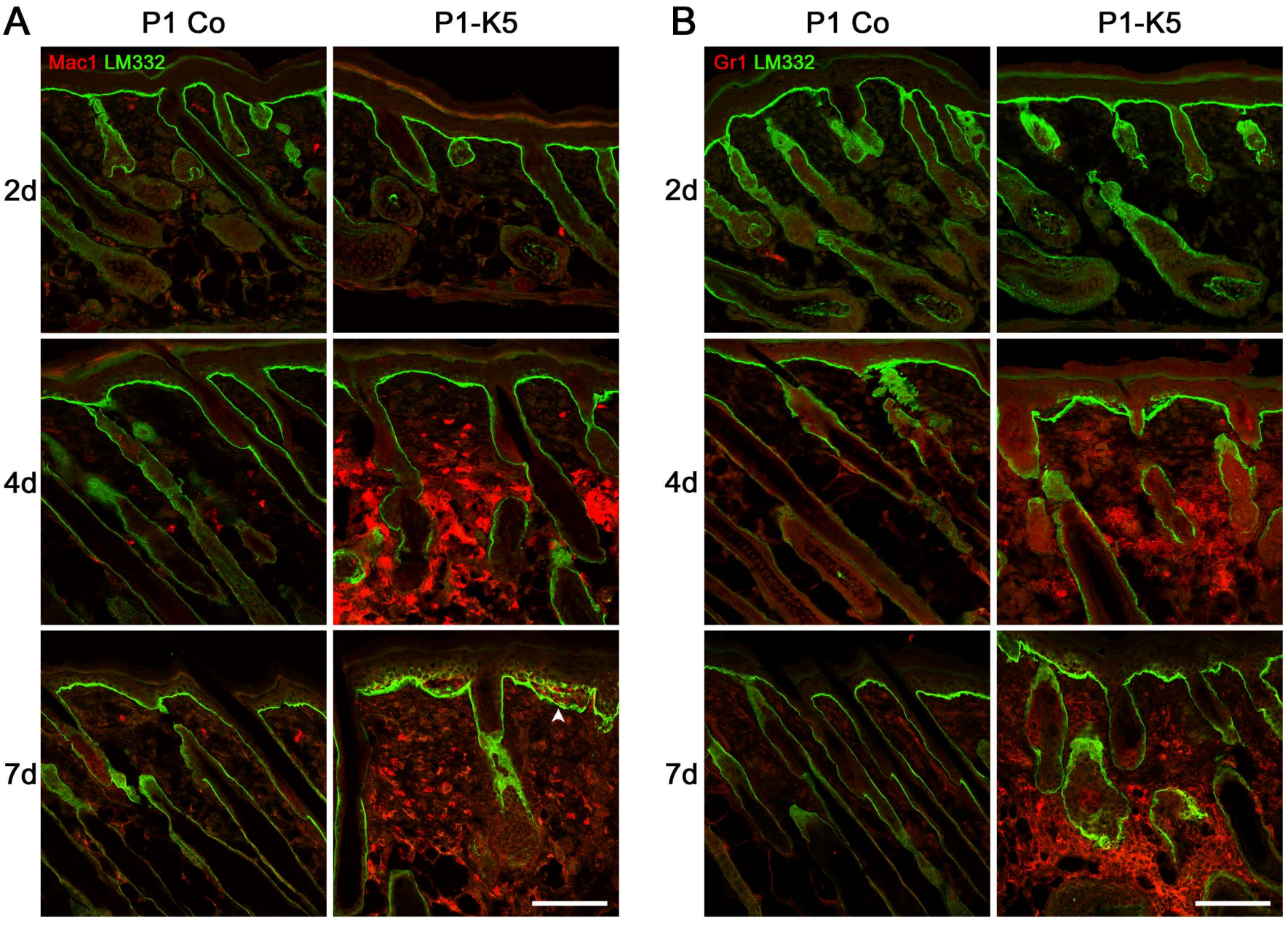

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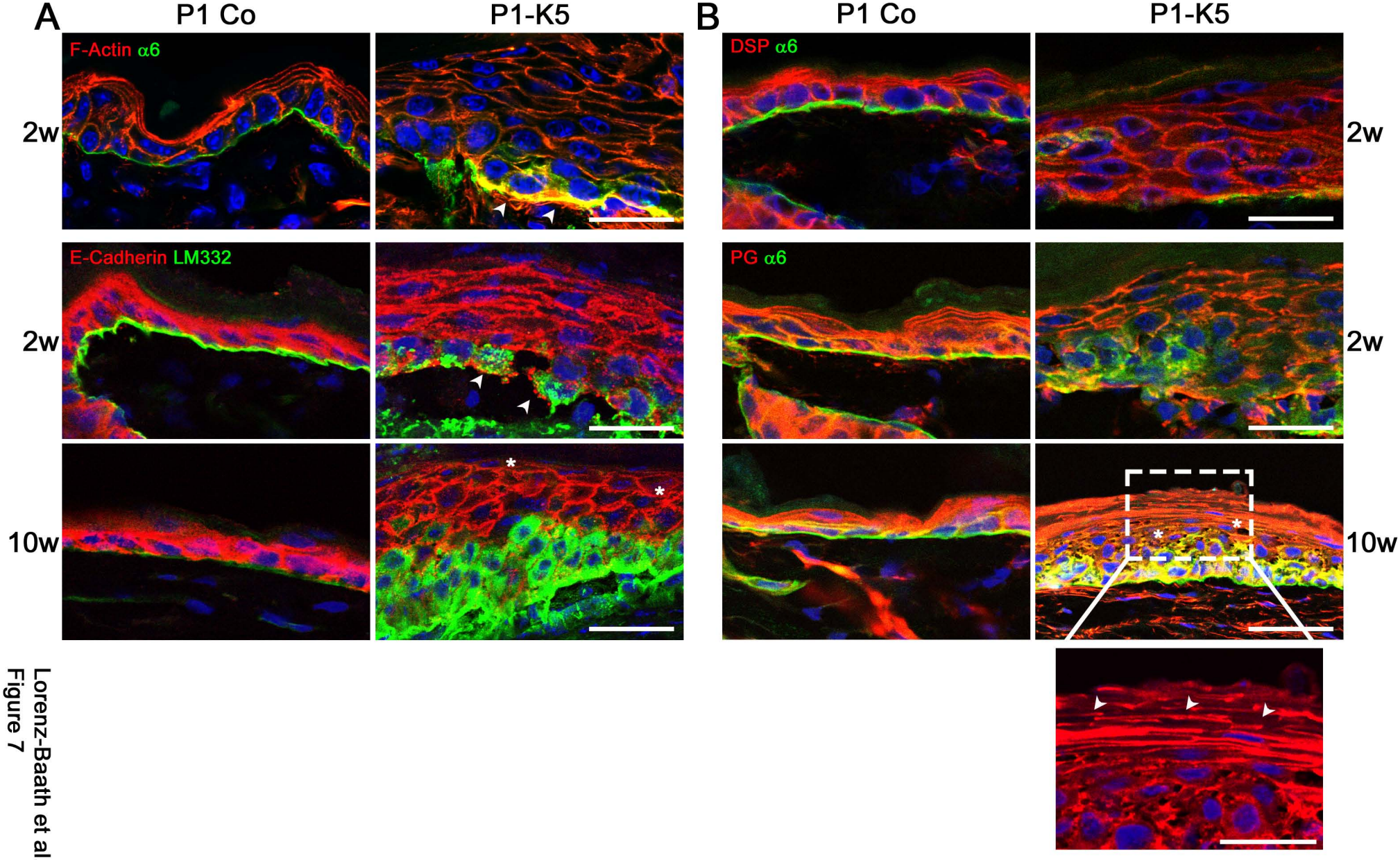

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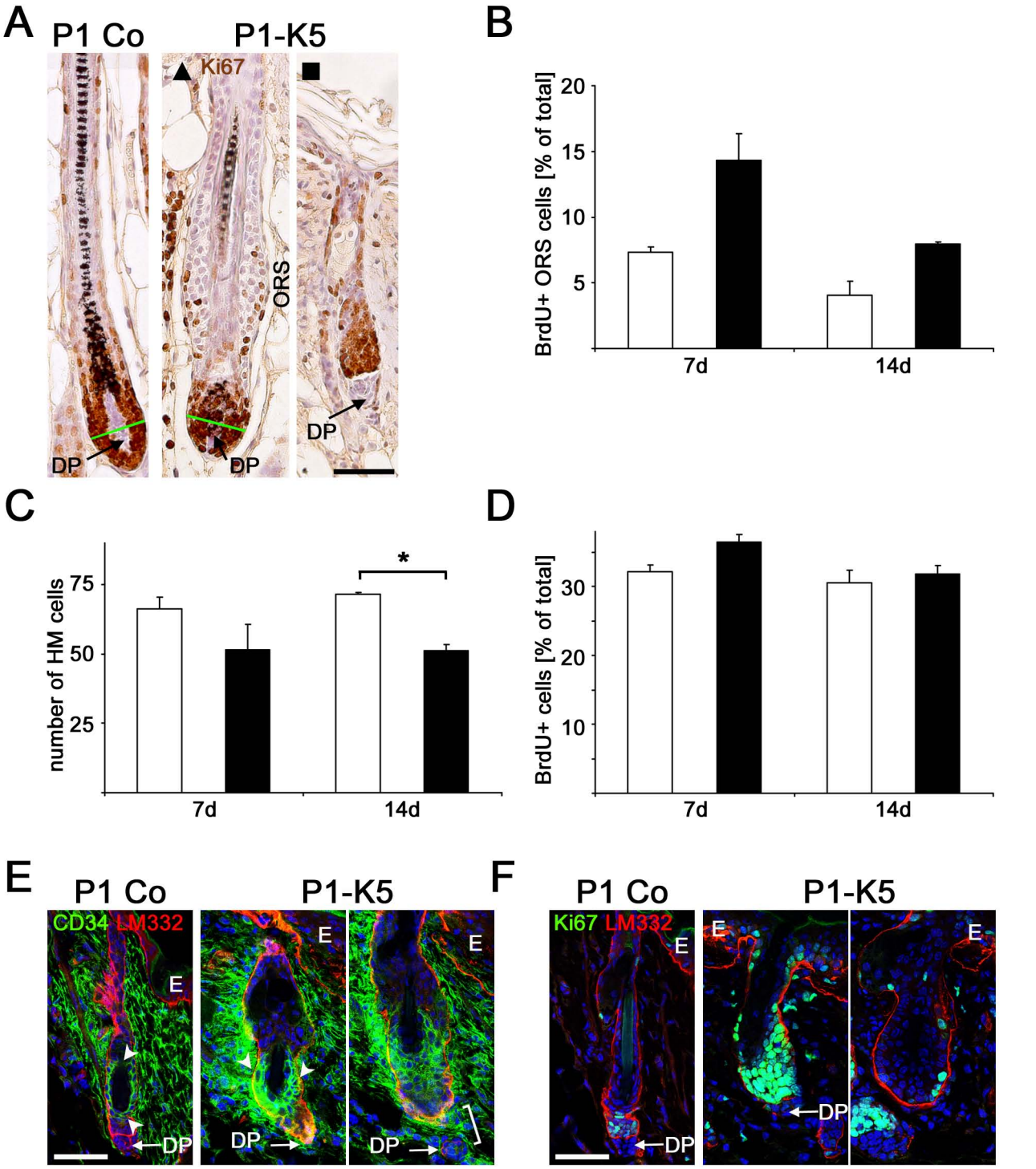

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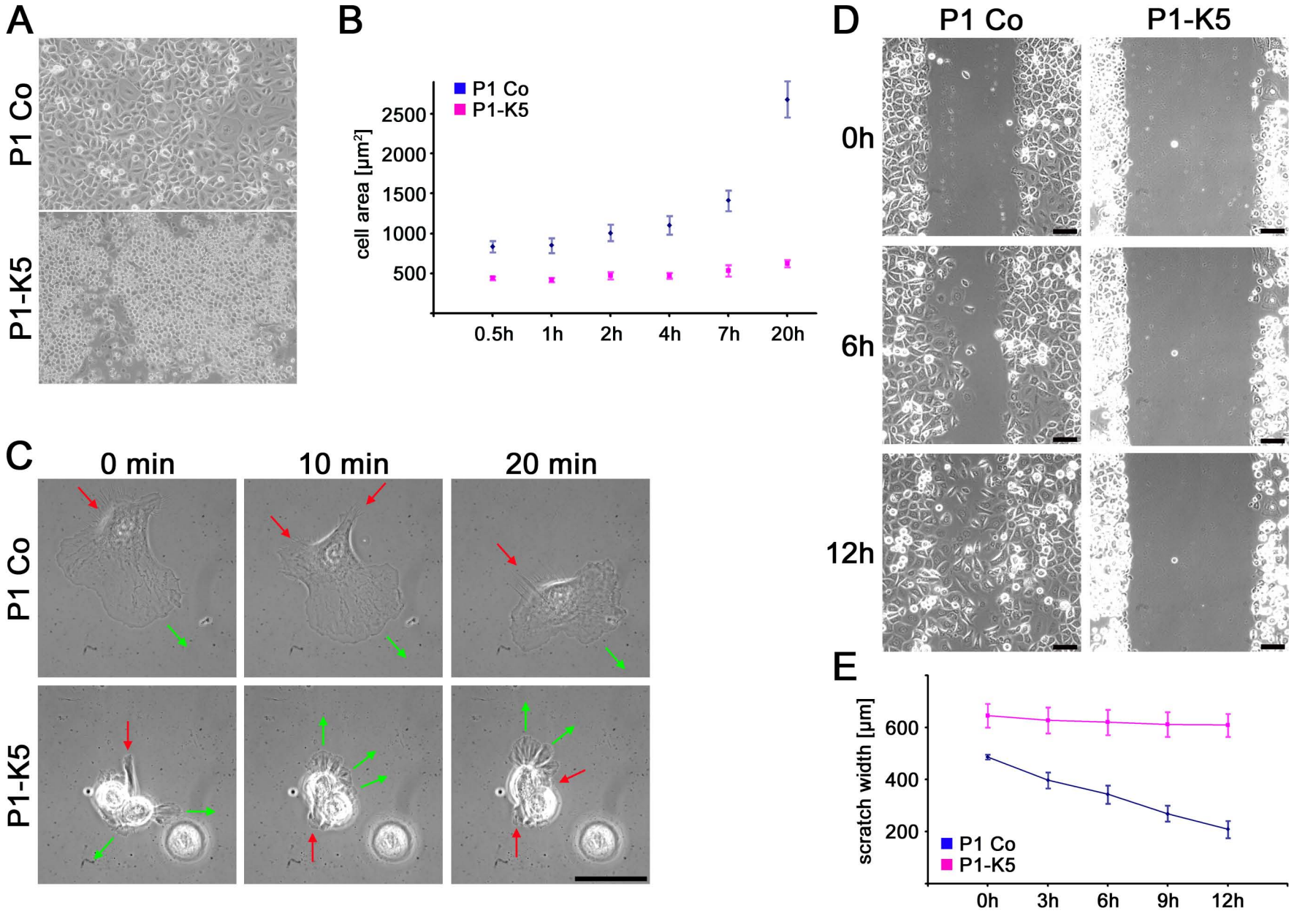

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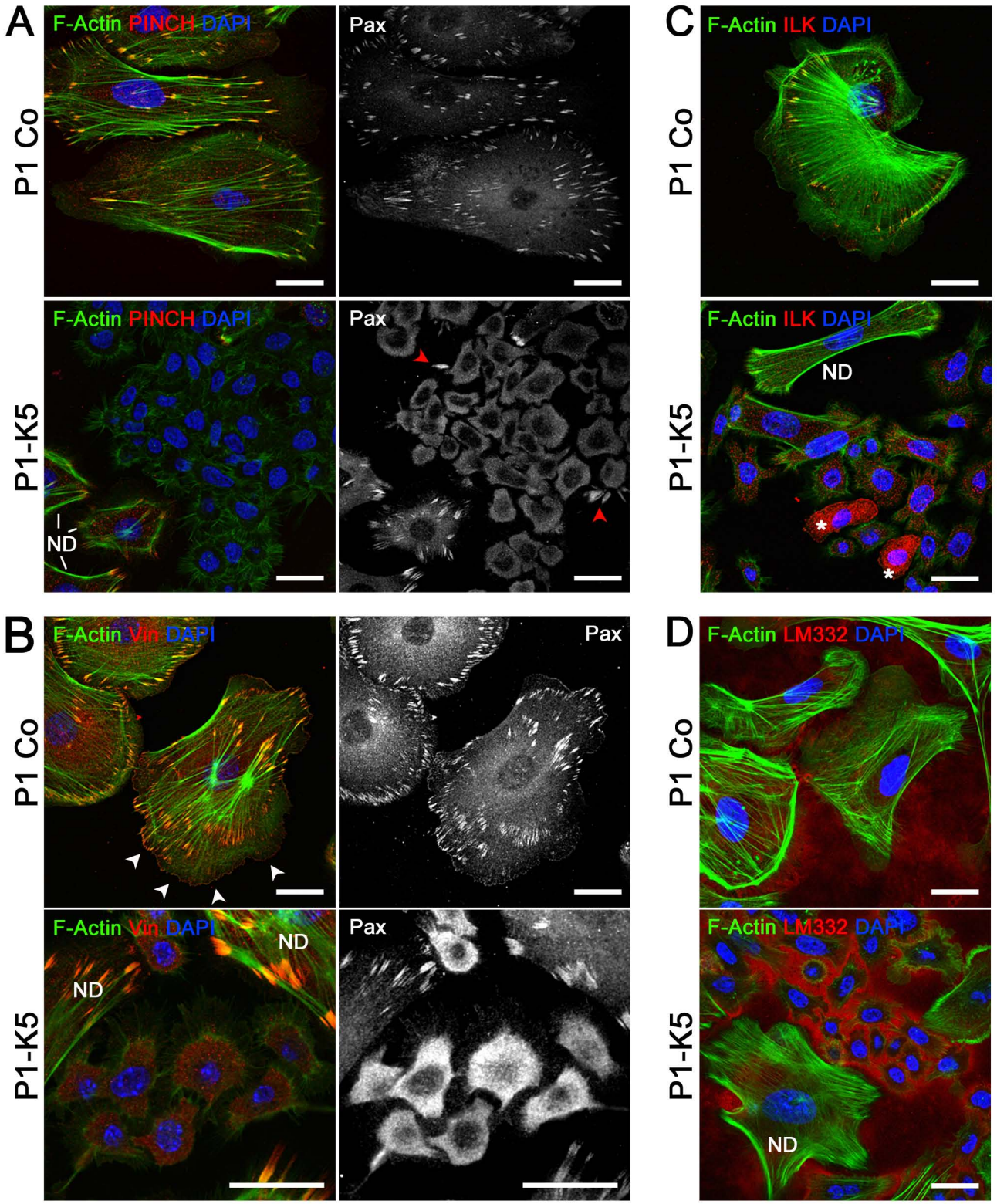

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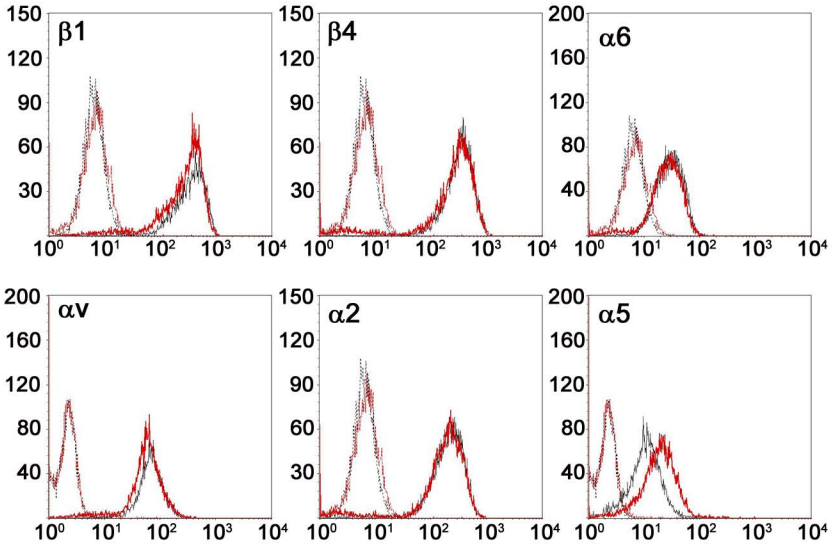

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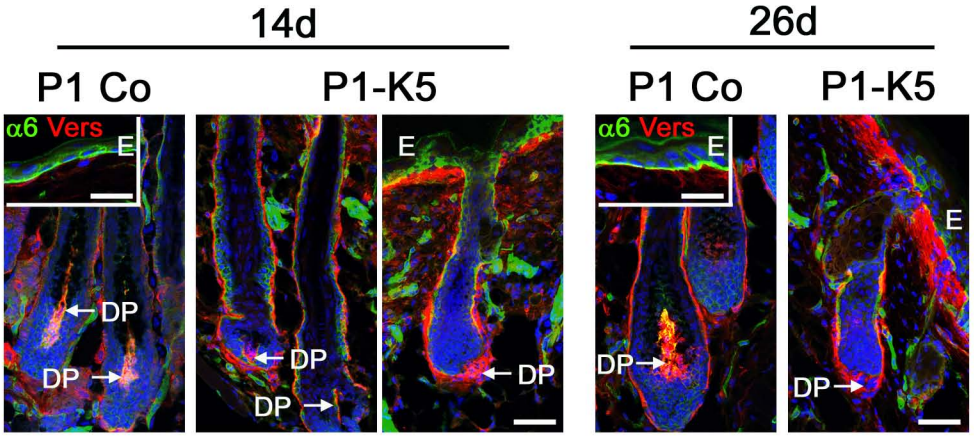
Supplementary Figure 1



Lorenz-Baath et al.,
Supplementary Figure 2

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Ausbildung

Promotion — ***"Comparative Analysis of the Functions of Integrin Adaptor Molecules ILK and PINCH1 in the Skin Epithelium"*** — **seit Nov. 2003**
Abteilung Molekulare Medizin
Max-Planck-Institut für Biochemie
Martinsried

Studium — **Diplom-Biotechnologe / Diplôme d'Ingénieur de Biotechnologie** — **September 2003**
Ecole Supérieure de Biotechnologie de Strasbourg (ESBS), Strasbourg, Frankreich

Vordiplom, Biotechnologie — **September 2000**
Technische Universität Braunschweig

Schule — **Allgemeine Hochschulreife** — **Juni 1998**
Gymnasium Grafing
Grafing b. München

Praktika / Erfahrung

Diplomarbeit — ***"Analysis of Human Breast Cancer Cells in Three-Dimensional Context"*** — **Jan. – Sept. 2003**
Lawrence Berkeley National Laboratory
Berkeley, CA, USA

Praktikum — ***"Expression of Angiogenesis Promoting Factors in the Liver of Nude Mice in Response to MMP Inhibitor Treatment"*** — **Jul. – Aug. 2001**
Institut für Experimentelle Onkologie
Technische Universität München