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SPECIFIC ROLES OF EPITHELIAL INTEGRINS IN CHEMICAL AND PHYSICAL SENSING OF THE EXTRACELLULAR MATRIX TO REGULATE CELL SHAPE AND POLARITY
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Abstract

Integrins are a large family of $\alpha\beta$-heterodimeric cell adhesion receptors of which the cell type specific expression defines the extracellular matrix (ECM) binding properties of different adherent cell types. In addition to various growth factors and their receptors, epithelial morphogenesis is also executed by dynamic changes in the chemical composition and physical properties of the ECM that controls the shape and behavior of the associated cells via integrin mediated adhesion and signalling. Epithelial cell polarity and contractility are central mechanisms of epithelial shape determination and are established upon spatially, mechanically and chemically sensitive integrin signals of the microenvironment. The functional hierarchy between different integrin heterodimers and their ECM ligands in organizing these tasks has not been systematically addressed.

In order to study the relative roles of different integrins, we set up a loss-of-function screen of co-expressed integrin subunits in the Madin-Darby canine kidney (MDCK) epithelial cell line. By analyzing MDCK cystogenesis in three-dimensional (3D) ECMs, we were able to establish a model of how epithelial polarity is organized: cell adhesion either by $\alpha_2\beta_1$- or $\alpha_6\beta_4$-integrins defines the orientation of cell polarity and coordinated functions of $\alpha_2\beta_1$- and $\alpha_3\beta_1$-integrins mediate the establishment of epithelial lumens via cavitation and hollowing, respectively. By analyzing the spreading of MDCK cells, we established that epithelial cell contractility is based on synergistic functions of $\beta_1$-integrins that mediate cell adhesion and $\alpha V$-integrins that facilitate ECM rigidity sensing. We also discovered that the hemidesmosomal integrin $\alpha 6$ and integrin $\beta 4$ did not require heterodimerization to be transported to the plasma membrane (PM) and that integrin $\beta 4$ may support laminin assembly to the basement membrane (BM) independently of integrin $\alpha 6$.

Keywords: epithelial cell polarity, extracellular matrix, integrin, lumen formation, mechanotransduction


Asiasanat: epiteelisulon polarisatio, integriini, mekaaninen signaalinvälitys, rakkularakenteen muodostuminen, soluväliaine
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Abbreviations

AJ               adherens junction
aPKC             atypical protein kinase C
BM               basement membrane
BME              basement membrane extract
BPAG2            bullous pemphigoid antigen 2 (collagen XVII)
Cdc42            cell division control protein 42 homolog
Col-I            type I collagen
Col-IV           type IV collagen
CRB              crumbs homolog 3
2D               two-dimensional
3D               three-dimensional
DA               dominant active
DAG              α-dystroglycan
DLG              discs large homolog
DN               dominant negative
E                embryonic day
EB               embryoid body
ECM              extracellular matrix
EMT              epithelial to mesenchymal transition
ER               endoplasmic reticulum
FA               focal adhesion
FAK              focal adhesion kinase
FN               fibronectin
GAP              GTPase activating protein
GEF              guanine nucleotide exchange factor
GF               growth factor
HD               hemidesmosome
HSP              heparin sulfate proteoglycan
ICM              inner cell mass
IF               intermediate filament
ILK              integrin-linked kinase
KD               knockdown
KO               knockout
LN-111           laminin-111
LN-332           laminin-332
LN-511 laminin-511
LGL lethal giant larvae homolog
LGN G-protein signalling modulator 2
MDCK Madin-Darby canine kidney
MET mesenchymal to epithelial transition
MMP matrix metalloproteinase
PAR-1 MAP/microtubule affinity-regulating kinase 2
PAR3 partitioning defective 3 homolog
PAR6 partitioning defective 6 homolog
PINCH-1 LIM and senescent cell antigen-like-containing domain protein 1
PIP phosphoinositide
PM plasma membrane
Podxl podocalyxin
PTEN phosphatase and tensin homolog on chromosome 10
qPCR quantitative PCR
Rac1 ras-related C3 botulinum toxin substrate 1
RhoA ras homolog family member A
RM Reichert’s membrane
ROCK1 rho-associated protein kinase 1
RTK receptor tyrosine kinase
SGL sulphated glycolipid
shRNA short hairpin RNA
Src SRC proto-oncogene, non-receptor tyrosine kinase
TIRF total internal reflection fluorescence
TJ tight junction
List of original articles

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


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7 Summary

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1 Introduction

Integrins are a large family of non-covalently associated \( \alpha \beta \)-heterodimers that function as cell surface adhesion receptors for proteins of the extracellular matrix (ECM) and cell adhesion molecules at the surface of other cells (Hynes 2002). Integrins are an integral part of the molecular arsenal by which cells can physically interact with other cells and with the ECM. The ECM ligands of integrins are co-assembled by integrins into a complex supramolecular structure, which is composed of multiple ECM proteins and displays remarkable structural and biochemical diversity across tissues (Mouw et al. 2014). Cells modify their behaviour in response to the composition and physical state of the ECM, which can be detected by the sophisticated and multifaceted integrin signalling machinery (Geiger & Yamada 2011, Legate et al. 2009). This enables cells to function as a collective coordinated by the ECM, which is the basis for multicellular architecture and function (Rozario & DeSimone 2010).

The aim of this study was to investigate the molecular mechanisms underlying the formation of simple epithelial structures, specifically those regulated by integrins and their ECM ligands. Integrins guide the orientation of epithelial cell polarity by transmitting ECM-sensitive spatial cues to organize epithelial cells into lumen-enclosing epithelial spheres (Lee & Streuli 2014, Overeem et al. 2015). These cues originate from specialized ECMs called basement membranes (BM) that epithelial cells associate with (Yurchenco 2011). Another aim was to study how integrins transmit mechanical signals in response to ECM rigidity, thereby promoting epithelial cell contractility and cell spreading (Geiger et al. 2009). As epithelial cells interact with the ECM via multiple integrins, the emphasis in these studies was on understanding how the different integrin heterodimers contribute to the chemical and mechanical sensing of the ECM to orchestrate the above mentioned cellular behaviours.

This thesis will begin with a review of the literature, where the first chapter will describe the basic molecular mechanisms by which epithelial polarity is established and how orientation of this polarity is regulated by spatial cues from the ECM. The second chapter will describe how BMs are assembled and regulate epithelial development and morphogenesis and the final chapter will focus on the functions of integrins. The major aims of this study will be then introduced and the obtained results described, followed by a discussion about the significance of the results in relation to what has been reported in literature.
2 Review of the literature

2.1 Apicobasal polarity is the fundamental design principle of epithelial tissues

Epithelial tissues cover the external surfaces and internal cavities of all metazoans and constitute the major protective and communicative interface of the body towards the outside environment (Roignot et al. 2013). The epithelia can be comprised of a single cell layer (simple epithelium) or multiple layers (stratified epithelium) with distinct cell morphologies (squamous, cuboidal and columnar), and take the shape of sheets or lumen-enclosing tubules and cysts (termed acini in the mammary gland, alveoli in the lung and follicles in the thyroid) (Roignot et al. 2013, St Johnston & Sanson 2011). The basic form and function of all epithelia is based on the polarization of the epithelial cell plasma membrane (PM) into two opposing domains with specific structure, function and spatial orientation relative to the biological compartments. The apical membrane domain, facing the lumens of tubular organs and covering the external surfaces, mediates the exchange of ions and biomolecules between body compartments and with the external environment (Roignot et al. 2013). The apical surface domain is responsible for organ-specific functions, such as secretion, excretion, absorption, filtration and transport (Roignot et al. 2013). The combined apical domains of epithelial cells unfold into large continuous surface areas, further elaborated in branched organs, where the exchange of substances meets physiological requirements. The basolateral membrane domain interacts with other cells and with the underlying tissue via a myriad of cell adhesion receptors that bind counter receptors in neighbouring cells or ligands of the ECM. The basolateral domain is subdivided into lateral and basal regions of cell-cell and cell-ECM contact that fix the position of the epithelial cell within the epithelium. Different types of adhesions are arranged into junctions that display a discrete organization along the apicobasal axis. The surface polarity of epithelial cells in vertebrates is maintained by intercellular tight junctions (TJ); molecular seals formed at the apical-basolateral boundary between two cells by claudins, occludins and junctional adhesion molecules that are homophilic cell adhesion receptors (Wang & Margolis 2007). TJs prevent the intermixing of the protein and lipid contents of the apical and basolateral domains within the outer leaflet of the PM (fence function) while also restricting the paracellular diffusion of solutes across the
epithelium (barrier function). Intercellular adhesion is mediated by cadherin, nectin and nectin-like transmembrane proteins at adherens junctions (AJ) directly beneath the TJ at the lateral domain (Wang & Margolis 2007). Together the TJs and AJs comprise the apical junctional complex, which is an important organizing center for apicobasal polarity (Wang & Margolis 2007).

2.1.1 Molecular toolbox for generation of plasma membrane asymmetry

The surface polarity of epithelial cells is complemented by asymmetric organization of cytoplasmic components, including organelles and cytoskeletal elements (Bryant & Mostov 2008). Importantly, the membrane trafficking system is routed to supply cargo to the apical and basolateral domains separately, which requires the assembly of specialized molecular machinery to the PM-cytosol interface (Apodaca et al. 2012). Cell polarization is established upon the dynamic interplay between multiple classes of membrane proteins, lipids and membrane-proximal proteins of the cytosolic face of the PM that are engaged in reciprocal crosstalk with cell adhesion molecules (Rodriguez-Boulan & Macara 2014). Cell adhesion induces a symmetry breaking event, leading to segregation of initial polarity landmarks at the membrane cortex, followed by elaboration of this asymmetry by engagement of the cytoskeleton and the membrane trafficking system (McCaffrey & Macara 2012). The underlying principle is that components become enriched within a specified domain and depleted from beyond the boundaries of this domain (McCaffrey & Macara 2012). Initial membrane asymmetry is established in combination by positive feedback loops and mutual antagonism of apical and basolateral polarity determinants (Goehring 2014). This dynamic balance is then enforced by physical diffusion barriers set in place by cell junctions coupled to the cortical actin cytoskeleton and amplified by polarized membrane trafficking. The precise interrelationships between polarity determinants and their hierarchy in the molecular cascade of cell polarization remains incompletely understood.

Polarity complexes

The formation of preliminary cell-cell adhesions is thought to trigger the apicobasal polarization process by recruitment of a conserved group of universal polarity regulators called the polarity complexes (Roignot et al. 2013). The
polarity complexes then distribute into non-overlapping regions at the PM based on their mutual antagonism and drive further segregation of downstream components. Their localization is regulated by protein-protein interactions and post-translational modifications that promote membrane association and dissociation, respectively (McCaffrey & Macara 2009).

The roles of polarity complexes in the establishment of epithelial apicobasal polarity varies between invertebrates and vertebrates mainly due to the different arrangement of intercellular cell-cell junctions to which polarity complexes become localized (St Johnston & Ahringer 2010). Three different polarity complexes are known, but their positions differ due to the aforementioned reasons. In mammals, the partitioning defective (Par) complex, composed of partitioning defective 3 homolog (PAR3), partitioning defective 6 homolog (PAR6) and atypical protein kinase C (aPKC also known as PKCα), resides at the TJ. The Crumbs complex, composed of crumbs homolog 3 (CRB), a protein associated with lin seven 1 (PALS1 also known as MPP5) and the PALS1-associated tight junction protein (PATJ also known as INADL), demarcates the apical membrane. The scribble complex, formed by protein scribble homolog (SCRIB), lethal giant larvae protein homolog (LGL also known as LLGL) and discs large homolog (DLG), associates with the AJ and demarcates the basolateral domain together with MAP/microtubule affinity-regulating kinase 2 (PAR-1 also known as MARK2) (St Johnston & Ahringer 2010). The two kinases aPKC and PAR-1 are key players that facilitate mutual exclusion between apical and basolateral polarity determinants, respectively (McCaffrey & Macara 2012).

The polarity complexes are not constitutive and there are crossovers during different stages of the apicobasal polarization process. Initially PAR3 is recruited to the early adhesions and facilitates targeting of PAR6/aPKC. However, the tripartite Par complex is destabilized upon phosphorylation of PAR3 by aPKC, which leads to redistribution of the PAR6/aPKC pair to the apical surface. Therein, the PAR6/aPKC complex may interact with CRB and/or the small GTPase cell division control protein 42 homolog (Cdc42), thereby outcompeting PAR3, which remains confined to the TJ (Atwood et al. 2007, Harris & Peifer 2005, Morais-de-Sa et al. 2010, Nagai-Tamai et al. 2002). Destabilization of the Par complex is required for the definition of the apical/lateral border. In the early adhesions, LGL also competes with PAR3 for binding of PAR6/aPKC before it is phosphorylated by aPKC and expelled to the basolateral domain to form the scribble complex (Yamanaka et al. 2003, Yamanaka et al. 2006). The Par complex is excluded from the basolateral domain by the phosphorylation of PAR3 by PAR-
Therefore, the restriction of PAR-3 to the TJ between the apical and basolateral domains is the combined outcome of its exclusion from the apical surface domain by CRB and aPKC and from the basolateral surface domain by PAR-1 (Benton & St Johnston 2003, Hurd et al. 2003). PAR-1 on the other hand is repelled from the apical surface domain by direct or indirect phosphorylation by aPKC (Hurov et al. 2004, Watkins et al. 2008). Removal of aPKC and PAR-1 substrates is mediated by recruitment of PAR-5 also known as 14-3-3 from the cytoplasm, the binding of which uncouples membrane interaction (Benton & St Johnston 2003, Hurd et al. 2003, Watkins et al. 2008).

Precise balancing of these apical and basolateral exclusion mechanisms is needed as overexpression of key factors in the system leads to expansion of one domain over the other (Chalmers et al. 2005, Roh et al. 2003). Aside from the transmembrane protein CRB, the polarity complexes associate with the membrane via lipids or other membrane proteins, frequently junctional cell-cell adhesion molecules (Nelson 2003). Polarity complexes in turn regulate the maturation of cell-cell adhesions into distinct junctional complexes and thereby enhance the molecular sorting and compartmentalization at the cell surface (Ooshio et al. 2007). Segregation of cell-cell junctions and polarity complexes during apicobasal polarization is illustrated in Figure 1. Although cell adhesion molecules are required for initial targeting of the polarity complexes, cells have been shown to polarize in the complete absence of cell-cell adhesion, suggesting that there is remarkable plasticity in the organization of the polarity program (Baas et al. 2004).

**Phosphoinositides**

Phosphoinositides (PIPs), the phosphorylated products of phosphatidyl inositol, are a special class of signalling lipids that play fundamental roles in membrane recognition by promoting the assembly of membrane-associated factors that mediate membrane trafficking (Di Paolo & De Camilli 2006). In eukaryotes seven different PIP species are known that each become specifically enriched to a discrete membrane compartment. PIPs can be rapidly converted from one species to another by removal or addition of phosphate groups at positions 3, 4 and 5 of the inositol ring by specific phosphatases and kinases (Di Paolo & De Camilli 2006). Specification of the membrane by PIPs is based on their selective affinities towards effector proteins containing different PIP-binding modules (Shewan et al. 2011). Their relatively weak binding is potentiated by co-clustering and...
recruitment of additional proteins and/or lipids. Spatiotemporal synthesis and turnover of PIPs across cell membranes is the basis for vectorial vesicle transport by ensuring that appropriate factors are assembled to facilitate transport, docking and fusion of the vesicles to the acceptor membranes and then dissipated once they are no longer needed (Shewan et al. 2011).

The enrichment of specific PIPs also plays a role in specifying the apical and basolateral PM domains during cell polarization. The PIP species associated with the PM are PI(3,4,5)P₃, which signifies basolateral identity (apical identity in drosophila retinal epithelium (Pinal et al. 2006), and PI(4,5)P₂, which signifies apical identity (Gassama-Diagne et al. 2006, Martin-Belmonte et al. 2007). When the balance of these PIPs is experimentally manipulated by feeding of ectopic PIPs to either compartment, thereby exchanging one species with the other, the membrane identity is reversed. This suggests that PIPs are essential to defining the identities of the apical and basolateral membrane domains (Gassama-Diagne et al. 2006, Martin-Belmonte et al. 2007). PI(3,4,5)P₃ is metabolized into PI(4,5)P₂ by the action of phosphatase and tensin homolog on chromosome 10 (PTEN), which regulates apical membrane biogenesis (Martin-Belmonte et al. 2007). PTEN is recruited to cell-cell junctions by PAR3 (Feng et al. 2008), where it starts to synthesize PI(4,5)P₂ over the apical surface and helps to target the apical Cdc42/PAR6/aPKC complex (Figure 1) (Martin-Belmonte et al. 2007). The apical complex is important for the establishment of apical vesicle transport (Apodaca et al. 2012). The reverse process is catalysed by phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), which resides in the adherens junction at the basolateral membrane domain where it is targeted by DLG and E-cadherin and serves as a source of lateral PI(3,4,5)P₃ (Figure 1) (Laprise et al. 2004). The role of PIPs may be to integrate cortical polarity with polarized membrane trafficking by controlling exocytosis of apical and basolateral cargo vesicles to the appropriate membrane domain (Figure 1) (St Johnston & Ahringer 2010).

**Rho GTPases**

A major class of PIP effectors is the Ras superfamily of small GTPases, further subdivided into five subfamilies (Ras, Rab, Ran, Rho and Arf), that function by switching between GTP-bound active and GDP-bound inactive states (Shewan et al. 2011). Signalling is induced by guanine nucleotide exchange factors (GEF) that exchange GDP for GTP and terminated by GTPase activating proteins (GAPs) that promote GTP hydrolysis. Many of these factors specific to a
particular subfamily contain PIP-binding domains by which they promote membrane association and activation of GTPases in a spatiotemporally controlled manner. Most small GTPases also contain lipid anchors that regulate their membrane-association (Wennerberg et al. 2005). These lipid anchors in Rho and Rab GTPases can be masked by binding of GDP dissociation inhibitors (GDI) that thereby prevent these GTPases from associating with membranes (An et al. 2003, Di-Poi et al. 2001).

Of particular interest are the Rho family of GTPases, which is composed of 22 members including the most studied members ras homolog family member A (RhoA), ras-related C3 botulinum toxin substrate 1 (Rac1) and Cdc42 that regulate cortical actin polymerization and also interface with the membrane trafficking system by regulating exocytosis and endocytosis in coordination with other GTPases (de Curtis & Meldolesi 2012). Rho GTPases are known to occupy several subcellular pools simultaneously in distinct signalling modules that regulate different cellular processes (Pertz 2010). This is achieved by more than 70 different GEFs, a similar number of GAPs and three GDP dissociation inhibitors (GDI) that control their localized activation and by 70 different effectors including enzymes and scaffold proteins that specify the output signalling (Cherfils & Zeghouf 2013, Iden & Collard 2008). Classically, Rho GTPases induce nucleation of different types of actin filaments at cytoplasm membrane interfaces, either branched networks or linear bundles that can be contractile, thereby promoting localized deformation of the membrane into distinct protrusions or invaginations (Burridge & Wennerberg 2004). Rho GTPases are engaged in mutual antagonism of each other and display non-overlapping patterns of activation throughout the cellular membranes, whereby the contractile forces generated by RhoA activity stand in contrast to the protrusive forces generated by Rac1 and Cdc42 (Iden & Collard 2008). In polarized cells, Rho GTPases are intimately linked with cell-cell junctions, where they act in concert with the polarity complexes to control the assembly and maintenance of junctions (Figure 1) (Mack & Georgiou 2014). Interaction of Cdc42 with the Par and Crumbs complexes is crucial for apical membrane biogenesis and definition of the apical-lateral border, whereas Rac1 is known to transmit integrin-mediated signals from the ECM to regulate maturation of the basal surface domain (Bryant et al. 2010, Morais-de-Sa et al. 2010, O’Brien et al. 2001, Yu et al. 2005, Yu et al. 2008). Globally, the coordinated functions of different Rho GTPases recruited to distinct PM domains and junctions organize
the cortical cytoskeleton according to the underlying membrane asymmetry thereby constructing the overall shape of the cell (Figure 1).
Fig. 1. Molecular and structural organization of apicobasal polarity in simple epithelial cells in vertebrates. A) The polarization process is initiated by recruitment of the Par and Scribble complexes to early cell-cell adhesions (boxed area, molecules and interactions elaborated in the insert on the left). The two complexes transiently overlap as LGL and PAR3 compete over binding of PAR6. PAR and Scribble complexes interact with lipid metabolizing enzymes that begin to segregate PM species of PIPs to generate membrane asymmetry. aPKC is activated by Rho GTPases and phosphorylates PAR3 and LGL, which inhibits their association with PAR6, thereby promoting the interaction of PAR6-aPKC with the Crumbs complex. This enables PAR6-aPKC to position apically while PAR3 is positioned to the maturing TJ and the Scribble complex to the AJ. Structural features of the pre-polarized state are illustrated in the cell on the right. B) Upon destabilization of the PAR complex, PAR3 associates with TJs via JAM and helps to define the apical-lateral border. PAR3 interacts with PTEN that synthesizes PIP2 over the apical membrane domain, thereby promoting apical membrane association of Cdc42. Active Cdc42 interacts with PAR6-aPKC to regulate apical membrane transport, thus promoting apical membrane identity. aPKC also phosphorylates CRB, thereby enhancing its association with PALS1-PATJ that are anchored to the TJ via claudin. The apical complexes are excluded from the basolateral domain by PAR-1-mediated phosphorylation. aPKC on the other hand excludes the Scribble complex and PAR-1 from the apical domain. Polarity proteins and junctional molecules contribute to the asymmetric organization of the cortical cytoskeleton and polarized membrane trafficking illustrated in the polarized cell on the right. Solid dash may represent either direct or indirect interactions. Assembled from McCaffrey & Macara (2012), Rodriguez-Boulan & Macara (2014) and Roignot et al. (2013). Abbreviations: MT = microtubule, MTOC = microtubule organizing center, TV = transport vesicle, TGN = trans-golgi-network, E-cad = E-cadherin, ITG = integrin, JAM = junctional cell adhesion molecule.

2.1.2 Cell adhesion dictates orientation of apicobasal polarity

Apicobasal polarity is part of the inherent cell-autonomous genetic programming of epithelial cells but requires instructions from the microenvironment to be correctly implemented during development (O’Brien et al. 2002). Individual cells must co-orient their intrinsic polarity with other cells and do so relative to the ECM for higher order multicellular organization. It is generally believed that cell-cell and cell-ECM adhesion receptors transmit guidance cues that inform the cell of the relative position of the neighbouring cells and the ECM, thereby defining the axis of apicobasal polarity (Roignot et al. 2013). Most adhesion receptors are known to possess signalling functions and are activated in a localized fashion in response to different cell surface or ECM ligands so that each signal carries
within a specific chemical and spatial signature (Yeaman et al. 1999). This input is deciphered by the cell in a concept of surface detection, which is used as a reference for orientation of the intrinsic polarity program. A model has emerged in which individual epithelial cells are subject to a constant geometric constraint to possess three surfaces and if not satisfied, to engage in compensatory behaviour up until coalescence into epithelial type architecture (O'Brien et al. 2002, Zegers et al. 2003). Cell-cell and cell-ECM interactions are said to impart lateral and basal identity for the membrane at which they are positioned. Lack of adhesions on the other hand in so-called free surfaces is thought to signify apical identity (O'Brien et al. 2002). The basic rules that define orientation of cell polarity are largely based on studies conducted in cultured Madin-Darby canine kidney (MDCK) cells (Yeaman et al. 1999).

Polarity from anisotropy

Both cell-cell and cell-ECM interactions in combination are required for complete expression of apicobasal polarity. However, a level of asymmetry between surface molecules is observed in single cells adhering to an ECM so that molecules distribute differently to adherent and non-adherent surfaces (Figure 2A) (Meder et al. 2005). A cell doublet in suspension similarly displays molecular asymmetry between cell-cell contacting and non-contacting surfaces (Figure 2A) (Wang et al. 1990a). Cell adhesion thus represents a symmetry breaking event that triggers cell polarization (Yeaman et al. 1999). The dichotomy between adherent and non-adherent surfaces dictates the axis of polarity as seen in standard two-dimensional (2D) monolayer cultures of epithelial cells where the apical domain forms invariably to the adhesion-free surface exposed to the culture media and the basolateral domain towards the solid support where the cells sit upon (Zegers et al. 2003). Similarly, epithelial cells cultured as a liquid suspension form spherical cysts with apical domains facing the culture medium and basolateral domains towards the center, where an internal ECM-filled cavity is formed (Chambard et al. 1984, Wang et al. 1990a, Wang et al. 1990b). If free surfaces are blocked by either embedding of cysts or overlaying of monolayers with exogenous ECM, such as type I collagen (Col-I), the polarity is reversed in both conditions (Chambard et al. 1984, Wang et al. 1990b, Zuk & Matlin 1996). Cysts relocate apical proteins to an apical lumen and monolayers undergo extensive remodelling into two layers with a lumen in between. These experiments indicate that orientation of polarity is defined differently depending on whether or not free
surfaces are available. Importantly, a lack of free surfaces triggers mechanisms of lumen formation that converge with mechanisms of surface detection and epithelial remodelling for correct positioning.

**Polarity from isotropy**

*In vivo* free surfaces are not always available, and cells must generate space in the form of lumens *de novo* to accommodate the apical domain and to acquire the polarized phenotype. Referred to as mesenchymal to epithelial transition (MET), this phenomenon is observed throughout development whereby non-polarized precursors give rise to polarized lumen-forming cells (Bryant & Mostov 2008). Developmental MET involves cell fate decisions, but other aspects can be recapitulated by organotypic culture of epithelial cells within three-dimensional (3D) ECM gels (Bissell et al. 2003, O’Brien et al. 2002). Initially without apparent polarity, cells are required to resolve the axis of polarity based on cues from the ECM and cell neighbours to determine where the apical lumen should form (Figure 2A). 2D-cultured cells with fixed apical domains may bypass the requirement for these cues. For example β1-integrins (integrin heterodimers containing the integrin β1 subunit) that comprise a major family of ECM-binding receptors are not required for polarization of cells in monolayer cultures (Matlin et al. 2003). However, in the absence of free surfaces, β1-integrins regulate orientation of polarity and apical lumen formation in MDCK cells (Ojakian & Schwimmer 1994, Yu et al. 2005, Zuk & Matlin 1996). Similarly, Cdc42 is dispensable for apicobasal polarization in monolayer cultures but is essential for apical lumen formation (Martin-Belmonte et al. 2007, Mertens et al. 2005, Mertens et al. 2006). Based on these observations, the molecular machinery that regulates orientation of polarity is called upon in conditions that require *de novo* lumen formation but may be ignored when the axis of polarity has been pre-established. The 3D ECM culture model is therefore more informative for the study of how polarity is guided by spatial cues of the ECM. Different mechanisms for lumen formation exist *in vivo*, but a common denominator is that the lumen will be positioned away from the ECM. ECM interactions are therefore particularly important for apical guidance.
2.1.3 Reciprocal cell-extracellular matrix interactions regulate orientation of cell polarity

In 3D ECM cultured epithelial cells the ECM-derived cue by which apical domains are guided to a central lumen is most likely mediated by the β1-integrin subfamily (Akhtar & Streuli 2013, Yu et al. 2005, Zovein et al. 2010). During cystogenesis of kidney epithelial cells apical markers initially appear at the membrane opposite the ECM but are quickly transcytosed and accumulate to a site at the cell-cell boundary (Figure 2B) (Bryant et al. 2010, Ferrari et al. 2008). Inhibition of β1-integrins in 3D ECM culture leads to the formation of cysts with the apical domain facing outward at the ECM in a similar orientation as with cysts cultured in liquid suspension, indicating a failure in ECM detection and orientation of polarity (Yu et al. 2005). A series of experiments have uncovered a pathway in which the β1-integrin mediated cue is transmitted via activation of the small GTPase Rac1 and controls deposition of laminin, a major scaffold of the epithelial BM, to the ECM interface, triggering an autocrine signal back to the cell to orient polarity (Figure 2B) (O'Brien et al. 2001, Yu et al. 2005). The function of Rac1 has been suggested to inhibit actomyosin contractility at the basal cell cortex by antagonism of RhoA and its effectors rho-associated protein kinase 1 (ROCK1) and myosin II (Yu et al. 2008). Inhibition of RhoA by integrin-mediated focal adhesion kinase (FAK) signalling is required for destabilization and subsequent endocytosis of the apical proteins from the ECM-facing surface (Figure 2B) (Bryant et al. 2014). An alternative mechanism, whereby active ROCK1 signalling is required for basal localization of PAR-1b, which guides BM positioning and subsequent β1-integrin mediated orientation of polarity has been described in ex vivo cultured submandibular salivary glands (Daley et al. 2012). Assembly of the LN-rich BM is also essential for polarized organization of the mammary acini (Muschler et al. 1999, Weir et al. 2006). In mammary epithelial cells β1-integrins control organization of the microtubular network via the effector integrin-linked kinase (ILK) to orient polarity during acinogenesis, whereas Rac1 seems to be dispensable (Akhtar & Streuli 2013). β1-integrin and α-dystroglycan (DAG) mediated signals from the laminin-rich BM also mediate epiblast polarization and formation of the proamniotic cavity, which can be recapitulated by culture of embryonic stem cells in suspension into embryoid bodies (EB) (Li et al. 2002, Smyth et al. 1998). The BM downstream signalling in EBs probably involves integrins and focal adhesion (FA) components including ILK, LIM and senescent cell antigen-like-containing domain protein 1 (PINCH-1)
and kindlin-2 as well as Rho GTPases (He et al. 2010, Li et al. 2005a, Montanez et al. 2008, Sakai et al. 2003b, Wu et al. 2007). These studies point towards a common principle whereby assembly of the BM structure adjacent to the cell membrane and β1-integrin-mediated ECM signals at the basal cell cortex facilitate acquisition of basal identity most likely via Rho GTPase induced remodelling of the actin cytoskeleton and anchorage of microtubules to reposition the apical domain (Figure 2B).
Fig. 2. Orientation of apicobasal polarity. A) The juxtaposition of adherent surfaces (either cell-cell or cell-ECM) and free surfaces defines the orientation of the polarity axis (marked by an arrow) in cells cultured in standard 2D tissue culture conditions or in suspension. In the absence of free surfaces, when cells are embedded into a 3D ECM, apical surfaces are oriented away from the ECM and into a lumen that forms at the cell-cell contacting surface. B) Sequential reciprocal cell-ECM interactions are required for apical lumen formation. In the absence of BM components cells deposit laminin (LN) to the ECM in a polarized manner which requires integrin-mediated Rac1 activation via PI3K signalling. PAR-1 restricts laminin-receptors to the basal surface to promote laminin assembly. Laminin-derived Integrin signalling via ILK guides anchorage of microtubules. In addition, FAK mediated inhibition of basal RhoA activity enables apical proteins, such as podocalyxin (Podxl) and CRB, to be transcytosed from the basal surface along microtubules towards the apical membrane initiation site (AMIS). Assembled from Li & Streuli (2014) and Overeem et al. (2015).

2.1.4 De novo lumen formation

Two mechanisms are utilized by epithelial cells to generate lumens de novo. These include hollowing, which is established upon polarized membrane trafficking, and cavitation, which relies upon apoptosis (Figure 3) (Lubarsky & Krasnow 2003). The two are not mutually exclusive, but are utilized in a context dependent manner depending on the balance of cell proliferation and cell polarization (Martin-Belmonte et al. 2008). Both mechanisms rely upon cell polarization induced by a cue from the laminin-rich BM as a prerequisite (Andrew & Ewald 2010). Physical dimensions and mechanical properties of the ECM are also important factors in regulating lumen formation. Increased cell confinement that restricts cell contractility and thereby inhibits cell spreading is permissive for lumen formation (Rodriguez-Fraticelli et al. 2012).

In hollowing, the coalescence of exocytic vesicles carrying anti-adhesive proteins to the apical membrane initiation site at the cell-cell apposing surface promotes membrane repulsion and initiates lumen formation (Figure 3A). Coupling of the vesicular transport system to the apical cortex by polarity landmarks underlines de novo lumen formation (Bryant et al. 2010, Martin-Belmonte et al. 2007). Enrichment of PIP2 at the apical surface domain by the function of PTEN serves to recruit Cdc42 via the PIP2-binding protein annexin-2. Active Cdc42 couples the PAR6-aPKC complex to the apical surface domain, which is required for apical targeting of vesicles transporting apical proteins such as podocalyxin (Podxl) and CRB (Bryant et al. 2010, Martin-Belmonte et al. 2007). The docking of these vesicles is regulated by the exocyst in collaboration
with the PAR3/aPKC complex (Bryant et al. 2010). MDCK cells cultured in conditions that favour rapid cell polarization over cell proliferation, such as in the presence of BM laminins, form lumens preferentially via hollowing (Martin-Belmonte et al. 2008). The process can be observed *in vivo* for example in the zebrafish gut tube (Bagnat et al. 2007).

Cavitation involves polarization of peripheral cells in a larger cell cluster, which leads to selective apoptosis of the entrapped central cells and results in clearance of the lumen. Formation of the proamniotic cavity in the epiblast during mammalian embryogenesis occurs by programmed cell death of the inner cell mass (ICM) as exemplified in studies conducted in EBs (Li et al. 2001, Murray & Edgar 2000). Mammary epithelial MCF10 cells also utilize cavitation as a means for generating lumens during acinogenesis (Mailleux et al. 2008). In the mammary acini, the apoptosis regulator Bcl-2 family of factors plays a role in regulating luminal cell apoptosis, but additional mechanisms such as autophagy are also involved (Debnath et al. 2002, Mailleux et al. 2007). The selective apoptosis of luminal cells is thought to be triggered by loss of ECM contact, which may involve loss of integrin-mediated survival signals (Mailleux et al. 2008). Cavitation is triggered in conditions where cells proliferate to a large enough cluster prior to acquisition of polarity so that there are distinct central and peripheral cell populations (Figure 3B). MDCK cells can be conditioned to undergo cavitation instead of hollowing by compromising the polarity status of the cells either by culture in an unfavorable ECM, in which cells must first deposit an endogenous BM, or by inhibition of certain polarity determinants (Martin-Belmonte et al. 2008).
2.1.5 Maintenance of lumens during oriented cell division

Lumen integrity requires spatial control of the cell division axis so that dividing cells stay confined to the epithelial layer and do not intrude the lumen. This is maintained in so-called symmetric cell division as opposed to asymmetric cell division in which one of the daughter cells escapes the epithelium to undergo differentiation (Siller & Doe 2009). Cell division must be coordinated with the cell’s intrinsic polarity program to be correctly oriented. Therefore, the same molecular machinery that controls apicobasal polarity also guides oriented cell division. Cell division is not only controlled in the apicobasal dimension, but also in the planar dimension by the planar cell polarity program, which is essential for epithelial morphogenesis. Symmetric cell division of epithelial tissues is established upon rotation of the mitotic spindle perpendicular to the apicobasal axis during metaphase, so that the cleavage plane is aligned with the epithelial plane (Reinsch & Karsenti 1994). Spindle rotation involves linkage of the astral microtubules of the mitotic spindle to the cell cortex by cortical cues and coupling of the dynein-dynactin motor complex to generate pulling forces along
microtubuli. A complex composed of G-protein signalling modulator 2 (LGN), nuclear mitotic apparatus protein 1 (NuMA) and the membrane anchor guanine nucleotide-binding protein G(i) subunit alpha 1 (GαI) regulates spindle orientation in various model organisms by anchoring astral microtubules (Morin & Bellaiche 2011). Restriction of the microtubule-capturing complex to the lateral cell cortex is necessary for correct spindle orientation (Peyre et al. 2011, Zheng et al. 2010). The lateral targeting of the LGN complex may be regulated by DLG of the scribble complex (Saadaoui et al. 2014). Members of the apical polarity apparatus are also involved in the spatial partitioning of the complex. LGN is a substrate of αPKC, which facilitates its PAR5-mediated apical exclusion, thereby preventing apical microtubule capture (Hao et al. 2010, Zheng et al. 2010). Apical targeting of αPKC via interplay of PAR3, PAR6 and Cdc42 is essential for symmetric cell division and single lumen maintenance (Durgan et al. 2011, Hao et al. 2010, Jaffe et al. 2008). Inhibition of Cdc42 or Cdc42-specific GEFs leads to defects in lumen integrity due to loss of cell division control (Jaffe et al. 2008, Qin et al. 2010, Rodriguez-Fraticelli et al. 2010). Cdc42 helps regulate epithelial morphogenesis firstly, by generating the apical surface domain, and secondly, by maintaining it via oriented cell division.

2.2 Basement membranes provide instructions for epithelial morphogenesis

Epithelial cells sit upon BMs, which are specialized ECMs composed of different ECM proteins that together form a supramolecular cell-associated sheet-like structure (Li et al. 2003b). The BM is both a structural and a signalling entity perceived by the cells via specialized cell adhesion receptors that send signals for cells to carry out behaviour such as cell adhesion, migration, differentiation, polarization, survival and proliferation. Globally the BMs serve as cell scaffolds that organize epithelial sheets and tubes and delimit cell populations by serving as semipermeable barriers between epithelial cells and the underlying stromal cells or in some cases other epithelial cells (e.g. glomerular and alveolar BMs). Cells at both sides of the BM contribute to the deposition of its ECM protein components (Spenle et al. 2013). BMs promote MET and are thought to stabilize the polarized epithelial phenotype, whereas cancer cells turn off BM assembly by blocking cell anchoring of laminins (Akhavan et al. 2012). In some cases BMs constitute barriers that divert or bring to a halt migrating cells or require disassembling to allow cell migration to proceed (Rozario & DeSimone 2010). Remodelling of the
BM by ECM deposition and degradation is important for cell rearrangements in complex developmental processes such as branching morphogenesis during which cells must alternate between motile and stationary behaviour by transitioning between differentiated states (Nistico et al. 2012). The chemical composition of the BM and the repertoire of ECM receptors expressed by the cell are not the only factors defining cell behaviour, but also local concentration gradients, mechanical properties and embedded growth factors (GFs) greatly influence cellular responses to a particular ECM (Rozario & DeSimone 2010).

2.2.1 Structure and assembly of the basement membrane

All BMs display the same core structure in which polymerized laminins and type IV collagen (Col-IV) trimers form a molecular scaffold incorporating other stabilizing components such as nidogens and heparin sulfate proteoglycans (HSP) (Figure 4). Tissue specific expression of different isoforms of the core molecules and of different supplementary components accounts for diversity of the final structure, signalling capacity and stability of the BMs (Yurchenco 2011). The glomerular BM, composed of altogether 35 distinct ECM proteins contributed by secretions of both podocytes and endothelial cells at each side, exemplifies the complexity of specialized BMs (Byron et al. 2014). The BM is organized asymmetrically, so that each side has distinct cell-binding properties. The epithelial side is laminin-rich and the stromal side rich in fibrillar collagen and fibronectin (FN) (Halfter et al. 2013). In between is a transitional matrix rich in collagens XVII, XV and VI that integrates the two faces of the BM (Yurchenco 2011).

Laminins, bound by specific integrins and non-integrin receptors, are essential for BMs, and BM assembly is initiated by formation of the cell-anchored laminin polymer in a process of cell-assisted self-assembly (Hohenester & Yurchenco 2013). Other BM components contribute to the integrity of the BM but are not essential for BM assembly (Bader et al. 2005, Costell et al. 1999, Gautam et al. 1996, Poschl et al. 2004). Type IV network forming collagen is a ubiquitous component of all BMs and bound by collagen-binding integrins α2β1 and α1β1 and discoidin domain receptors (DDRs) (Hudson et al. 1993, Leitinger & Hohenester 2007). In addition to their structural role, HSPs are involved in ECM sequestering of many GFs, serve as co-receptors for receptor tyrosine kinases (RTK) in GF-binding, potentiate ECM-binding of integrins, and thus promote crosstalk between cell adhesion and RTK signalling (Sarrazin et al. 2011).
Laminin-assembly

Laminin heterotrimers are formed by 16 different combinations of 5α, 4β and 3γ chains\(^1\). Laminins show tissue-specific patterns of expression accompanied by their laminin-binding receptors. BMs can only form on so-called competent cell surfaces that express suitable receptors for laminin because cell anchorage is required to reach the local threshold concentration that allows laminin polymerization to occur (Li et al. 2002, Li et al. 2005b, McKee et al. 2007, McKee et al. 2009, Smyth et al. 1998). The laminin heterotrimer is a cross-shaped molecule of which the three chains form a coiled coil α-helix at the long arm and separate to form the short arms. The N-terminal LN-domain at the ends of the short arms facilitates polymerization with LN-domains of adjacent molecules, which is essential for BM assembly (Figure 4) (McKee et al. 2007). The laminin heterotrimers containing the α3 and α4 chains do not possess intact LN-domains in all of the three short arms and are incapable of polymerization and therefore do not support BM assembly (Aumailley et al. 2005, Hohenester & Yurchenco 2013). BM assembly is mediated by the prototypical laminins containing the α1-, α2 or α5 chains that are competent in polymerization. The C-terminus of the α-chain at the base of the coiled coil domain contains LG-domains 1-5 that facilitate cell binding by integrins α3β1, α6β1, α7β1, α6β4, DAG, heparin sulphates, sulfated glycolipids (SGL) and the Lutheran blood group antigen/basal cell adhesion molecule, which show distinct ligand-binding specificities towards different laminin isoforms (Hohenester & Yurchenco 2013). Cell binding via the LG-domain is thought to co-orient and align laminins in two dimensions such that the LN-domains of adjacent molecules are presented for polymerization (Figure 4) (Hohenester & Yurchenco 2013). The N-terminal LN-domain of the α-chain at the short arm contains a second site for cell-binding via integrins α1β1, α2β1, known primarily as collagen receptors, α3β1, sulphatides and heparin, the significance of which is poorly understood (Yurchenco 2011).

The relative roles of specific laminin-binding receptors in regulating laminin-assembly seem to be tissue specific, and co-expressed receptors probably cooperate in laminin-anchorage, but a comprehensive view of their unique contributions is lacking. SGLs have been suggested to provide the laminin anchoring function in Schwann cells and when sulphatides are incorporated to the

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\(^1\) Laminin heterotrimers are identified using three Arabic numerals, based on the α, β, and γ-chain numbers (e.g. laminin-511 is a heterotrimer formed by the α5, β1 and γ1 chains) (Aumailley et al. 2005).
PMs of non-BM-forming fibroblasts they become competent for BM assembly (Li et al. 2005b). The BMs of the mammary epithelium, skeletal muscle and the extra-embryonic Reichert’s membrane (RM) on the other hand depend upon DAG for cell surface anchoring of laminins (Colognato et al. 1999, Henry & Campbell 1998, Weir et al. 2006, Williamson et al. 1997). β1-integrins have been proposed to mediate BM assembly in the skin (Raghavan et al. 2000). In EBs, β1-integrins regulate laminin expression, but neither integrins nor DAG are uniquely required for laminin anchorage (Li et al. 2002). Recently the mechanophysical properties underlying laminin-assembly in mammary epithelial acini have been characterized, whereby rotational movement of epithelial cells involving polarity complexes and cytoskeletal dynamics is required for weaving of the laminin matrix (Wang et al. 2013). The process of weaving is unique for BM components and is lost in cancer-derived cells. These results suggest that BM assembly is not a passive process delimited to the extracellular interface of the outer cell membrane but requires linkage of the BM to the cytoskeleton and BM-derived signalling to the cell.
Fig. 4. Structure and assembly of the BM. BM assembly is initiated by polymerization of laminin at the cell surface. Cell surface anchorage is provided by laminin receptors, including integrins, DAG and SGLs that bind to laminin LG domains. Additional linkages may be provided via the LN-domain of the α-chain. The αLN, βLN and γLN-domains of adjacent laminin molecules form ternary nodes that facilitate polymerization. A second, covalently cross-linked network is formed by self-assembly of type IV collagen. The laminin and collagen networks are integrated via nidogens. HSPs (Agrin and Perlecan) serve as collateral linkages to cell surfaces by binding to both ECM molecules and cell surface receptors. HSPs also tether GFs, thereby promoting RTK-signalling. From Peter D. Yurchenco (2011). Reprinted with permission from Cold Spring Harbor Laboratory Press.
2.2.2 Regulatory roles of basement membrane interactions in epithelial development inferred from loss-of-function studies

The role of BMs in guiding epithelial morphogenesis has been long appreciated by the use of function blocking antibodies in organotypic systems (Klein et al. 1988, Schuger et al. 1990, Streuli et al. 1995). Genetic studies in mouse models combined with ex vivo organ culture models have unravelled the highly complex cell-ECM interactions of the BM that orchestrate development of different epithelial systems. Loss of core BM components leads to severe consequences that manifest early during development, while loss of specific receptors leads to more restricted tissue-specific effects (Hynes 2002, Yurchenco et al. 2004). Some examples of the roles of laminins and their integrin receptors in the development of epithelial tissues will be highlighted in the next couple of paragraphs. The major epithelial specific laminin heterotrimers include laminin-111 (LN-111), laminin-511 (LN-511) and laminin-332 (LN-332), whose main integrin receptors are α3β1, α6β1 and α6β4 (Ekblom et al. 1998, Gilcrease 2007).

Peri-implantation

Embryonic BMs contain LN-111, LN-511, Col-IV, nidogens and perlecan (Li et al. 2003b). LN-111 is prevalent in embryonic BMs but becomes more restricted during organogenesis and is ultimately replaced by LN-511 in specific tissues (Ekblom et al. 1998). LN-111 is the only isoform that is uniquely required at the peri-implantation stage, while LN-511 becomes critical later at the onset of organogenesis (Miner et al. 1998, Smyth et al. 1998). LN-111 is essential for epiblast differentiation and polarization, whereby the outer layer of the ICM differentiates into the primitive endoderm and deposits a BM that promotes polarization of the adjacent ICM cells into ectodermal cells and apoptosis of the central ICM cells to form the proamniotic cavity. Mouse embryos lacking Lamb1 (laminin β1) and Lamec1 (laminin γ1) genes fail to develop the embryonic BM and the extra-embryonic RM, arresting embryonic development at embryonic day (E) 5.5 (Miner et al. 2004, Smyth et al. 1998). LN-511 can compensate for the loss of LN-111 in the assembly of the embryonic BM, whereby the embryonic ectoderm is able to undergo cavitation, but this replacement does not occur in the RM of mice deficient for the Lama1 (laminin α1) gene leading to lethality at E7 (Miner et al. 2004). The major cellular receptors mediating BM interactions at this time are β1-integrins and DAG. DAG is essential for the development of the RM
because null mice die at E6.5, but it is not for the embryonic BM (Williamson et al. 1997). Loss of β1-integrin heterodimers upon deletion of the Itgb1 (integrin β1) gene in mice leads to absence of the embryonic BM and deterioration of the ICM of the epiblast at E5.5 (Fassler & Meyer 1995, Stephens et al. 1995). In Itgb1 knockout (KO) EBs the endodermal cells fail to mature and do not trigger laminin expression (Li et al. 2002, Liu et al. 2009). Later studies argue that the endoderm deposits BM components, but the adjacent ICM cells fail to retain the ECM, leading to dissemination of the endodermal layer (Moore et al. 2014). The major heterodimers formed by β1 integrin at the epiblast side are the laminin-binding α6β1 and FN-binding α5β1 with additional heterodimers expressed by extra embryonic tissues (Liu et al. 2009). Even though the α6β1-receptor is the only laminin-binding integrin heterodimer in the blastocyst at this stage, integrin α6 does not share the same requirement as integrin β1 for embryogenesis since Itga6 (integrin α6) KO mice survive until birth (Georges-Labouesse et al. 1996).

One possible explanation is that the laminin-binding function of α6β1 is not needed to regulate laminin expression by β1-integrins as reported by Li et al. (2002). This would explain the more severe phenotype of Itgb1 KO embryos. Stabilization of the BMs by Col-IV becomes essential at E10.5 (Poschl et al. 2004), but the collagen-binding integrins are not uniquely required for embryogenesis or organogenesis, suggesting that they may be functionally redundant (Chen et al. 2002, Gardner et al. 1996, Holtkotter et al. 2002).

Organogenesis

The specific requirement of laminins containing the α5 chain becomes critical at E14-17 onwards for neural tube closure, digit separation, placenta, kidney, lung, salivary gland, hair follicle and tooth development (Spenle et al. 2013). LN-511 is found in nearly all BMs during the somitogenesis stage, explaining the wide-ranging consequences of Lama5 (laminin α5) gene deletion (Miner et al. 2004, Timpl & Brown 1994). Some of the Lama5 KO-specific defects can be attributed to the loss of scaffolding and barrier functions of the BM, especially at sites that experience mechanical forces such as neural folds during neural tube closure and limb buds during digit separation (Miner et al. 1998). However, α5-lamins also provide morphogenetic signals to regulate branching morphogenesis in the salivary gland and kidney, as well as morphogenesis of the tooth and hair follicle (Fukumoto et al. 2006, Li et al. 2003a, Miner & Li 2000, Rebustini et al. 2007). Double homozygous deletion of Itga3 (integrin α3) and Itga6 genes in mice leads
to a developmental phenotype similar to that of Lama5-deletion, which in many respects suggests that these integrins synergistically regulate cell interactions with α5-laminins in many developmental processes (time of death not specified) (De Arcangelis et al. 1999). Single homozygous Itga6 KO mice die at birth with severe skin blistering and abnormal cortical and retinal lamination, whereas Itga3 KO mice, also neonatal lethal, show defects in the kidneys, lung, salivary gland and mild skin blistering (DiPersio et al. 1997, Georges-Labouesse et al. 1996, Georges-Labouesse et al. 1998, Kreidberg et al. 1996, Menko et al. 2001). Deletion of Lama5 in mice leads to perturbed branching morphogenesis and lumen formation of the salivary gland, a phenotype also observed in the Itga3/Itga6 double-deficient mice (Rebustini et al. 2007). Another example of possible synergy is the dual role of α6β4- and α3β1-integrins in regulating branching morphogenesis of the ureteric bud during kidney development as demonstrated in vitro by studies using function blocking antibodies (Zent et al. 2001). In vivo however, the Itga3/Itga6 double-deficient mice develop normal kidneys, though the ureters are missing (De Arcangelis et al. 1999, Georges-Labouesse et al. 1996). Itga3 KO mice show a diminished number of collecting ducts in the papilla region of the kidney, corroborating the in vitro evidence (Kreidberg et al. 1996). Conditional deletion Itgb1 in the ureteric bud in mice leads to a branching defect more severe than that of the Itga3 global deletion, suggesting that additional β1-heterodimers are required (Zhang et al. 2009). Global deletion of Lama5 and kidney-specific deletion of Lamg1 both abrogated the assembly of the collecting duct system and the underlying BM, convincingly demonstrating the morphogenetic role of LN-511 (Liu et al. 2009, Miner & Li 2000, Yang et al. 2011). The loss of laminins not only discontinues integrin-mediated adhesion, but also perturbs the integrity of the BM structure and its capacity to tether relevant GFs (Yang et al. 2011). Moreover, β1-integrins have been shown to engage in crosstalk with GF-mediated signals that stimulate the branching process (Zhang et al. 2009). A role for α3-laminins has also been described for the collecting system (Ryan et al. 1999, Yazlovitskaya et al. 2015). Moreover, the ureteric bud cells express a unique integrin ligand called nephronectin, which is bound by cells of the metanephric mesenchyme across the BM via integrin α8β1 and constitutes a type of intercellular crosstalk necessary for the branching process (Muller et al. 1997).

Because integrin α3β1- and α6-heterodimers (α6β1 and α6β4) also bind laminin isoforms other than LN-511, such as LN-332, the Itga3 and Itga6 null phenotypes have Lama5-independent features, including blistering of the skin.
The skin blistering phenotype is also observed upon loss of laminin α3 and integrin β4, the heterodimer partner of integrin α6, which together comprise the cell adherent part of the epidermal-dermal junction (Dowling et al. 1996, Ryan et al. 1999, van der Neut et al. 1996). In epidermal-dermal junctions LN-332 is bound by the α6β4 integrin in specialized cell adhesions called hemidesmosomes (HD) and anchored to the fibrillar collagen network of the underlying dermal connective tissue via so-called anchoring fibrils composed of type VII collagen of the reticular matrix (Yurchenco 2011). In the absence of these junctional components, the BMs appear to be ultrastructurally normal, even though the HD structures are missing. Conditional ablation of Itgb1 in the skin leads to BM deformation, reduced epidermal proliferation and failure in hair follicle morphogenesis, which are related to the skin-specific defects of Lama5 deletion (Li et al. 2003a, Raghavan et al. 2000). The Itga3 null mice also have similar skin-related characteristics (Conti et al. 2003, Kreidberg et al. 1996). Therefore, α3β1 integrin is likely to mediate cell adhesion to and morphogenetic signalling from the LN-511-composed BM, whereas α6β4 integrin mediates LN-332-dependent anchorage between the epidermal and dermal cell compartments through the epidermal-dermal junction. The α6β4 integrin is also expressed in simple epithelia such as in the kidney, mammary gland and intestine, but the adhesions formed are distinctly different from the specialized HDs observed in the skin (Fontao et al. 1999, Uematsu et al. 1994, Zent et al. 2001). The roles of α6β4 not related to the skin are still poorly characterized, however.

2.2.3 Dynamic remodelling of the basement membrane during epithelial morphogenesis

Dynamic deposition, assembly and degradation of ECM are required for epithelial morphogenesis, whereby cells must expand beyond the boundaries of the existing BM. Dynamically executed changes in molecular composition, concentration and physical properties of the ECM are all factors that define the morphogenetic behaviour of cells (Lu et al. 2011). During development cells migrate long distances along tracks of ECM to form new tissues in distant locations of the embryo (Rozario & DeSimone 2010). To do so the epithelial cells undergo an epithelial to mesenchymal transition (EMT), whereby the polarized stationary epithelial phenotype is replaced by the mesenchymal motile phenotype, which is characterized by disassembly of cell-cell adhesion and degradation of the surrounding BM (Rozario & DeSimone 2010). For example, the epithelial cells of
the neuroectoderm in neural folds are induced by nearby tissues to undergo EMT into neural crest cells that travel in streams along specific ECM routes to redifferentiate into neurons and glial cells of the peripheral nervous system and different structures of the head (Christiansen et al. 2000). Different ECM molecules have been implicated in the control and guidance of migratory cells in a cell type specific manner; some of which are permissive and some non-permissive (Rozario & DeSimone 2010). Degradation of the ECM by different proteases of the matrix metalloproteinase (MMP) and A disintegrin and metalloproteinase (ADAM) families is another important regulatory mechanism during cell migration (Alfandari et al. 2001, Monsonego-Ornan et al. 2012).

During epithelial branching morphogenesis, epithelial buds and tubes invade the surrounding embryonic mesenchyme and bifurcate or form clefts to branch (Kim & Nelson 2012). Reciprocal signalling between the mesenchymal and epithelial cell populations via tissue-specific morphogens is essential for branching morphogenesis. The ECM interfacing both cell populations has an indirect role in regulating how these morphogens are presented to the cells but also possesses intrinsic signalling functions as well as mechanical and adhesive properties important for shape determination. Specific ECM proteins display distinct patterns in their distribution over the different subcompartments of the branching structure, which translates into differences in the behavioural responses of the associated cells (Rozario & DeSimone 2010). The proliferative and migratory bud tips associate with a thinner ECM than the more polarized ducts surrounded by mature BM, which in part relates to matrix degradation by proteases of the MMP family (Fata et al. 2004). ECM composition and concentration is under tight spatiotemporal control and experimental manipulation of the intrinsic chemical balance by reducing or promoting ECM turnover or deposition can lead to perturbations in the branching system (Rozario & DeSimone 2010).

FN is not considered a constitutive BM component but is important in conditions of epithelial remodelling. FN binding in epithelial cells is mediated by α5β1-integrin and integrin αV-heterodimers (Hynes 2002, Margadant & Sonnenberg 2010, Matlin et al. 2003, Moyano et al. 2010, Zuk et al. 2001). FN is specifically required for development of mesodermal tissues, but is important also for epithelial branching morphogenesis (George et al. 1993, Liu et al. 2010, Sakai et al. 2003a). FN is deposited to the cleft region in the salivary gland and induces partial EMT in the associated epithelial cells that take on a motile phenotype with reduced cell-cell and increased cell-ECM adhesion, thereby promoting cleft
progression (Sakai et al. 2003a). Fibrillar collagens are also associated in the
clefts of salivary glands and support their formation, indicating a common
requirement for stromal matrix components (Fukuda et al. 1988, Nakanishi et al.
1986, Nakanishi et al. 1988). Progressive assembly of the FN matrix into a
wedge-like structure may provide mechanical forces for advancement of the cleft
(Larsen et al. 2006). Indeed, cellular actomyosin contractility that acts upon the
FN matrix via integrin adhesions may drive cleft propagation (Daley et al. 2009,
Daley et al. 2011). Contractile actin cytoskeleton, coupled to the ECM via
adhesions, creates tension within the epithelium, which is also essential for
epithelial branching morphogenesis in bifurcating systems (Kim & Nelson 2012).
Fibrillar matrix molecules such as FN and fibrillar collagens are particularly
important for determining the mechanical properties of the ECM and as such,
modulate cell contractility (Erler & Weaver 2009, Rozario & DeSimone 2010).
Cells are known to undergo differential morphogenetic behaviour and undertake
different cell fate decisions depending on the matrix stiffness, which is translated
largely by integrins known as mechanoreceptors in integrin-based adhesions
(Rodriguez-Fraticelli & Martin-Belmonte 2014).

2.3 Integrins integrate signals from the extracellular matrix

In the previous chapters integrins have already been introduced. This chapter is
dedicated to describing their functions in more detail. The integrin family is
composed of altogether 18 α and 8 β subunits that can combine into 24 different
heterodimers (Figure 5). Adherent cells express a distinct subset of the 24
different possible integrin αβ-combinations, largely defining the ECM-binding
properties and therefore the extent to which the cell can sense the chemical nature
of the ECM. Epithelial cells are specialized towards binding of BM proteins but
can also bind proteins of the interstitial matrix since both types of matrices
contribute to the development of epithelial systems as discussed previously.
Different ECM proteins can be bound by multiple different integrin heterodimers
that each possess broad and partially overlapping ligand-binding specificities
towards different isoforms of a particular ECM protein (Humphries et al. 2006).
An integrin can also bind completely unrelated ECM proteins. The multivalency
of integrins is based on their binding affinity towards the same consensus
sequence found at different frequencies in different ECM proteins (Figure 5). For
example, the widely distributed RGD-peptide, found both in ECM proteins and
soluble ligands, explains the broad binding specificities of αV-integrin
heterodimers (αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8) (Humphries et al. 2006). The same consensus sequence is also bound by α5β1- and α8β1-heterodimers. The αI-domain containing β1-integrins (α2β1, α2β1, α10β1 and α11β1) recognize a GFOGER motif in collagens but are able to recognize a different undefined binding site in laminins (Humphries et al. 2006). The precise consensus sequence for laminin-binding non-αI-domain-containing integrins (α3β1, α6β1, α7β1 and α6β4) has yet to be identified (Humphries et al. 2006). Although the biological relevance of the reported binding interactions is incompletely understood, it is clear that the multivalent binding of integrins to ECM-proteins diversifies the intrinsic signalling capacity of the ECM.

Fig. 5. Map of mammalian integrin heterodimers and ligand-binding specificities. The RGD consensus sequence is found in various soluble integrin ligands and ECM proteins. The β2- and β7-integrins are restricted to leukocytes and bind the LVD motif, which is prevalent in soluble integrin ligands and cell surface counter receptors. The αIIβ3 heterodimer is restricted to platelets. Heterodimers that bind ECM proteins such as fibronectin, laminins and collagens as ligands are expressed variably in multiple cell types. Adapted from Humphries et al. (2006) and Richard O. Hynes (2002)
The particular $\alpha\beta$-combination defines not only the ligand-binding specificity of the heterodimer (Hynes 2002) but also specifies what type of signalling input is transmitted into the cell as the cytoplasmic tails support distinct binding interactions with various adaptor proteins (Morse et al. 2014). Although the interactions of the integrin $\beta$-tails are well characterized, it is currently not known how the different integrin $\alpha$-subunits that distinguish the different $\beta_1$- and $\beta_2$-heterodimers contribute to signalling (Legate & Fassler 2009). Therefore, the signalling specificities of integrins are less well understood than the corresponding ligand-binding specificities. Moreover, many of the integrins are subject to alternative splicing at the C-terminus, yielding variants with different cytoplasmic sequences and distinct binding properties (de Melker & Sonnenberg 1999). Some of the integrin subunits, such as $\alpha V$, $\alpha 6$, $\beta 1$, $\beta 2$ and $\beta 7$ are shared between different heterodimers that therefore support similar interactions. Moreover, the cytoplasmic tails of integrins encode for protein binding motifs that are conserved between different subunits (Morse et al. 2014). Therefore, binding of different ECM proteins can induce both unique and overlapping responses within the cell. For example, almost all integrins share the capability of interacting indirectly with the actin cytoskeleton in response to binding to different ECM molecules, which is perhaps the most central of integrin functions.

2.3.1 Integrin heterodimerization

The cell type-specific expression of integrin chains is not the only factor in determining which heterodimers form within the cell. For example, integrins $\alpha V$ and $\beta 1$ are frequently co-expressed, but may not always form heterodimers with each other. These two promiscuous subunits are synthesized in excess over their multiple heterodimer partners, whose relative levels define the cellular pools of heterodimers (Heino et al. 1989, Sheppard et al. 1992). As integrins $\alpha V$ and $\beta 1$ are both abundantly expressed, they should be able to form heterodimers together if availability alone defined the patterns of heterodimerization. It has been suggested however that there is a hierarchy between the different $\beta$-subunits in binding of $\alpha V$, whereby the other $\alpha\beta$-combinations are prioritized over $\alpha V\beta 1$. Therefore, $\alpha V\beta 1$ only forms when there are sufficiently high quantities of both subunits left after pairing with other subunits (Koistinen & Heino 2002). Similarly, integrins $\alpha 6$ and $\beta 1$ do not always form heterodimers when co-expressed. When integrin $\beta 4$ is available, integrin $\alpha 6$ preferentially forms the $\alpha 6\beta 4$-heterodimer (Hemler et al. 1989, Kennel et al. 1989). Unlike the
heterodimer partners of integrins $\alpha V$ and $\beta 1$, integrin $\beta 4$ is synthesized in excess over its unique heterodimer partner integrin $\alpha 6$ (Sonnenberg et al. 1990). It is clear that the matchmaking of integrin subunits is in part established by their rates of synthesis and also by their relative affinities. A detailed view of the binding hierarchy between co-expressed integrin subunits, which defines the cellular pools of heterodimers, has not been established.

Integrin heterodimers form at the endoplasmic reticulum (ER) by non-covalent association of the $\alpha$- and $\beta$-subunits, which is thought to facilitate their correct folding and entry to the secretory pathway (Ho & Springer 1983, Huang & Springer 1997, Kishimoto et al. 1987, Lu et al. 1998, Sonnenberg et al. 1988, Tiwari et al. 2011). This theory is largely based on the observations that maturation coincides with heterodimer formation in pulse-chase experiments. Further support has come from mutational studies where heterodimeric association between $\alpha$- and $\beta$-subunits has been disrupted. A conserved GFFKR motif in the membrane-proximal domain of integrin $\alpha 6$ was found to mediate heterodimerization with integrin $\beta 1$ and when mutated, retained integrin $\alpha 6$ at the ER (De Melker et al. 1997). A similar requirement for the motif has been reported for the maturation of $\alpha L\beta 2$ (Pardi et al. 1995). The GFFKR motif is important, however, for stabilization of the low affinity inactive conformation of integrins which they first adopt whilst in the biosynthetic pathway (O'Toole et al. 1994, Tiwari et al. 2011). The GFFKR motif has also been suggested to serve as an ER-export signal for integrins and thereby facilitate integrin secretion (Martel et al. 2000). Whether the GFFKR motif is required for correct folding of the heterodimer to pass the ER quality control or to serve specifically as an export signal is currently unclear. Specific N-glycosylation sites in the $\beta$-propeller domain of integrin $\alpha 5$ and in the I-like domain of integrin $\beta 1$ facilitate heterodimerization of $\alpha 5\beta 1$ and block surface expression when mutated (Isaji et al. 2006, Isaji et al. 2009). N-glycosylation was not required for surface expression or heterodimerization of $\alpha 6\beta 4$, however (Kariya & Gu 2011). The interactions that mediate heterodimerization may vary between heterodimers and are known to change considerably during signalling, but the view that integrins are obligate heterodimers remains undisputed (Ulmer 2010).

2.3.2 Conformational basis for integrin signalling

Integrins transmit signals in a bidirectional manner across the PM: from extracellular ligands to induce cellular responses and from intracellular activators
to promote extracellular ligand-binding. Integrins possess no intrinsic enzymatic activity and transmit signals based on conformational changes that propagate throughout the length of the receptor and involve alterations in αβ-interactions. The structure of integrins is composed of a globular head domain standing on two legs inserted into the membrane and terminating at the C-terminal cytoplasmic tails (Luo et al. 2007). Integrins transition between an active high affinity conformation, which may or may not involve a bound ligand, and an inactive low affinity conformation (Figure 6) (Askari et al. 2009). When inactive, the TM-domains and cytoplasmic tails of the legs are closely associated and the head domain bent towards the membrane into a v-shape. Upon activation, the head domain becomes unbent and the legs stand separated extending the conformation relative to the bent inactive conformation (Kim et al. 2003, Takagi et al. 2002). Activation of integrins can be regulated from the inside-out by binding of the allosteric effectors to the cytoplasmic tails in a process referred to as “priming”, which enhances ligand-binding affinity of individual receptors. Cytoplasmic interactions that activate integrins serve to disrupt the inactive conformation by separating the association between the TM and cytoplasmic domains of the α- and β-subunits (Luo et al. 2007). Deletions or mutations that similarly disrupt the αβ-interface lead to constitutively active integrins (Hughes et al. 1996, Lu & Springer 1997, O'Toole et al. 1991, O'Toole et al. 1994). Integrins can also be activated from the outside-in as extracellular ligand binding, dependent on co-binding of divalent cations, stabilizes the active conformation (Luo & Springer 2006). The relationship between conformation and the ligand-bound or primed states is not absolute, and intermediate “semi-active” conformations exist that are functionally relevant (Askari et al. 2009). Most likely, the different occupied states of integrins affect the dynamic balance between different possible conformers in a heterodimer-specific manner (Luo & Springer 2006). Some studies even suggest that integrins can be ligand-occupied while in the bent conformation (Adair et al. 2005, Xiong et al. 2002, Ye et al. 2008).
Fig. 6. Conformational changes associated with integrin activation. Integrins can be activated either by binding of intracellular activating proteins to the integrin β-tail (inside-out activation) or by extracellular ligand binding (outside-in activation). Both induce the bent inactive conformation (1.) to become extended (2-3.). Inside-out activation leads to enhanced affinity for extracellular ligands (2.). Outside-in activation, induced by extracellular ligand binding (3.), leads to a second conformational change: the β-I domain is switched from a closed into an open conformation accompanied by a swing out of the hybrid domain (4.). This enables integrin outside-in signalling via binding of multimolecular complexes to the integrin cytoplasmic tails (4.). Adapted from Bouvard et al. (2013). Abbreviations: ACTN = α-actinin, IPP = ILK-PINCH-1-parvin complex, VCL = vinculin.

Integrin inside-out and outside-in signalling are often discussed separately, because in leukocytes and platelets, used widely as a model to study integrin
activation, inside-out signalling precedes ligand-dependent outside-in signalling. In adherent cells these signalling modes are more integrated. Priming refers exclusively to inside-out activation by allosteric regulators rather than activation of integrins by extracellular binding. The extent to which priming regulates ligand-binding affinity of integrins depends upon the level of control imposed by the cell-type on integrin activation (Hynes 2002). Moreover, different heterodimers display structural differences that affect their relative responses to activation via agonists and allosteric effectors (Anthis et al. 2010, Askari et al. 2009, Bazzoni et al. 1998). Integrins in non-adherent cells such as leukocytes and platelets are constitutively inactive and require priming, triggered by a signalling cascade downstream of other cell surface receptors, to bind ligands during platelet aggregation and leukocyte extravasation (Evans et al. 2009). Activation of integrins in adherent cells is not as stringently regulated since they display a constitutive ability to bind the ECM, likely because allosteric regulators are not restricted. Moreover, integrins in adherent cells may not require priming at all for initial ligand-binding. In fact, many of the events associated with priming occur as a response to outside-in activation (Margadant et al. 2011).

Integrins can be activated or inactivated by specific C-terminal interacting proteins (Morse et al. 2014). Competitive binding of these proteins to two conserved motifs in the tails of all integrin β-subunits, except for integrin β4 and β8, constitutes a major mode of integrin activity regulation. The membrane-proximal NPxY motif is more conserved and promotes similar interactions in different heterodimers whereas the membrane-distal NxxY motif is divergent and discriminates between heterodimers in regulating adaptor-binding (Calderwood et al. 2003). Tyrosine phosphorylation of these sites promotes additional interactions with PTB domain-containing proteins and protects β-tails from calpain-cleavage (Calderwood et al. 2003, Xi et al. 2006). In addition, the intermediary serine/threonine motif between the NPxY and NxxY sites coordinates binding of some adaptors (Takala et al. 2008). β4- and β8-integrin subunits do not share the NPxY and NxxY motifs in their cytoplasmic tails and are as a result regulated by distinctly different mechanisms. The integrin α-tails possess a common membrane-proximal GFFKR-motif that supports binding of integrin inactivators that either stabilize the αβ-chain interaction of the inactive conformation or prevent the binding of activators to the β-tails (Morse et al. 2014). Mutation of this motif locks some integrins in the active conformation (Hughes et al. 1996, Imai et al. 2008, Lu & Springer 1997, Peter & Bode 1996) but has also been implicated in the maturation of integrins as earlier discussed. The α-tails distal to
the GFFKR-motif are divergent and support integrin-specific binding interactions. The instructions for the recycling of integrins are also encrypted in the cytoplasmic tails of the integrins, which is important for the turnover of integrin-based cell adhesions during cell motility, shape changes and cell rearrangements (Margadant et al. 2011).

Binding of talin to the membrane-proximal NPxY motif in all β-tails aside from integrins β4 and β8 dissociates the inhibitory αβ-association of the integrin legs and triggers integrin activation (Kim et al. 2012). Cells can utilize different mechanisms to control talin-mediated integrin activation, including regulation of talin membrane-recruitment, proteolysis, phosphorylation, conformation and competitive binding of other factors such as filamin and docking protein 1 (DOK1) to the β-tail binding site, which results in integrin inactivation (Morse et al. 2014). Inactivating factors that compete with talin for β-tail binding are in competing with other integrin activating proteins, constituting a complex multi-layered regulatory system. However, talin binding is considered to be the final step in integrin activation (Tadokoro et al. 2003). Integrins in non-adherent cells are kept inactive by restriction of talin-availability. In adherent cells talin-mediated integrin activation is not a prerequisite for ligand binding or adhesion but is required for efficient outside-in signalling in integrin adhesions (Giannone et al. 2003, Zhang et al. 2008). Another important class of integrin activating proteins that bind the membrane-distal NPxY motif of β-tails are kindlins that cooperate with talin in integrin activation (Calderwood et al. 2013).

2.3.3 Clustering of active integrins into cell adhesions

The complete signalling response of integrins cannot be fully realized by conformational changes and requires clustering of activated receptors in the lateral dimension, which greatly increases the valency of ligand-binding. Individual weak ligand-coupled integrin links are clustered into focused cell adhesions that may contain perhaps thousands of integrins. This mode of regulation is central to affinity modulation in adherent cells, whereas priming is mainly utilized by circulating blood cells that require an on/off-system (Shattil et al. 2010). Integrin activation and clustering together trigger outside-in signalling via assembly of macromolecular protein complexes to the cytoplasmic tails of integrins that anchor the cytoskeleton. These adhesion complexes include signalling and scaffold molecules capable of inducing a vast number of structural and signalling changes both locally and globally (Margadant et al. 2011).
Clustering is independent of TM-domain interactions between heterodimers (Wang et al. 2011) and is driven instead from both sides of the cell membrane via interactions of the ectodomains and cytoplasmic tails with multivalent ligands and protein complexes, respectively. Clustering is initiated by the binding of integrins to multivalent ligands such as the ECM (Buensuceso et al. 2003, Cluzel et al. 2005, Kim et al. 2004). The β-tails of the integrins possess intrinsic targeting information to be recruited to pre-existing cell adhesions due to the conserved motifs that regulate protein-protein interactions (Akiyama et al. 1994, LaFlamme et al. 1992). These sequences are masked by the α-subunit in inactive integrins and require activation to be exposed (Briesewitz et al. 1993, Ylanne et al. 1993). Integrin β-tail-binding proteins such as talin form multivalent links with actin filaments that upon cross-linking and bundling drive clustering of integrins from within the cell (Cluzel et al. 2005). Local enrichment of signalling lipids such as PIPs that coordinate the binding of talin may further support clustering (Wehrle-Haller 2012).

The molecular composition, structure and dynamics of integrin-mediated cell adhesions have been characterized in cultured cells adhering to a specified ECM molecule coated onto a stiff 2D surface (Geiger & Zaidel-Bar 2012, Kanchanawong et al. 2010, Worth & Parsons 2010). The properties of cell adhesions depend upon the chemical and physical features of the adhesive surface as well as the type of molecular connection mediated by the integrins (Geiger et al. 2009). The ECM-molecule defines which integrins are engaged leading to formation of characteristic adhesions. However, as ECMs in vivo are co-assembled from multiple ECM-molecules into a supramolecular structure, cells are likely to display many types of adhesions and adhesions with integrated ECM-binding properties (Geiger & Yamada 2011). The composition of adhesions, including different integrins within, undergoes changes over time as cells modify their ECM (Pankov et al. 2000, Rossier et al. 2012, Zamir et al. 2000). The cellular cytoskeleton and cell adhesions are intimately interdependent structures where alterations in one affect the composition of the other (Geiger et al. 2009). Coordinated co-assembly of distinct cell adhesions with different ECM-binding and cytoskeletal anchoring elements are essential for the structural organization of the cellular phenotype in the natural environment.
Focal adhesions

FAs are integrin-mediated adhesive structures in which the integrins form connections with the actin cytoskeleton. These types of adhesions have been observed in many different types of cells with a different subset of integrins but are mainly associated with the functions of β1- and β3-integrins in binding fibrillar matrix molecules such as Col-I and FN in fibroblasts (Geiger & Yamada 2011). Integrins that possess the conserved motifs of the β-tails can support binding of FA-components, but the adhesions formed would display distinct differences in both composition and structure. For example, FN engages both integrins αVβ3 and α5β1, but they each contribute differently to the assembly and signalling capacity of FAs (Danen et al. 2002, Danen et al. 2005, Katz et al. 2000, Schiller et al. 2013). Different ECM-ligands can support FA-type interactions, but FAs on laminins that form networks rather than fibres would be arranged differently from FAs on FN or collagen (Geiger & Yamada 2011). The unique cytoarchitecture of the cell type as well as the characteristic expression pattern of adhesion related molecules would also influence the constitution of FAs. As it stands the contributions of different ECM-molecules and integrins to the formation of FAs have not been systematically addressed.

FA assembly is triggered by outside-in signalling from ECM-bound integrins. Up to ~180 protein constituents have been associated with the formation and regulation of FAs, referred to collectively as the “adhesome” (Zaidel-Bar et al. 2007, Zaidel-Bar & Geiger 2010). Not all the adhesome components are present at the same time nor do they contribute to every adhesion. The adhesome consists of scaffolding molecules and signalling/regulatory proteins that together form the integrin signalling platform as well as the structural basis for the actin cytoskeletal linkage. Many interactions in the adhesome network are switchable as they can be regulated by signalling events such as phosphorylation and signalling lipids (Geiger et al. 2009). A subset of ~30 proteins are involved in mediating the connection with actin, of which some bind directly to integrins, others directly to actin and the remaining bind to both with additional stability provided by adaptors that bridge these factors together (Wolfenson et al. 2013). The assembly of proteins to the developing adhesions follows a hierarchical model in which the presence of certain proteins is required for the addition of others based on which different transient and more stable stages of assembly can be distinguished (Zaidel-Bar et al. 2004). These different stages can co-exist within a single cell and are in a constant state of turnover, each with a
characteristic lifespan. FAs are associated with actin filaments and their life-cycle is intimately connected to the turnover of actin filaments and the forces they generate. The hierarchical assembly of FAs is depicted in Figure 7.

![Diagram of adhesion maturation](image)

Fig. 7. Maturation of FAs. Nascent adhesions form beneath the lamellipodia and are dependent on actin polymerization. FAK, PXN and talin are recruited early to nascent adhesions. Recruitment of VCL and ACTN facilitates maturation of nascent adhesions, whereas Zyxin and VASP demarcate mature FAs exclusively. Maturation of FAs requires myosin-II generated traction forces along F-actin cables. FAs are situated at the lamellipodia-lamella border. FAs can further develop into fibrillar adhesions, located at the central region of the cell and associate with fibronectin fibrils. Fibrillar adhesions are characterized by replacement of talin with tensin as the actin-linkage and a switch in integrin-engagement. Assembled from Bouvard et al. (2013), Geiger & Yamada (2011) and Zaidel-bar et al. (2004). Abbreviations: PXN = paxillin, VCL = vinculin, NM-II = non-muscle myosin-II, VASP = vasodilator-stimulated phosphoprotein, ACTN = α-actinin, VCL = vinculin, IPP = ILK-PINCH-1-parvin complex.

The earliest type of adhesions, called nascent adhesions or focal complexes, have been proposed to consist of unstable links between actin and ligand-bound integrins mediated by talin and are dependent on actin polymerization (Geiger et al. 2009). Recruitment of FAK and the adaptor protein paxillin, which do not
directly interact with actin, to nascent adhesions has been suggested to precede that of talin (Lawson et al. 2012). A subset of adhesions can evolve into FAs depending on their association with the contractile actomyosin machinery of actin stress fibres, which leads to an increase in their molecular complexity (Choi et al. 2008, Riveline et al. 2001). Recruitment of vinculin, which interacts with both integrin and talin, stabilizes the actin-linkage and promotes clustering of activated integrins (Galbraith et al. 2002, Gallant et al. 2005, Humphries et al. 2007). An actin crosslinking protein α-actinin, which interacts with both talin and vinculin, is essential for force-dependent strengthening of the integrin-cytoskeleton linkages (Choi et al. 2008, Roca-Cusachs et al. 2013). FAs can further develop into fibrillar adhesions, elongated structures closely associated with FN fibrils. This involves a switch from integrin αVβ3 to integrin α5β1 engagement and the replacement of talin with tensin (Pankov et al. 2000, Zaidel-Bar et al. 2003, Zamir et al. 2000).

The nascent adhesions have a high turnover rate, whereas FAs and fibrillar adhesions are stabilized by the cytoskeleton and are therefore more persistent, although the protein constituents within are constantly recycled (Wolfenson et al. 2009). Rho GTPases regulate the formation of the different species of actin filaments at adhesions and spatiotemporal activities of different Rho GTPases are important for the transition of one type of adhesion into another (Wolfenson et al. 2013). Disassembly of FAs is less well understood but may involve endocytosis, calpain-cleavage of talin, loss of contractility and ECM degradation (Wehrle-Haller 2012). Microtubules are known to be essential for FA-disassembly and facilitate the turnover of FAs during cell migration (Ezratty et al. 2005, Wu et al. 2008).

**Hemidesmosomes**

HDs are specialized cell adhesions entirely dependent on the functions of the integrin α6β4 and the specialized binding properties of the unusually large cytoplasmic tail of integrin β4, which is unique among integrins. Unlike FAs, HDs are restricted to the epithelia. The cytoplasmic tail of integrin β4 supports binding of intermediate filaments (IFs) indirectly via the plakin family of proteins that contribute to HDs in a cell-type specific manner. Type I HDs found in the skin and other complex epithelia are composed of the integrin α6β4 that binds LN-332 of the ECM, the plaks plectin and bullous pemphigoid antigen 1 (BPAG1e), also called dystonin isoform 1e, that connect the tail of integrin β4 to
keratin IFs 5 and 14 and the transmembrane components bullous pemphigoid antigen 2 (BPAG2), also known as collagen XVII and CD151 (Figure 8) (Zhang & Labouesse 2010). Integrin α6β4 is also expressed in simple epithelial cells where it makes similar but less elaborate connections with IFs termed type II HDs that have been characterized in the intestine. These connections consist of a plectin-mediated linkage between α6β4 and keratins 8 and 18 (Orian-Rousseau et al. 1996, Uematsu et al. 1994). The interaction between integrin α6β4 and plectin represents an essential first step in the assembly of HDs (Koster et al. 2004). HDs are the central components of the dermal-epithelial junction that maintains the cohesion between the dermis and epidermis, the loss of which can be caused by mutations in the HD-genes and is manifested in several skin blistering disorders in human patients (Margadant et al. 2008). Dynamic breakdown of the HDs is required for cell migration during epithelial regeneration and carcinoma cell invasion (Litjens et al. 2006).

Fig. 8. Structure of the type I HD. Integrin α6β4 and BPAG2 interact with laminins of the BM. The extracellular domain of integrin α6 binds the transmembrane proteins CD151 and BPAG2. The cytoplasmic tail of integrin β4 interacts with BPAG1e, BPAG2 and plectin, the latter two of which provide anchorage to IFs. Adapted from dePereda et al. (2009).

Unlike other integrins, the clustering of α6β4 and subsequent HD-assembly does not require ligand-binding and can be mediated solely by its interaction with plectin (Homan et al. 1998, Nievers et al. 1998, Nievers et al. 2000). However, both plectin-mediated cytoskeletal anchoring and ligand-coupling contribute to the stability and turnover of HDs (Geuijen & Sonnenberg 2002). The binding hierarchy of different HD-components is built upon the primary interaction of the integrin β4 tail with plectin (Koster et al. 2003, Koster et al. 2004). The signalling
of the $\alpha 6\beta 4$ may not be regulated based on the same conformational principle as other integrins as the ability of the integrin $\beta 4$ tail to support protein-protein interactions seems to be uncoupled from ligand-binding. A tail-less mutant of integrin $\beta 4$ can mediate ligand-binding, suggesting that ligand-binding may also be uncoupled from cytoplasmic interactions (Spinardi et al. 1995). Whether $\alpha 6\beta 4$ is engaged in bidirectional signalling is unknown despite significant advances in understanding the conformational basis for the integrin $\beta 4$ tail interactions (de Pereda et al. 2009). The integrin $\beta 4$ tail protein-protein interactions can be mediated allosterically by phosphorylation, which imparts conformational changes to the $\beta$-tail. Serine and tyrosine phosphorylation by different kinases, activated downstream of GF signalling, promotes dissociation of the plectin-interaction, thereby promoting HD-disassembly (Rabinovitz et al. 2004, Wilhelmsen et al. 2007). The phosphorylation sites uncovered in these studies do not directly overlap with the known binding sites for plectin and instead serve to disrupt an intramolecular bond between the C-terminus and the connecting segment of the integrin $\beta 4$ tail that provides a binding platform for plectin and other HD-interactions (Margadant et al. 2008). HDs may in turn be stabilized by phosphatases that dephosphorylate the $\beta 4$-tail (Litjens et al. 2006).

2.3.4 Integrin-integrin crosstalk

Different integrins can function in a synergistic or an antagonistic manner, which contributes to their complex signalling scheme. The concept of transdominant regulation, also called crosstalk, whereby integrins modulate the activities of each other, has been introduced based on loss-of-function studies. Inhibition of one integrin heterodimer by function blocking antibodies or by silencing may indirectly inhibit or promote the ligand-binding activities of a second heterodimer (Gonzalez et al. 2010). Crosstalk not only exists between integrins that bind different ligands but also concerns integrins that bind the same ligand. This type of regulation contributes to the normal physiological responses of cells. For example, transdominant inhibition of the collagen-binding $\alpha 2\beta 1$ integrin by ligand binding of $\alpha II\beta 3$ is required for platelet aggregation in response plasma proteins while preventing adherence to vessel walls (Riederer et al. 2002). The underlying mechanisms are diverse, ranging from integrin activation, recycling, expression and signal transduction (Blystone et al. 1999, Calderwood et al. 2004, Gonzalez et al. 2008, Kim et al. 2000, Retta et al. 2001, White et al. 2007). Crosstalk may be explained by the sequence similarity of the integrin tails in
promoting protein-protein interactions via similar motifs that bind regulatory factors. Ligand-binding of an integrin may affect how these factors are received by other integrins or induce signals that modify binding properties of other integrin tails. Transdominant inhibition of integrin activation results from competition between $\beta$-tails to recruit talin at the expense of others (Calderwood et al. 2004). $\beta$-integrins defective in talin-binding do not mediate transdominant inhibition. Moreover, the transdominant effect can be bypassed by overexpression of the integrin-binding and activating fragments of talin, suggesting that talin is limited and its sequestration by one integrin prevents others from being activated. Integrin inactivating proteins compete with talin for the $\beta$-tail binding site and may participate in talin-dependent integrin-integrin crosstalk (Gonzalez et al. 2010). Competitive binding of talin however, does not explain all cases of crosstalk. An alternative mechanism involves the coordinated functions of protein kinases together with counteracting phosphatases that impart selectivity of adaptor binding to phosphorylated versus non-phosphorylated integrin tails (Gonzalez et al. 2010). Transdominant mechanisms are likely to be highly diverse and cell-type specific due to the differential expression of integrins and their context-dependent regulatory mechanisms.

2.3.5 Environmental sensing by integrins

Adhesion-mediated signalling involves incorporation of various signalling molecules and their diverse substrates to the large cytoplasmic protein scaffold at integrin-based adhesions (Geiger & Yamada 2011). This signalling utility serves to regulate the immediate scaffolding activity of the adhesion itself as well as long term physiological responses downstream of transcriptional activities. The adhesions are sensitive to alterations in both chemical and physical properties of the ECM, which affects their composition and signalling capacity. This can be understood as the basis for the so-called environmental sensing, whereby integrins are said to mediate biochemical and biomechanical cues from the ECM into the cell. Chemical sensing relates to the engagement of ECM-specific integrins and their unique imprint to the adhesome-network that is committed to outside-in signalling (Geiger & Yamada 2011). The strength and complexity of the adhesive connection is directly related to the rigidity of the substrate due to the force-dependent nature of the assembly process (Schiller & Fassler 2013). Moreover, as integrins are activated in a polarized and localized manner, they also relay spatial information of the position, geometry, ligand-spacing and anisotropy
of the surrounding microenvironment (Geiger et al. 2009). These types of spatial cues regulate polarization at the cellular level as well as patterning of a collective of cells during tissue morphogenesis. In the long term integrins regulate gene transcription by regulating GF-mediated signal transduction pathways, which is the basis for adhesion-dependent control of many cellular processes such as cell proliferation, survival and differentiation (Legate et al. 2009). These events are also sensitive to the mechanical cues of the ECM, as ECM stiffness has profound effects on both cell fate and behaviour (Wolfenson et al. 2013).

The short term effects of integrin signalling are on cytoskeletal organization, whereby global morphological changes are induced in response to localized ECM-anchorage of actin filaments (Legate et al. 2009). This relates largely to the capacity of adhesion-associated signalling molecules such as FAK and ILK to mediate activities of Rho GTPases in a force-sensitive manner (Geiger et al. 2009). Cells generate myosin II-mediated contractile forces along actin filaments in response to substrate rigidity by pulling on the ECM-coupled FAs (Schiller & Fassler 2013). The adhesion complex contains mechanosensitive proteins that undergo conformational unwinding in response to force, thereby exposing cryptic binding sites to recruit additional proteins to adhesions, which leads to adhesion strengthening and cytoskeletal rearrangements (Schiller & Fassler 2013). The conversion of mechanical forces into biochemical signals in cell adhesions is referred to as mechanotransduction and allows cells to dynamically adapt to ECM stiffness. The mechanisms by which ECM rigidity is converted into myosin II-generated contractility all impinge on the phosphorylation of the myosin light chain, which is required for myosin II activity (Vicente-Manzanares et al. 2007). This depends on the activities of RhoA in promoting polymerization of bundled actin and inducing myosin II-activity. RhoA can be modulated by various signalling molecules stationed at FAs, the activities of which seem to require coordination of αV- and β1-class of integrins (Schiller et al. 2013).
3 Aims of the study

The purpose of this study was to elucidate the functional hierarchy between co-expressed integrin heterodimers in the regulation of epithelial cell polarity and contractility in response to different ECM-environments by means of systematic loss-of-function screening of individual integrin subunits in the MDCK cell model.

Epithelial cells express several $\alpha_{V}$-integrin heterodimers that bind the RGD consensus sequence in various ligands. $\alpha_{V}$- and $\beta_{1}$-integrin-mediated cell adhesions transmit mechanical signals from the ECM, allowing cells to adapt their shape by contracting in response to ECMs of different rigidities. In addition to mediating cell adhesion to its ligand fibronectin, we discovered that $\alpha_{V}$-integrins also regulated cell adhesion and cell contractility on ligands bound primarily by $\beta_{1}$-integrins. Our first aim therefore was to study the roles of $\alpha_{V}$-integrins in the maturation of FAs on collagen-I.

Epithelial cells also express specific $\beta_{1}$-integrins and $\alpha_{6}\beta_{4}$ integrins that together mediate adhesion to BMs. $\beta_{1}$-integrin heterodimers transmit BM-derived signals to regulate the organization of epithelial cell polarity, but the contributions of the specific heterodimers have not been analyzed in detail. Our second aim was to study how BM-interacting integrin heterodimers regulate the establishment of epithelial cell polarity during cystogenesis in 3D ECM culture.

Integrin $\alpha_{6}\beta_{4}$ is a lamin-binding integrin heterodimer that mediates stable adhesion of epithelial cells to the BM via HDs. The integrin $\beta_{4}$ subunit only forms a single heterodimer with the integrin $\alpha_{6}$ subunit. However, we discovered a role for the integrin $\beta_{4}$ in regulating the orientation of cell polarity during MDCK cystogenesis, which was not shared by integrin $\alpha_{6}$. Our third aim therefore was to study whether the integrin $\beta_{4}$ subunit can localize to laminin adhesions and regulate laminin assembly to the BM without integrin $\alpha_{6}$.
4 Materials and methods

The materials and methods used in this thesis are summarized in Table 1. Detailed descriptions of the experimental procedures are included in the original articles I-III.

Table 1. Materials and methods used in original articles I-III

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5 Results

5.1 Loss-of-Function RNAi screen to study the functional hierarchy of integrins in MDCK cells (I, II & III)

Functional studies in KO (KO) mice have demonstrated the significance of individual integrins and their ligands in diverse developmental processes part of various organ systems (Bouvard et al. 2001). Multiple integrins with varied ligand-binding specificities are co-expressed within a single mammalian cell. We set out to interrogate how their combined functions would orchestrate the behaviour of an epithelial cell. The MDCK cell line is a well-established epithelial cell model that displays apico-basal polarity and partially recapitulates epithelial morphogenesis in vitro when cultured in 3D ECM gels (Mostov et al. 2000, Zegers et al. 2003). To study integrin function we set up an RNAi-based loss-of-function screen, where we systematically silenced every integrin subunit expressed in the MDCK cell line. We utilized a previously established retrovirus-mediated short hairpin RNA (shRNA) expression system to produce stable integrin knockdown (Itg-KD) cell lines (Manninen et al. 2005, Schuck et al. 2004). Specificity of our loss-of-function phenotypes was confirmed with two independent shRNA-constructs against each subunit and in some cases by rescue-experiments using cDNA-complementation as discussed by Cullen (2006).

5.1.1 Characterization of the MDCK cell type-specific integrin expression profile (I)

In order to identify the subset of integrins expressed in MDCK cells, we measured the relative mRNA levels of all canine integrin α- and β-subunits by quantitative PCR (qPCR) (Figure S1 in I). The results indicated abundant expression of integrins β1, β3, β4, β5, β6, β8, α2, α3, α6 and αV that together form heterodimers summarized in Figure 9. These include laminin-binding and collagen-binding receptors (studied in I and III) as well as receptors binding RGD-peptides in various ligands (studied in II).
5.1.2 Determination of integrin-specific knockdown efficiencies (I & II)

To confirm successful silencing of expressed integrin subunits, the mRNA-content was measured from the KD and control cells relative to a reference gene by qPCR and the KD-efficiencies (% reduction) were calculated (Tables S1 in I and II) using the \( \Delta \Delta CT \)-method (Livak & Schmittgen 2001). The corresponding protein levels were determined by western immunoblotting, immunofluorescence or metabolic labelling and immunoprecipitation using integrin-specific antibodies (Figures S1 in I and II).
5.1.3 Validation of integrin knockdowns (I & II)

To validate our integrin KD cell lines and to assess their ligand-binding properties we tested their adherence to various ECM-ligands by a standard adhesion assay as described by Matlin et al. (2003). Results are shown in Figure 1 in I and Figure S2 in II. Tested ligands included Col-I and Col-IV, LN-511, FN and BM extract (BME), all of which promote adhesion of normal MDCK cells. The KD of integrins $\alpha_2$ and $\beta_1$ efficiently inhibited adhesion of cells to both types of collagens and the BME rich in both collagen and laminins. Cell adhesion to the BME was also reduced by KD of integrins $\alpha_6$ and $\beta_4$, but to a lesser degree. These results confirm that integrin $\alpha_2\beta_1$ mediates cell adhesion to collagens and that cells may adhere to the BME both via $\alpha_2\beta_1$-mediated collagen interactions and $\alpha_6\beta_4$-mediated laminin interactions. Adhesion to LN-511 was partially abrogated in Itg$\beta_1$-KD, Itg$\alpha_3$-KD or Itg$\beta_4$-KD cells, but no significant adhesion defect was observed in Itg$\alpha_6$-KD or Itg$\alpha_2$-KD cells. Therefore, adhesion to LN-511 may be mediated by both $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins. FN adhesion was dramatically reduced by KD of integrin $\alpha_V$ and partially affected by KD of the heterodimer partner $\beta_6$. KD of the other integrin $\alpha_V$ heterodimer partners did not affect FN adhesion, suggesting that they are either redundant or dispensable. Adhesion to FN was unaffected by silencing of integrin $\beta_1$ or $\alpha_5$, which is consistent with the absence of integrin $\alpha_5$ expression in MDCK cells. Unexpectedly, KD of integrin $\alpha_V$ significantly reduced cell adhesion to both Col-I and LN-511, known to be ligands for other integrins.

5.2 Mechanotransduction at $\beta_1$-integrin-based cell adhesions is regulated by $\alpha_V$-integrins (II)

$\alpha_V$- and $\beta_1$-integrins cooperate in fibroblasts to regulate assembly and mechanotransduction of FAs formed on a fibronectin substrate. The role of $\alpha_V$-integrins in regulating adhesion of MDCK cells to other $\beta_1$-integrin ligands raised the possibility that this cooperation also takes place in epithelial cells adhering to collagens and laminins that are not directly bound by $\alpha_V$-integrins. Here we have investigated the possible explanations as to how $\alpha_V$-integrins might contribute to cell adhesion on ligands of $\beta_1$-integrins.
5.2.1 αV-integrins do not influence availability or avidity of β1-integrins at the plasma membrane

To investigate the expression of β1-integrin heterodimers in ItgαV-KD cells, β1-integrins were immunoprecipitated from metabolically labelled control and KD cell lysates. The results show that KD of αV had no effect on the steady state levels β1-heterodimers (Figure S1G in II). To further address the surface availability of β1-integrins, we isolated surface biotinylated proteins from control and ItgαV-KD cells and determined the amounts of integrin α2 and β1 relative to the whole cell lysates by western immunoblotting (Figure 1E-F in II). The levels of integrins α2 and β1 were unchanged, indicating that their availability at the PM is not regulated by αV-heterodimers.

Avidity of integrins for ligand-binding is mediated by affinity- or valency-based mechanisms, both of which contribute to adhesive strength (Carman & Springer 2003). αV-integrins may regulate avidity of β1-integrins or, in theory, directly contribute to ligand-binding via cryptic RGD-sites in collagens and laminins (Aumailley et al. 1990, Davis 1992, Montgomery et al. 1994, Sasaki & Timpl 2001). To precisely quantify adhesive forces at the single-cell level, single cell force spectroscopy with an atomic force microscope was performed (Friedrichs et al. 2010, Helenius et al. 2008). Short term adhesive forces of control and ItgαV-KD cells towards a Col-I substrate were measured with Itgα2-KD cells as a negative control (Figure 1A-B in II). Loss of the α2β1-receptor in Itgα2-KD cells correlated with reduced Col-I binding capacity, confirming that it is the principal collagen-binding receptor in MDCK cells. Forces generated by control and ItgαV-KD cells were similar, suggesting that αV-integrins play no role either directly or indirectly in mediating collagen-binding. Furthermore, the ItgαV-RFP fusion protein accumulates at FN-cell-matrix adhesions, but not at Col-I-cell-matrix adhesions, suggesting that Col-I does not visibly trigger clustering of αV-integrins, which remain diffusely distributed (Figure S1H in II). In conclusion, αV-integrins do not directly or indirectly contribute to the initial adhesive strength to Col-I.

5.2.2 αV-integrins are required for cell spreading on fibronectin, collagen and laminin-511

Cell adhesion is a complex bidirectional process as part of which inside-out and outside-in signals cooperatively promote ligand binding and the strengthening of
adhesions, respectively. Adhesion strengthening or maturation involves formation of elaborate connections between the cytoplasmic tails of ligand-bound integrins and the intracellular actin cytoskeleton via complex interactions (Zaidel-Bar et al. 2007). Turnover of cell adhesions, where nascent peripheral adhesions become anchored to the cytoskeletal network while old central ones are deployed, facilitates cell spreading (Webb et al. 2002). As cell spreading depends upon maturation of cell adhesions, we analyzed spreading of ItgαV-KD and different Itgβ-KDs on various ECM-ligands to determine their contribution to FA assembly (Figure 2A-C in II). Spreading on FN was strongly inhibited in ItgαV-KD cells and partially inhibited in Itgβ3- and Itgβ6-KD cells. The spreading of ItgαV-KD cells, similar to Itgβ1-KD cells, was impaired on Col-I and LN-511, but none of the Itg-KDs for heterodimer partners of αV replicated the spreading defect of ItgαV-KD on these ligands. As cell spreading most likely improves the adhesion of cells by distribution of more adhesions to a larger surface area, a spreading defect would explain the previously observed poor adhesion of ItgαV-KD cells in the standard adhesion assay. These results suggest that αV-integrins may regulate maturation of FAs on Col-I and LN-511. To confirm the specificity of the ItgαV-KD spreading phenotype, we transduced the human ItgαV-RFP-expressing cell line with both ItgαV-targeting shRNA-constructs. The human construct was resistant to one of the canine ItgαV-targeting shRNAs and was able to rescue the related spreading defect, confirming the specificity of the loss-of-function phenotype (Figure 2D-E in II).

5.2.3 Silencing of αV-integrin abrogates assembly of β1-integrin-based focal adhesions

Maturation of cell adhesions is characterized by increasing molecular complexity as the nascent adhesions evolve into FAs and, eventually, fibrillar adhesions (Zaidel-Bar et al. 2004). As individual components are recruited in a hierarchical manner, the different stages of maturation can be distinguished (Zaidel-Bar et al. 2003). Talin mediates the initial link between integrins with actin and recruitment of vinculin further reinforces this link upon transition to FAs (Tadokoro et al. 2003, Humphries et al. 2007, Ginsberg et al. 2005, Galbraith et al. 2002). Reporter cell lines expressing vinculin-GFP and talin-GFP were employed and fluorescent foci at the edges of spreading cells, demarcating adhesion sites, were monitored by live cell imaging (Figure 3A in II). KD of β1- and αV-heterodimers both reduced the lifetime of talin-GFP and vinculin-GFP -positive adhesions that
persisted for >60min on average in control cells. β1-integrin positive foci, absent in Itgβ1-KD cells, were less numerous in ItgαV-KD cells than in control cells as determined by immunofluorescence staining, indicating reduced clustering of β1-integrins (Figure 3B-C and Figure 4 in II). Consequently, reduced numbers of fluorescent foci were also measured for specific proximal interacting molecules (talin and paxillin), associated molecules (vinculin and zyxin) and signalling molecules (FAK and ILK). Subtle differences were observed in how different FA components accumulated to FAs in ItgαV-KD and Itgβ1-KD cells. FAK and ILK accumulated in smaller foci in ItgαV-KD cells and the talin-foci were smaller in Itgβ1-KD cells. However, as ItgαV-KD and Itgβ1-KD cells both formed very few adhesions in general, assembly of adhesion complexes is probably arrested at an elemental early stage in both cases, even though the underlying reasons are likely to be different.

FAK is targeted to the adhesions via the FAT domain containing binding sites for talin and paxillin (Chen et al. 1995, Hildebrand et al. 1993, Hildebrand et al. 1995). Clustering of integrins induces autophosphorylation of FAK at Tyr397, creating docking sites for multiple signalling molecules, including cellular proto-oncogene tyrosine-protein kinase (Src), which is activated at Tyr416 (Eide et al. 1995). Lack of FAK or its phosphorylation inhibits turnover of adhesions and inhibits cell migration (Ilic et al. 1995, Webb et al. 2004). We measured the levels of phosphorylated FAK and Src in ItgαV-KD cells relative to Itgβ1-KD and control cells (Figure 5B-C in II). In ItgαV-KD cells the levels of phosphorylated FAK were slightly but still significantly reduced, while in Itgβ1-KD cells pFAK levels remained strikingly unchanged. Levels of phosphorylated Src were unaffected by either KD of αV- or β1-integrins. These results indicate that αV-integrins may stimulate phosphorylation of FAK independently of β1-integrins. To identify which of the key FA-components would replicate the loss-of-function phenotype of αV-integrins, the spreading of talin-1-, FAK- and ILK-KD cell lines on LN-511 and Col-I was measured. Efficient silencing was confirmed by qPCR and western immunoblotting (Table S1A and Figure 6D-E in II). Silencing of the above mentioned FA-components all perturbed cell spreading on Col-I and LN-511, suggesting that loss of these components is analogous to the loss of αV-integrins.
5.2.4 Cell-adaptation to extracellular matrix rigidity is controlled by $\alphaV$-dependent traction forces generated at $\beta1$-integrin-based focal adhesions upon mechanical stimuli

Biomechanical forces stimulate formation of initial adhesions as well as maturation of FAs (Geiger et al. 2009). Myosin II-mediated contraction of actin stress fibers in response to resistant ECMs induces traction forces at FAs, promoting their maturation (Even-Ram et al. 2007, Vicente-Manzanares et al. 2007). Traction at FAs can be experimentally measured with the atomic force microscope by coupling an ECM-coated bead to a cantilever which is adhered by a cell and then oscillated to mechanically stress the adhesion. We measured the traction forces of control and Itg$\alphaV$-KD cells in response to a Col-I-coated bead oscillated at a frequency of 0.25Hz and amplitude of 200ns for 20 minutes (Figure 6C in II). Traction forces generated by control cells during the procedure increased, indicative of adhesion strengthening, while the corresponding response in Itg$\alphaV$-KD cells decreased, suggesting a defect in transduction of mechanical signals.

Mechanotransduction at FAs leads to global responses at the cellular level, based on which the cell is thought to sense the physical properties of the ECM. One aspect of this is the morphological adaptation of cells to matrix rigidity seen in cell spreading and which is maximised on stiff flat surfaces. To better elucidate the process, we studied the adaptation of control and Itg$\alphaV$-KD cells to polyacrylamide gels of different rigidity coated with the same concentration of Col-I (Figure 6A in II). Control cells adapted a spread morphology on stiff gels and a round morphology on soft polyacrylamide gels, whereas Itg$\alphaV$-KD cells were insensitive to changes in rigidity. Morphological changes are facilitated by reorganization of the cytoskeleton which alters the elastic properties of cells (Solon et al. 2007). When the elasticities of control and Itg$\alphaV$-KD cells on stiff surfaces were measured, Itg$\alphaV$-KD cells were found to be significantly softer than control cells, suggesting that they were incapable of responding to mechanical cues (Figure 6B in II). A similar mechanosensory defect was also verified for talin-1-KD cells, but not for FAK- or ILK-KD cells (Figure 6F in II). Therefore, reduced signalling via FAK or ILK by itself would not explain the defects observed in ItgaV-KD cells. However, as talin was essential for transduction of mechanical signals, it might contribute to the loss-of-function phenotype of $\alphaV$-integrins.
5.3 Laminin- and collagen-binding integrins regulate distinct complementary processes that control apical lumen-formation in MDCK cells (I)

Integrins and their ECM-ligands of the BM guide apicobasal polarization during epithelial morphogenesis. β1-integrins and laminins regulate the orientation of cell polarity and apical lumen formation during cystogenesis of MDCK cells. The aim of this study was to utilize the MDCK cyst model to systematically dissect the roles of specific integrin chains that form laminin- and collagen-binding interins in these processes.

5.3.1 Integrins are differentially utilized to ensure lumen formation in type I collagen- and basement membrane-composed extracellular matrices

We utilized the Itg-KD cell lines of laminin- and collagen-binding integrins α3β1, α6β4 and α2β1 that are likely to regulate BM-interactions in MDCK cells. Control and Itg-KD cells were embedded in 3D Col-I gels and cultured for 10 days, during which the parental MDCK cells formed polarized lumen-enclosing cysts. Cyst phenotypes were characterized based on criteria that normal cysts contain a dilated circular lumen positive for apical membrane marker Podxl and delineated by a single layer of epithelial cells as visualized by actin- and nuclei-staining (Figure 2A in I). Control cells as well as Itgα3-KD and Itgα6-KD cells formed normal cysts at a ~80% frequency (Figure 2B in I). Conversely, a majority of Itgα2-KD, Itgβ1-KD and Itgβ4-KD cysts lacked organized epithelial architecture and formed misshapen lumenless cell aggregates with apical markers mislocalized to the basal ECM-contacting membrane surface. These results suggest that collagen-binding α2β1-integrin is essential for the orientation of cell polarity in 3D Col-I. As cell adhesion and spreading on 2D Col-I is α2β1-dependent (Figure 1 and S2 in I), integrin α2β1 is likely to trigger orientation of cell polarity by mediating the initial contact between the cells and the extracellular matrix. Surprisingly, although integrin α6 and β4 form a single heterodimer in MDCK cells, their silencing resulted in different phenotypes, suggesting that the β4 subunit but not the α6 subunit is required for orientation of cell polarity. Similarly, silencing of integrin β4 but not integrin α6 reduced the spreading of single cells on 2D Col-I (Figure 1 and S2 in I). These non-overlapping phenotypes indicate a complex non-linear relationship between the
functions of the α6- and β4-integrin subunits that cannot be explained by their role as heterodimers.

Supplementation of exogenous laminin can partially rescue the inverted polarity of cysts in which the function of β1-integrins is blocked (Yu et al. 2005). We cultured different Itg-KD cells in 3D BME gels rich in laminin to test whether exogenous laminin supplementation would similarly reverse the inversion of polarity observed in specific Itg-KD cell lines. The Itgα2-KD and Itgβ4-KD phenotypes were fully rescued in the 3D BME gels. Conversely, Itgβ1-KD cells formed cysts where lumen formation was severely abrogated and many small rudimentary lumens were observed (Figure 2A in I). However, apical markers were effectively cleared away from the basal ECM-contacting membrane, thereby restoring basal identity. To verify whether integrin α6β4 might compensate for the ECM-derived cue in Itgβ1-KD cells to facilitate basal clearance of apical membrane proteins, we introduced β1-integrin function blocking antibodies into control and Itgβ4-KD cells cultured in 3D BME (Figure 2C in I). The addition of β1-function-blocking antibodies to the control cells led to a phenotype comparable to Itgβ1-KD cells in 3D BME gels. In Itgβ4-KD cells addition of β1-integrin function-blocking antibodies led to the formation of cysts with inverted polarity with ECM-facing apical surfaces, suggesting that either β1- or β4-integrins may facilitate clearance of apical proteins from the basal surface domain in 3D BME gels. Curiously, Itgα3-KD cells that had formed normal cysts in 3D Col-I gel now formed cysts with multiple rudimentary lumens similar those observed in Itgβ1-KD cysts in 3D BME gels (Figure 2A in I). However, Itgα3-KD cells had a more polarized cuboidal appearance and formed larger lumen compartments compared to Itgβ1-KD cells. Taken together, these results suggest that while both α2β1 and β4-integrins promote acquisition of basal membrane identity in 3D Col-I, either β1- or β4-integrins alone may do so in 3D BME.

5.3.2 Integrin-mediated basal cue for the establishment of basal-identity proceeds via Rac1-induced antagonism of RhoA

β1-integrins activate the small GTPase Rac1 to direct basal laminin assembly, which is required for the orientation of cell polarity in 3D Col-I (Yu et al. 2005, Yu et al. 2008). Orientation of polarity downstream of laminin assembly has been suggested to be independent of Rac1-activation (O’Brien et al. 2001). To confirm this we induced expression of a dominant negative (DN) mutant form of Rac1 (Rac1T17N-Tet-Off) in both 3D Col-I and BME cell cultures by withdrawal of
doxycycline. Rac1-DN perturbed orientation of polarity also in 3D BME (Figure S3 in I), pointing towards a more general Rac1-dependency for the integrin-mediated basal cue. When Rac1 activation is artificially enforced by expression of the dominant active (DA) form, the requirement of β1-integrins in 3D Col-I is overcome (Yu et al. 2005). We expressed Rac1-DA (GFP-Rac1G12V) in Itgα2-KD and Itgβ1-KD cells cultured in 3D Col-I gels to verify whether this would rescue the inversion of polarity. Although Rac1-DA expression severely disturbed lumen morphogenesis of the control cells, the identity of the basal surface domain was rescued in Itgα2-KD and Itgβ1-KD cells as visible by absence of apical markers and consistent deposition of laminin to the matrix-exposed periphery of the cyst (Figure 4A-B in I). These results suggest that localized activation of Rac1 by integrins is likely to confer basal identity, but systemic activation by overexpression of Rac1-DA leads to the perturbation of lumen morphology.

Rac1 activity is required for alleviation of the RhoA/ROCK-kinase/myosin II induced contractility at the basal-associated actin-cytoskeleton. Inhibition of the RhoA/ROCK/myosin II pathway counteracts effects of both β1-integrin function blocking and Rac1-DN to cystogenesis (Yu et al. 2008). To confirm whether this involves the α2β1-heterodimer, we treated the Itgα2-KD and Itgβ1-KD cells with a ROCK-inhibitor (Y27632). We observed that ROCK-inhibition of Itgα2-KD and Itgβ1-KD cells indeed rescued the polarity and led to the formation of dilated lumens similar to the control cells (Figure 4C-D in I). These results suggest that in 3D Col-I gels, the collagen-binding α2β1-integrin is likely to induce Rac1-dependent repression of the RhoA/ROCK/myosin II pathway, which relates to the acquisition of basal identity and the establishment of apical lumens.

5.3.3 Integrin α3β1 directs orientation of the cell-division axis during apical lumen formation via hollowing

Culture of MDCK cells in 3D BME gels promotes rapid polarization of cells that rely solely upon polarized vesicle transport to form lumens (Martin-Belmonte et al. 2008). We observed that Itgα3-KD cysts cultured in 3D BME frequently contained apoptotic cells in their lumens that were positive for cleaved caspase-3 (Figure 3B in I). These apoptotic cells were significantly enriched in the lumens of both Itgα3-KD and Itgβ1-KD cysts compared to the control cells, which were mostly apoptosis-free (Figure 3C in I). Upon prolonged culture we observed that Itgα3-KD cells were able to form larger lumens supposedly by eliminating luminal cells via apoptosis (Figure 3A). Indeed, when Itgα3-KD cells were
treated with an apoptosis-inhibitor (QVD-OPh) to block apoptosis, massive accumulation of cells to the lumens was observed (Figure 3D). These results suggest that in the absence of α3β1-integrins, lumens can form via cavitation.

When the acquisition of cell polarity is delayed by silencing of the small GTPase Cdc42, a multiple lumen phenotype is observed (Martin-Belmonte et al. 2007, Martin-Belmonte et al. 2008). Cdc42 has been linked directly to apical surface biogenesis and indirectly to the maintenance of apical lumens via control of the cell division axis, both of which contribute to the observed multiple lumen phenotype (Jaffe et al. 2008, Martin-Belmonte et al. 2007). Because the Itgα3-KD phenotype closely resembled that of Cdc42, we analyzed angles of cell division in cysts by measuring the positions of post-metaphase nuclei from 3D confocal stacks (Figure 5A in I). Indeed, silencing of integrin α3 and β1 led to randomization of cell division angles (Figure 5B in I). These results indicate that misdirected cell divisions in Itgα3-KD and Itgβ1-KD cysts likely lead to an accumulation of cells into the lumen, thereby obstructing lumen-integrity. To establish whether there is a functional link between integrin α3β1 and Cdc42 which would explain the observed phenotype, we examined whether silencing of α3- or β1-integrins affects the activity of Cdc42. To this end, we measured GTP-loading of Cdc42 in Itgα3-KD and Itgβ1-KD cells (Figure 6B in I). Surprisingly, we observed a higher GTP-loading of Cdc42 in Itgα3-KD cells. This suggests that α3β1 may instead repress the activity of Cdc42. In agreement, the expression of Cdc42-DA (GFP-Cdc42Q61L) in Itgα3-KD cells did not rescue the observed defects in lumen-formation and in fact also compromised the lumen integrity of the control cells (Figure 6A in I). However, the localization of Cdc42-DA in Itgα3-KD cells was altered to such a degree that it could be observed at the basal surface domain, while in the control cells it was restricted to the lateral and apical surface domains. This correlated with an abnormally strong actin-staining at the basal cortex of Itgα3-KD cells, while in control cells a strong actin-staining is observed sub-apically, which may be related to the increased Cdc42 activity. These results suggest a putative role for integrin α3β1 in regulating localized inhibition of Cdc42 at the basal surface domain.
5.4 Integrin β4 is targeted to putative hemidesmosomes in the absence of integrin α6 and contributes to basement membrane laminin assembly (III)

The discrepant loss-of-function phenotypes of integrin α6 and integrin β4 in cystogenesis led us to speculate that β4-integrin may have intrinsic α6-independent functions. Here we further investigated the possibilities for the integrin α6-independent functions of integrin β4, including its biosynthetic trafficking to the PM and capacity to promote HD assembly and laminin organization to the BM.

5.4.1 Integrin β4 supports basal laminin assembly in an integrin α6-independent manner, but is not necessary for laminin secretion

Because assembly of laminins to the ECM is essential for BM formation and has been shown to guide the orientation of cell polarity (O'Brien et al. 2001, Yu et al. 2005), it is possible that the role of integrin β4 in regulating cystogenesis is inherent to its capacity to regulate laminin assembly. To characterize laminin adhesions formed by α6β4 and to distinguish between laminin-associated integrin β1-positive and α6β4-positive adhesions, we analyzed co-localization of integrins with laminins by immunofluorescence staining and confocal microscopy in confluent MDCK cells (Figure 1 in III). Integrin β4 co-localized with integrin α6 at the basal cell membrane in parallel array-like structures (Figure 1A & B in III). The α6β4-positive areas and actin stress fibers appeared to be mutually exclusive, which is consistent with the known association of α6β4 with keratin filaments in HDs (Figure 1G in III). Distinct from integrin β4, integrins α6 and β1 could be visualized at the lateral cell membrane and co-localized with the cortical actin cytoskeleton (Figure 1G & H in III). Of the laminin isoforms expressed in MDCK cells, LN-332 had a more diffuse staining pattern compared to LN-511, which more clearly correlated with α6β4-positive array structures (Figure 1C & E in III). However, both laminins co-localized with α6β4-integrins in these putative HDs. Conversely, very little basal labelling was observed for β1-integrins.

To study the relative roles of integrin α6 and integrin β4 in regulating laminin assembly at the basal cell surface we utilized the Itg-KD cell lines introduced in I. The efficiency of Itgα6-KD and Itgβ4-KD was confirmed by western immunoblotting and qPCR. We measured a better KD efficiency for integrin β4 based on mRNA levels (75-80%) compared to the corresponding protein levels.
(50-60%), while efficiency of Itgα6-KD was equally efficient at both mRNA and protein levels (75%) (Figure 2A & B in III). It is now generally understood that mRNA transcripts do not fatefully predict corresponding protein levels, which can be partially explained by translational and post-translational factors (Vogel & Marcotte 2012). It is possible that Itgβ4-KD is either exceptionally stable as a protein or is efficiently translated from a relatively small pool of mRNA transcripts. To study the contribution of α6- and β4-integrins in regulating the assembly of laminins to the mature BM, we labelled laminins in Itgα6-KD and Itgβ4-KD cells and visualized their basal organization (Figure 2C & D). Both laminin isoforms were deposited into parallel arrays in control and Itgα6-KD cells, coinciding with Itgα6-staining in control cells, whereas punctate laminin deposits that lacked organization were visible in Itgβ4-KD cells (Figure 2B & C in III). To verify how much of the observed defects can be explained by differences in laminin secretion we isolated secreted proteins from the medium and ECM of control and Itgβ4-KD cells and probed for laminins by western immunoblotting. No difference in the abundance of ECM laminins between Itgβ4-KD cells and control cells could be observed (Figure 3 A & B in III). The same conclusion was made based on immunofluorescence staining of ECM-laminins in non-permeabilized control and Itgβ4-KD cells (Figure 3C & D in III). These results suggest that integrin β4 may be competent in supporting laminin assembly to the BM without affecting laminin secretion.

5.4.2 Integrin β4 contributes to cell spreading on Col-I independently of integrin α6, while both subunits contribute to cell spreading on laminin-511

To analyze the contributions of α6- and β4-integrins to laminin adhesion, we analyzed spreading of KD and control cells on exogenous laminins by live cell imaging. To assess whether α6β4-integrins contribute to cell spreading on non-laminin-containing ECMs possibly by regulating matrix assembly, we also studied spreading of control and KD cells on Col-I substrate. MDCK cells responded poorly to recombinant LN-332 (data not shown), which was therefore omitted from the analysis. The onset of cell spreading of Itgα6-KD and Itgβ4-KD cells was slightly delayed compared to the control cells, which may be due to a lowered laminin-binding capacity (Figure 4A in III). Itgα6-KD and Itgβ4-KD cells also spread more slowly on LN-511 relative to the control cells (Figure 4C in III). Contrary to Itgα6-KD cells, Itgβ4-KD cells also displayed reduced
spreading kinetics on Col-I, although both Itgα6-KD and Itgβ4-KD cell lines initiated spreading without delay (Figure 4A & B in III). These results suggest that integrin β4 is required for sustained MDCK cell spreading on Col-I substrate independently of integrin α6.

Cell spreading is mediated by localized remodeling of the actin cytoskeleton via cell-ECM adhesions. Turnover of lamellipodial protrusions and contractile actin stress fibers is required for advancement of the cell edge during both cell spreading and cell migration. We analyzed the distribution of these actin-rich structures in Itgα6-KD and Itgβ4-KD cells relative to control cells by staining of filamentous actin. The percentage of cells with well-resolved actin stress fibers and lamellipodia was reduced in Itgβ4-KD cells relative to control and Itgα6-KD cells spreading on Col-I substrate (Figure 4D in III). Contrary to control and Itgα6-KD cells, Itgβ4-KD cells lacked lamellipodia and stress fibers appeared to be condensed into a single bundle directly adjacent to the cell edge (Figure 4F & G in III). However, when plated on LN-511 substrate, Itgβ4-KD cells displayed normal subcellular distribution of these structures (Figure 4E-F & H in III). According to these observations the role of integrin β4 in cell spreading is reflected in the organization of the actin cytoskeleton specifically on Col-I substrate.

### 5.4.3 Integrin β4 exists as heterodimeric and integrin α6-independent full-length and truncated C-terminal forms

Integrins α6 and β4 form a single heterodimer with each other in MDCK cells (Schoenenberger et al. 1994). To exclude the possibility of other heterodimers being formed and to investigate the heterodimeric ratios of integrin α6 and β4, we immunoprecipitated integrins α6 and β4 from steady state metabolically labelled cells and visualized the SDS-PAGE resolved immunoprecipitated proteins by autoradiography (Schoenenberger et al. 1994). In integrin α6-immunoprecipitations we detected two bands corresponding to the expected molecular weights for integrin α6 (120kDa) and β4 (200kDa) (Figure 5A in III). In addition to these two bands, an additional ~150kD band was visualized in integrin β4-immunoprecipitations (Figure 5A in III). To confirm the identity and specificity of the observed bands, we performed the same immunoprecipitations from Itgα6-KD and Itgβ4-KD cell lysates. The KDs reduced the intensity of the bands expected to represent the corresponding integrin (Figure 5B & C in III). Notably, no additional bands appeared upon KD of either integrin subunit,
suggesting the specificities of heterodimer formation are not altered in these conditions. Integrin β4 co-precipitated with integrin α6 at the expected heterodimeric ratio of 1:1 whereas integrin α6 co-precipitated with integrin β4 at a much lower ratio, suggesting that only a fraction of total integrin β4 forms a heterodimer with integrin α6 (Figure 5A in III). These results suggest that a large integrin α6-free pool of integrin β4 exists at steady state in MDCK cells. To examine how much of this integrin β4 occupies the ER, we treated immunoprecipitations with endoglycosidase-H, which specifically cleaves N-linked sugars of ER-bound proteins, thereby changing their mobility in SDS-PAGE. Upon endoglycosidase-H treatment, a faster migrating band of integrin β4 was observed confirming the existence of and ER resident pool of integrin β4 (Figure 5G in III). Although the quantity of the ER form was seemingly unaffected by Itgα6-KD, the mature form appeared to be reduced.

To identify the unknown protein that migrated at ~150kD along with other components in integrin β4 -immunoprecipitations, we analyzed the excised coomassie-stained bands by mass spectrometry (Figure 5D & E in III). The band corresponded to sequences mapping to the C-terminal portion of integrin β4 and as it was immunoprecipitated with an antibody against the C-terminus of integrin β4, it is likely a truncated form that lacks parts of the ectodomain. A C-terminal product of the observed size could result from extracellular proteolysis catalysed by MMPs, as has been previously reported (Pal-Ghosh et al. 2011, von Bredow et al. 1997). To test whether MMPs are involved in the processing of integrin β4 in MDCK cells, we treated cells in culture with increasing concentrations of a general MMP-inhibitor (GM6001) and studied its effects upon the C-terminal form by SDS-PAGE and western immunoblotting (Figure 5F in III). The C-terminal form was sensitive to the treatment, suggesting that it is likely to be produced by MMP-cleavage. These results suggest that integrin α6 forms a single heterodimer with integrin β4, but integrin β4 is also found as full-length and truncated C-terminal forms that do not associate with integrin α6, hereafter referred to as integrin β4-FL and integrin β4-C, respectively.

### 5.4.4 Integrin α6 and integrin β4 can be targeted to the plasma membrane independently of each other and β4 can contribute to basal laminin assembly without α6

To accommodate the integrin α6-independent function of integrin β4, it is reasonable to expect that integrin β4 should be able to mature and be transported
to the PM in the absence of integrin α6. To study the surface expression of α6- and β4-integrins we immunoprecipitated them from surface biotinylated control and KD cells then separated the immunoprecipitations by SDS-PAGE followed by detection of biotinylated proteins from western blots as previously described (Davis et al. 2001). To confirm the specificity of surface labelling, biotinylated proteins were captured by streptavidin precipitation and separated in SDS-PAGE followed by western immunoblotting of both cytosolic (β-tubulin) and PM proteins (E-cadherin) relative to whole cell lysates (Figure 6A in III). E-cadherin was detected in both avidin-precipitations and whole cell lysates while β-tubulin was only labelled from the whole cell lysate, confirming that membrane proteins are specifically biotinylated. Relative to the control cells, approximately half the integrin β4-FL and close to normal levels of integrin β4-C were detected at the surface of Itgα6-KD cells, suggesting that integrin β4 may indeed enter the secretory pathway to the PM in the absence of integrin α6 (Figure 6C in III). Surprisingly, the surface levels of integrin α6 and integrin β4-FL in Itgβ4-KD cells, where expression of total integrin β4 is reduced more than 50% by both KD constructs, were retained at control levels (Figure 6B & C in III). However, integrin β4-C was significantly reduced, suggesting that while Itgβ4-KD cells retain cell surface expression of the heterodimer, the truncated form is lost. Interestingly, Itgα6-KD and Itgβ4-KD cells displayed opposite cell surface expression profiles to the different forms of integrin β4, which may underlie the observed loss-of-function phenotypes.

To more conclusively address whether heterodimerization is a prerequisite for the cell surface expression of integrin α6 and integrin β4, we generated total KOs of both integrins by utilizing the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology incorporated into a lentivirus vector as previously described (Shalem et al. 2014). Two independent gRNA target sequences were chosen from exons 2 and 4 of ITGA6 and ITGB4, respectively. KO cells were generated by lentivirus infection, followed by puromycin selection and screening of clonal cell lines for loss of protein expression and presence of gene editing (Figure S2 in III). We assessed the surface expression of integrin α6 and integrin β4 in control and KO cells by surface biotinylation and immunoprecipitation as described above. Itgα6-KO cells expressed 20-30% of β4-FL and 80% of β4-C at the cell surface relative to control cells, but of the two different gRNA-expressing KO cell lines, Itgα6-KO1 expressed relatively more of β4-FL (Figure 6E in III). Strikingly, Itgβ4-KO cells expressed equal to control levels of integrin α6 at the cell surface (Figure 6D in
III). No additional bands were visible, suggesting that both integrins may be transported to the cell surface as single subunits.

To analyze whether the putative single subunit integrins could be targeted to cell-ECM adhesions, integrins α6 and β4 were visualized by total internal reflection fluorescence (TIRF) microscopy in control and KO cells (Figure 6F & G in III). Although integrin α6 was expressed at the surface of Itgβ4-KO cells, their targeting to the basal membrane was integrin β4-dependent as only faint background labelling was visible in TIRF-images (Figure 6F in III). Based on confocal microscopy, the surface expression of integrin α6 in these cells was restricted to the lateral cell-cell contacting membrane domain. Conversely, surface expression of integrin β4 in Itgα6-KO cells could be visualized at the basal surface domain at putative HDs that correlated with the LN-511 staining in TIRF-images (Figure 6G in III). However, only the Itgα6-KO1 cell line, which expressed relatively more of integrin β4-FL at the cell surface, displayed localization of integrin β4 to putative HDs (Figure 6G in III). Both Itgα6-KO cell lines however, displayed normal organization of LN-511 to the BM relative to Itgβ4-KO cells where LN-511 organization was abolished (Figure 6F & G in III). These results suggest that integrin β4 may possess an intrinsic capability to promote laminin assembly. However, further studies are required to establish whether the putative HDs visualized in Itgα6-KO cells are competent in mediating laminin anchorage.
6 Discussion

6.1 MDCK cell contractility in response to mechanosensing requires cooperation between β1- and αV-integrins (II)

We discovered that αV-integrins contribute to mechanotransduction at α2β1-integrin based FAs formed on Col-I since ItgαV-KD cells failed to reinforce and facilitate cytoskeletal coupling to FAs, leading to defective adaptation of MDCK cells in response to ECM rigidity. None of the heterodimer partners of integrin αV replicated the loss-of-function phenotype of integrin αV, suggesting that different αV-heterodimers may perform this task redundantly. αV- and β1-integrins are known to cooperate in FN adhesion and rigidity sensing, but both αV- and β1-heterodimers are assumed to co-localize to FAs and serve their relative functions via ligand-binding activities. In such conditions, α5β1 provides the bulk adhesive strength, whereas αVβ3 integrin facilitates mechanotransduction (Roca-Cusachs et al. 2009). Based on further characterization of the tasks of these integrin in fibroblasts, α5β1 generates force by stimulating myosin II activity, while αVβ3 promotes structural adaptation to force by mediating nucleation of actin stress fibers, both of which contribute to mechanotransduction (Schiller et al. 2013). In MDCK cells αV-integrins are uniquely required for adhesion to FN whereas the role of integrin α5β1 is negligible, since KD of the α5 or β1 chain did not affect FN adhesion and based on our qPCR analysis very little α5 integrin is expressed.

In our model system αV-integrins cooperate with β1-integrins to mediate mechanotransduction on ligands other than FN, but it is unclear how or whether αV can be recruited to FAs in the absence of its ligand, because αV-integrins did not contribute to binding of Col-I in our experiments. Moreover, recombinant integrin αV-mRFP was not visibly recruited to FAs formed on Col-I. However, we cannot rule out that even in the short time-scale of our analysis, endogenous deposition of FN and incorporation to the underlying Col-I substrate facilitates recruitment of αV-integrins to the FAs, thereby supporting their activities. αV-integrins also bind multiple ligands other than FN that might engage αV-integrins (Figure 9). In the absence of any significant contribution to cell adhesive strength, αV-integrin-mediated mechanical signals may still be necessary for FAs to sense force. Indeed, even small numbers of ligand-bonds by αVβ3 may be sufficient to support mechanical signal transduction while α5β1-integrins provide the adhesive
function (Roca-Cusachs et al. 2009). $\alpha V$– and $\beta 1$-integrins are likely to have different bond dynamics, allowing cells to generate internal traction forces at different ECM rigidities (Elosegui-Artola et al. 2014). Sufficient force must be applied by the individual integrin-ligand bonds to trigger the mechanotransduction processes on a rigid surface. Concomitantly a counter force needs to be provided by the cell-intrinsic actomyosin machinery, which might be differentially stimulated by $\alpha V$- and $\beta 1$-integrins (Schiller et al. 2013). $\alpha V$ and $\beta 1$-integrins may also show differences in how actin binding proteins that regulate the reinforcement of the cytoskeletal linkage between integrins and actin filaments bind (Roca-Cusachs et al. 2013). The combined external and internal forces lead to the conformational unwinding of mechanosensitive proteins in the FA, triggering signals and recruiting more proteins (Schiller & Fassler 2013). Any obstruction in this force transmission chain might lead to loss of traction and cell contractility. Rescue experiments with ligand-binding deficient mutant forms of $\alpha V$-integrin would allow us to determine whether ligand-binding by $\alpha V$-integrins is required for its role in mechanotransduction. Furthermore, blocking the binding of $\alpha V$-integrins to insoluble ECM-ligands such as FN with an RGD-peptide might indicate whether $\alpha V$-integrins serve as mechanoreceptors in our experimental setup.

We found that recruitment of multiple signalling and adaptor proteins to FAs in Itg$\alpha V$-KD cells similar to Itg$\beta 1$-KD cells was abrogated, but we could not establish a clear hierarchy. ILK, FAK and talin1 were required for the spreading of MDCK cells, but based on these findings a direct causal link to the integrin $\alpha V$-related loss-of-function defect cannot be established. Our studies support previous findings that cytoskeletal linkage of integrins and mechanotransduction at FAs is mediated by talin (Roca-Cusachs et al. 2009, Zhang et al. 2008). Talin regulates inside-out integrin activation (Calderwood et al. 1999) but is more likely to support integrin clustering and FA-assembly in adherent cells (Ellis et al. 2014). As recruitment of talin is an early step in FA-assembly and is required for clustering of integrins, Itg$\alpha V$-KD cells may fail to support talin recruitment, which would explain the low number of FAs observed. Similar to what we determined for $\alpha V$-integrins in our experimental setup, talin is also not required for initial cell adhesion in fibroblasts (Zhang et al. 2008), which is consistent with this hypothesis. As $\beta 1$-integrins are capable of interacting with talin (Calderwood et al. 1999), KD of $\alpha V$ might affect the capacity of $\beta 1$ integrins to support talin-binding. Such a mechanism might involve $\alpha V$-mediated signalling that modifies the binding properties of the integrin $\beta 1$ tail itself, which is mediated by
phosphorylation (Legate & Fässler 2009). Analysis of the phosphorylation status of the \( \beta_1 \)-integrin tail would allow us to determine whether \( \beta_1 \)-integrins are differently regulated in Itg\( \alpha V \)-KD cells. \( \alpha V \)-integrins might also induce signalling via protein kinases that regulate other FA components by phosphorylation (Zaidel-Bar & Geiger 2010). An example of this is the function of syndecan-4 as a co-receptor that stimulates activation of protein kinase C (PKC) by binding to the heparin-binding domain of FN, which is required for the formation of \( \alpha 5 \beta 1 \)-mediated FAs on FN (Mostafavi-Pour et al. 2003). The PKC in turn regulates maturation and mechanotransduction at FAs via Rho GTPases (Bass et al. 2008, Pasapera et al. 2015). The integrin \( \beta \) tails also support binding of trafficking factors, which may provide an alternative mechanism for integrin regulation. Indeed, ligand-binding and rapid recycling of \( \alpha V \beta 3 \) indirectly affects trafficking and surface exposure of \( \alpha 5 \beta 1 \) (White et al. 2007). We were unable to detect differences in the steady state surface exposure of \( \alpha 2 \beta 1 \)-integrin upon KD of \( \alpha V \), but this does not exclude the possibility that turnover of \( \alpha 2 \beta 1 \) may be altered. Additional experiments to study the trafficking of \( \alpha V \)- and \( \beta 1 \)-integrins might shed some light on how they interact.

6.2 Integrins \( \alpha 2 \beta 1 \) and \( \beta 4 \) transmit ECM-derived cues to guide orientation of apicobasal cell polarity (I)

Orientation of apicobasal polarity is directed by reciprocal signalling between epithelial cells and the ECM, which guides the establishment of apical lumens. Studies in MDCK cells have suggested that \( \beta 1 \)-integrins contact the ECM and induce an Rac1-mediated basal cue to direct polarized deposition of laminin to the ECM and to initiate laminin assembly via laminin receptors (O'Brien et al. 2001, Yu et al. 2005). Laminin-derived signals are then required for the correct orientation of apicobasal polarity and establishment of apical lumens. The identity of the \( \beta 1 \)-integrin heterodimers and ECM molecules that control the orientation of cell polarity has not been precisely defined. Our studies specify that the basal cue that guides orientation of polarity in MDCK cells is mediated by \( \alpha 2 \beta 1 \) and \( \beta 4 \) integrins when cells are cultured in a 3D Col-I gels. The role of integrin \( \alpha 2 \beta 1 \) is likely to contact the underlining ECM and to activate Rac1, while the role of integrin \( \beta 4 \) might be to regulate the assembly of laminin to the ECM. The role of integrin \( \beta 4 \) in this process seemed to be independent of integrin \( \alpha 6 \), which is controversial as \( \alpha 6 \) integrin is the only heterodimer partner of \( \beta 4 \). Based on this
evidence we can only conclude that integrin β4 has an intrinsic function unrelated to its role as the α6β4 heterodimer.

Exogenous laminin supplementation partially reverses the inversion of polarity in Itgβ1-KD cysts by clearing the apical proteins from the ECM-facing exterior of the cyst. Only when both α6β4 and β1-integrins were simultaneously inhibited, a total inversion of polarity was observed. It is therefore possible that in Itgβ1-KD cells α6β4 may regulate recognition of the BME by its laminin components, leading to the observed partial rescue of the Itgβ1-KD phenotype. However, as the Itgβ1-KD cysts are still grossly abnormal with disrupted lumen morphology and organization of apicobasal polarity, the fact that basal surfaces are cleared from apical proteins does not suggest that polarity is properly oriented. Indeed, apical cargo containing vesicles need to be targeted to the correct membrane domain, which requires β1-integrin mediated ILK signalling to establish apicobasal orientation of non-centrosomal microtubules as described in mammary epithelial cells (Akhtar & Streuli 2013). It is likely that different integrin-mediated signals regulate the recognition of the underlying ECM first, leading to endocytosis of apical proteins from the exterior of the cysts and their transport to the apical surface second, but it is unclear whether both of these events are strictly β1-integrin dependent. Indeed, we show that the first step can be mediated solely by α6β4.

Rac1 activation is not required when MDCK cells are already in contact with a BM rich in laminin (O’Brien et al. 2001). Contrary to previous evidence we find that Rac1 activation is also essential for orientation of polarity in an ECM composed of BM components. The role of Rac1, however, is cell-type specific as orientation of polarity in mammary epithelial cells is independent of Rac1-activation (Akhtar & Streuli 2013). In MDCK cells it is possible though that Rac1 serves as a more general adhesion-dependent cue that confers basal identity, whereby cells adhering to different types of ECMs may orient polarity. Forced activation of Rac1 rescued the inversion of polarity in Itgβ1-KD and Itgα2-KD cysts in 3D Col-I to the same extent as observed in Itgβ1-KD cells cultured in 3D BME. It is possible that a similar rescue would also occur in cysts cultured in 3D BME upon inhibition of both β1- and α6β4 integrins as orientation of polarity in these conditions was also Rac1-dependent. Integrin α6β4 can interact with Rac1 and contribute to its activation and might therefore also contribute to orientation of polarity by a Rac1-dependent mechanism (Hamill et al. 2009, Hamill et al. 2011, Sehgal et al. 2006). It is likely that the loss of cell adhesion upon KD of α2β1 integrins in 3D Col-I and both α2β1 and α6β4 integrins in 3D BME might
effectively convert the basal surface domain into a free surface where apical proteins are stabilized leading to the observed inverted polarity phenotype (Wang et al. 1990a, Wang et al. 1990b). This stabilization is dependent on high signalling activity of RhoA and ROCK1 that activates ezrin at the basal domain allowing it to bind Podxl and Na(+)/H(+) exchange regulatory cofactor NHE-RF1 (NHERF1) (Bryant et al. 2014, Yu et al. 2008). Integrin-mediated destabilization of this complex is dependent on FAK signalling to recruit Rho-GAPs basally to inhibit RhoA activity (Bryant et al. 2014). Our studies link the function of α2β1-integrin with this mechanism as the inversion of polarity in Itgβ1-KD and Itgα2-KD cells could be fully rescued by inhibition of ROCK1. However, in submandibular salivary glands RhoA/ROCK1-signalling is required for specification of the basal surface domain and BM-positioning, suggesting that the role of RhoA is also cell type specific (Daley et al. 2012, Yu et al. 2008).

Contrary to our findings Bryant and co-workers (2014) propose that both α2β1- and α3β1-integrins are uniquely required for switching the orientation of cell polarity from an inverted and front-to-rear type (partially inverted) into an apicobasal type during cystogenesis in 3D BME culture. The authors however noted the absence of cysts with fully inverted polarity, such as observed when β1-integrins are blocked in 3D Col-I culture, and instead described the prevalence of the so-called front-to-rear polarized, partially inverted phenotype. As our analysis focused on mature cysts at day 7 in 3D BME, we cannot rule out the presence of such intermediate phenotypes earlier during cystogenesis, but can conclude that these phenotypes do not persist. The roles of integrins in mediating different cellular processes during orientation of cell polarity are still somewhat unclear. To more precisely define the dependencies, independencies and redundancies of different integrin heterodimers, double KD cell lines where different integrin subunits are simultaneously inhibited could be utilized. The absolute requirement for laminin in regulating orientation of polarity in the MDCK cell model is based solely on the fact that it rescues the inversion of polarity caused by Rac1-DN and β1-integrin function blocking (O'Brien et al. 2001, Yu et al. 2005). Loss-of-function studies targeting specific laminin isoforms would also be beneficial in understanding how cells regulate endogenous BM assembly and which of these components are central to the establishment of apicobasal polarity.
6.3 Integrin α3β1 guides symmetric cell divisions to regulate maintenance of apical lumens (I)

Lumens in MDCK cells can be formed by two different mechanisms: either by hollowing or by cavitation (Figure 3), depending on the rate of cell polarization to cell proliferation (Martin-Belmonte et al. 2008). These mechanisms can be differentially utilized depending on ECM conditions. Interestingly, although Itgα3-KD cells formed cysts with normal lumens in 3D Col-I culture, they failed to do so in 3D BME culture. Instead, Itgα3-KD cells formed multiple small lumens similar to Itgβ1-KD cells in these conditions. Cells in collagenous ECM first deposit and organize an endogenous BM that then serves as a cue for the outer layer of cells in a cluster to orient apicobasal polarity, upon which lumens are formed by cavitation. In the presence of BMs, cells acquire apicobasal polarity more rapidly and lumens are formed by a hollowing mechanism. This process may be selectively regulated by α3β1 integrins. In the absence of α3-integrins we observed that lumen formation was compensated by cavitation as evident by the presence of apoptotic cells in the lumens of Itgα3-KD cysts. Upon prolonged culture apoptosis contributed to the formation of more dilated lumens in Itgα3-KD cells.

Simple epithelial cells divide symmetrically in order to maintain a central positioning of the lumen during hollowing (Overeem et al. 2015). This is established by orientation of the mitotic spindle perpendicular to the apicobasal axis via anchorage of astral microtubules to the lateral cell cortex (Hao et al. 2010, Zheng et al. 2010). Moreover, apical positioning of the midbody during cytokinesis serves as the site where the apical surface domain is established upon abscission (Jaffe et al. 2008). We observed that misoriented cell divisions significantly contributed to the multiple lumen phenotype of Itgα3-KD and Itgβ1-KD cells in 3D BME. A similar phenotype has been previously described upon loss of Cdc42-signalling and involves defective lumen biogenesis and loss of symmetric cell divisions (Jaffe et al. 2008, Martin-Belmonte et al. 2007). Precise control of Cdc42 cortical activity is required for symmetric cell divisions (Jaffe et al. 2008, Qin et al. 2010, Rodriguez-Fraticelli et al. 2010). Our results indicate that α3β1-integrins may serve to antagonize activation of Cdc42, thereby providing a basal exclusion mechanism as Cdc42 is normally activated at the apical surface domain (Martin-Belmonte et al. 2007). This might involve recruitment of factors such as Cdc42-specific GAPs to α3β1-mediated adhesions. In conclusion, our results describe a specific role for the laminin-binding α3β1-
integrin: it guides symmetric cell divisions, which is essential for single lumen formation. This defect can be compensated by integrin \( \alpha 2\beta 1 \) and \( \beta 4 \) mediated signals that trigger apicobasal polarization during cavitation. These results suggest that different sets of integrins can regulate lumen formation via two distinct mechanisms depending on ECM conditions (Figure 10).

**Fig. 10.** Role of integrins in orientation and maintenance of apical lumens. Collagen-binding \( \alpha 2\beta 1 \) is required for cell adhesion to the 3D Col-I ECM to induce Rac1-mediated basal laminin assembly together with \( \beta 4 \)-integrins. In the absence of these integrins apical proteins are not cleared from the ECM-facing surface, laminin (LN) assembly is defective and the lumen does not form. In 3D BME, \( \alpha 3\beta 1 \) is required to maintain the apical lumens by guiding the symmetric cell divisions. Lack of \( \alpha 3\beta 1 \) leads to the formation of multiple lumens. Adapted from Aki Manninen (2015).
6.4 Integrin β4 is targeted to putative hemidesmosomes and supports basement membrane laminin assembly independently of integrin α6 (III)

Our previous studies (I) indicated that integrin β4 may regulate orientation of polarity during MDCK cystogenesis independently of the integrin α6 subunit. As orientation of cell polarity depends upon deposition and assembly of laminin to the cell-ECM interface (O'Brien et al. 2001, Yu et al. 2005), an obvious assumption is that integrin β4 contributes to these processes during cystogenesis. Indeed, the integrin β4 loss-of-function phenotypes in both cystogenesis (I) and cell spreading (III) seemed to be alleviated in the presence of exogenous laminins. Previous studies show that β1-integrins do not contribute to the secretion of laminin chains in MDCK cells (Yu et al. 2005). Our data indicates that α6β4 integrin subunits are also dispensable for laminin secretion. However, integrins are more likely to regulate laminin assembly by promoting polymerization of laminins at the cell surface (Colognato et al. 1999, Li et al. 2005b, McKee et al. 2007, McKee et al. 2009). Previous evidence supports that β1-integrins are required for assembly of laminin to the cell-ECM interface during MDCK cystogenesis in 3D Col-I culture (Yu et al. 2005). It remains unclear whether β1-integrins both specify the site at which laminin should be deposited and actively participate in its assembly. Contribution of other laminin-binding receptors, such as integrin α6β4, cannot be ruled out. Indeed, we observed that integrin α6β4 more strongly colocalized with both laminin isoforms LN-332 and LN-511 in polarized MDCK cells. However, as the expression of these isoforms does not temporally overlap during cell polarization, they may differentially contribute to BM assembly and be differentially engaged by β1 and α6β4 integrins (Greciano et al. 2012, Mak et al. 2006, Moyano et al. 2010).

We observed that the integrin β4 subunit was synthesized in excess over the α6 subunit and exhibits distinct heterodimeric and α6-free pools, including a truncated C-terminal proteolysis product. Previous studies suggest that integrins α6 and β4 may also be expressed in non-stoichiometric quantities at the PM (Sonnenberg et al. 1990). Indeed, our data suggests that integrin β4 can occupy the PM in the absence of integrin α6, suggesting that integrin β4 may enter the secretory pathway and be targeted to the PM as a monomer. However, we observed that of the two KO cell lines, Itgα6-KO2 cells expressed less of the full length form of β4 relative to the Itgα6-KO1 cells at the cell surface. Early studies suggest that the maturation of integrin β4 is dependent on its association with α6,
but these results are based on pulse-chase experiments with an antibody directed against integrin α6 (Sonnenberg et al. 1988). Our results show that integrin α6 was also targeted to the PM in the total absence of integrin β4. However, it is possible that integrin α6 forms a heterodimer with integrin β1 in these conditions which facilitates its maturation. Formation of α6β1 could not be observed either in Itgβ4-KD or KO cells, but this needs to be further confirmed. A double KO cell line where both β4 and β1 integrins are depleted would allow us to determine whether the β4-independent surface expression of α6 is dependent on its heterodimerization with integrin β1. Itgβ4-KD cells that show 40-50% residual expression of the protein retain normal levels of the heterodimeric form of integrin β4 at the cell surface, which might contribute to the observed loss-of-function phenotypes. Further characterization of the Itgβ4-KO cells is required to verify the results obtained with the KD system.

Our studies indicated that integrin β4 by itself may be sufficient in regulating the assembly of laminins to the mature BM. Integrin β4 was essential for the arrangement of laminins into parallel array structures adjacent to the basal cell surface. These structures may represent type II HDs as described in other simple epithelial cells (Uematsu et al. 1994), but their composition needs to be further characterized by demonstrating the presence of other HD components such as plectin. These putative HDs formed normally in Itgα6-KO cells, suggesting that integrin α6 is not required for their formation. In contrast, formation of classical type I HDs in the epidermis is known to be dependent on both integrin subunits (Dowling et al. 1996, Georges-Labouesse et al. 1996, van der Neut et al. 1996).

We observed localization of integrin β4 to these HD-like structures only in the Itgα6-KO1 cell line that displayed relatively more surface expression of the full length form of integrin β4 than the Itgα6-KO2 cell line. As only the full-length form is likely to contain an intact ligand-binding domain, it is possible that association of integrin β4 with laminins is mediated by this domain also in absence of integrin α6. Rescue experiments with ligand-binding deficient mutant forms of integrin β4 in the Itgα6-KO background would clarify whether the basal organization of laminins is dependent on direct ligand-binding interactions between integrin β4 and laminins. Alternatively, integrin β4 may associate with a laminin-binding co-receptor or induce signals that regulate laminin-binding via other receptors (Figure 11). Indeed, integrin β4 is capable of regulating cell spreading and migration independently of ligand-binding (Nikolopoulos et al. 2005, Russell et al. 2003).
The functional relevance for the integrin β4 to support laminin assembly by itself is unclear as in normal conditions integrin α6 is available and both integrins equally colocalized with laminins. Deposition of laminins to the ECM is also required for cell migration and cell spreading (Greciano et al. 2012, Hamelers et al. 2005, Sehgal et al. 2006). Previous studies have implicated integrin α6β4 in the assembly of LN-332 to the ECM to promote cell migration (Sehgal et al. 2006). We speculate that the integrin α6-free pool of integrin β4 may be relevant under these conditions, as Itgβ4-KD cells that specifically lacked this pool were deficient in cell spreading in a matrix composed of Col-I. It is possible that incorporation of laminins to the Col-I-composed matrix facilitates normal cell spreading. In keratinocytes HDs are not fully disassembled but are more dynamically turned over during cell migration (Geuijen & Sonnenberg 2002, Goldfinger et al. 1999). These HDs may be structurally and functionally distinct from classical HDs and it is plausible that the α6-free pool may contribute to the formation of such less stable HDs. Turnover of HDs may in turn reflect upon turnover of FAs as it is likely that HDs and FAs and their associated filament systems need to be coordinately remodelled during cell migration and spreading. We observed that the abnormal spreading of Itgβ4-KD cells was accompanied by lack of actin stress fibers and lamellipodial protrusions, both of which are essential for turnover of FAs (Wolfenson et al. 2013). Moreover, we observed abnormal advancement of the IF system and ER that are normally restricted to the lamellipodia-lamella border (Lynch et al. 2013). Experiments with Förster resonance energy transfer (FRET) microscopy could be performed with recombinant α6 and β4-integrins to investigate whether they display non-overlapping distributions during cell spreading and migration.

In conclusion, our studies suggest that integrin β4 may regulate laminin assembly, but it remains to be established whether laminin assembly is the underlying cause for the observed loss-of-function phenotypes in cystogenesis and cell spreading. Moreover, the composition of the integrin β4-positive laminin structures should be better characterized and compared with those formed by α6β4 to determine whether they represent HDs.
Fig. 11. Different forms of integrin β4 and their role in laminin assembly. Integrin β4 exists at the cell surface as heterodimeric and integrin α6-independent full-length and truncated forms. Integrin β4 regulates laminin assembly in the absence of integrin α6, suggesting that the integrin α6-independent pools of integrin β4 possess intrinsic capacity to regulate laminin anchorage. The possible mechanisms may include direct ligand-binding or binding assisted by a laminin-binding co-receptor such as BPAG2. The cytoplasmic tail may also induce signals that promote laminin-binding via other receptors.
7 Summary

We have elucidated the principles of the functional hierarchy between integrins in mediating ECM-derived signals to organize of epithelial cell polarity and to promote epithelial cell contractility. These studies speak for the importance of understanding integrins as a collective with compensatory, complementary and synergistic functions.

In some cases the functions of integrins did not directly relate to cell adhesive duties, indicating that integrins mediate signals in conditions where cell adhesion is mediated by other receptors. This is exemplified by the αV-integrins that regulated mechanotransduction at the β1-integrin-based FAs, which enabled cells to spread by contracting their cytoskeleton in response to ECM rigidity. Our studies also indicated that α2β1 and β4-integrins which bind different ECM-molecules can compensate each other in mediating the basal cue for orientation of cell polarity. These integrins also mediated adhesion to the tested ECMs, suggesting that cell adhesion triggers the process. As a result, epithelial cells can orient their polarity in a robust and ECM-sensitive manner. Moreover, we observed that integrins which compensate each other can do so by regulating mechanistically distinct, but complementary cellular processes. Integrin α3β1 contributed to epithelial hollowing by directing symmetric cell divisions, while α2β1 and β4-integrins contributed to epithelial cavitation by directing the orientation of the apicobasal polarity axis. Therefore, integrins contribute to the overall plasticity by which the apical lumens can be established in response to various ECM-configurations. Integrin signals that regulate activities of Rho GTPases in a spatially restricted manner seem to be central to the establishment of properly oriented polarized membrane domains. We also describe a novel case in which integrin β4 could be transported to the cell surface in the absence of its sole heterodimeric partner integrin α6. In these conditions integrin β4 was capable of localizing to laminin adhesions and promoted laminin assembly to the ECM, which might explain its α6-independent role in regulating the orientation of cell polarity.
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SPECIFIC ROLES OF EPITHELIAL INTEGRINS IN CHEMICAL AND PHYSICAL SENSING OF THE EXTRACELLULAR MATRIX TO REGULATE CELL SHAPE AND POLARITY