Role of classical cadherins in epidermal junction and barrier formation

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Abstract

Classical cadherins mediate Ca^{2+}-dependent intercellular adhesion and are essential for tissue morphogenesis and maintenance. They are key components of adherens junctions (AJs). *In vitro* studies in simple epithelial cells indicated an essential role for E-cadherin not only in the formation of AJs but also other intercellular contacts, such as desmosomes and tight junctions. In contrast, *in vivo* tissue specific knockout studies did not reveal a necessity of E-cadherin in the formation of intercellular junctions, raising the question if classical cadherins are necessary or if other classical cadherins can compensate for the loss of E-cadherin. Therefore, the aim of this thesis was to ask how E-cadherin regulates tight junctions and if E-cadherin has a specific function in the formation of tight junctions. In addition, the question was asked if classical cadherin function is necessary for the formation of other intercellular contacts, such as desmosomes.

Using primary keratinocytes as a model for *de novo* junction formation, it was found that loss of E-cadherin prevents the formation of a functional tight junctional barrier. Surprisingly, the basic assembly of tight junctions was not affected, suggesting that E-cadherin regulates a late step in the formation of a functional barrier. One pathway through which E-cadherin may regulate the functional barrier is by controlling expression levels of the barrier promoting claudin-14. However, knockdown of claudin-14 is insufficient to reduce barrier function, suggestion that other mechanisms contribute to E-cadherin controlled barrier function.

Loss of E-cadherin in combination with knock down of the only other epidermal classical cadherin, P-cadherin, resulted in an almost complete loss of intercellular contacts showing that classical cadherins are crucial for desmosome formation. Re-expression of either E- or P-cadherin can rescue not only desmosome formation but also tight junction function, showing that levels but not specific classical cadherin expression is crucial for the functional formation of intercellular junctions.

E-cadherin is a tumor suppressor and found to be down regulated in many tumors. In this thesis it was found that loss of E-cadherin in primary keratinocytes is insufficient to enhance migration and proliferation, suggesting that in cancer cells E-cadherin interacts with other, E-cadherin independent pathways in the regulation of growth and migration.

Zusammenfassung

Klassische Cadherine sind Calcium abhängige Zelladhäsionsmoleküle und ein wichtiger Bestandteil der *zonula adherens*. Funktionale Studien zeigten eine wichtige Rolle für E-Cadherin bei der Initiierung und Erhaltung von Zell-Zell Adhäsion in einfachen Epithelien. Dabei hing nicht nur die Bildung von *zonula adherens*, sondern auch die von Desmosomen und *zonula occludens* von der funktionalen Aktivität von E-Cadherin ab. Im Gegensatz dazu war die Ausbildung von Desmosomen in der Epidermis nicht von E-Cadherin abhängig. Nur die Barrierefunktion der *zonula occludens* im *stratum granulosum* der Epidermis war nach genetischer Inaktivierung von E-Cadherin beeinträchtigt. Dies führte zur Frage, ob E-Cadherin für die Bildung epidermaler Desmosomen entbehrlich ist und wie die spezifische Regulation von epidermalen *zonula occludens* stattfindet.

Mithilfe primärer Maus Keratinozyten konnte in dieser Arbeit gezeigt werden dass E-Cadherin für die Bildung epidermaler Desmosomen entbehrlich ist, was auf eine funktionale Kompensation durch P-Cadherin zurück zuführen ist. Entweder E- oder P-Cadherin Expression wurde benötigt, um die Bildung von Desmosomen in primären Keratinozyten auszulösen.

Obwohl *zonula occludens* in Abwesenheit von E-cadherin strukturell vorhanden sind, konnte gezeigt werden dass diese Strukturen keine funktionelle Barriere bilden. Expression von entweder E- oder P-cadherin war erforderlich um eine funktionale Barriere in primären Maus Keratinozyten zu bilden. Die klassischen Cadherine E- und P-Cadherin zeigen funktionelle Redundanz bei sowohl Desmosomenbildung als auch bei der Bildung einer funktionalen *zonula occludens* Barriere.

Neben der Regulation von Zelladhäsion spielt E-cadherin eine wichtige Rolle bei der Regulation anderer zellulärer Prozesse wie Proliferation und Migration. E-cadherin gilt als Tumorsupressor und seine Expression ist in einer Reihe von Tumoren vermindert. Diese Arbeit zeigt das E-cadherin kein direkter Regulator der Keratinozyten Proliferation und Migration ist. Daher erscheint es wahrscheinlich, dass E-cadherin diese zellulären Prozesse im Zusammenspiel mit anderen, E-cadherin unabhängigen, Mechanismen reguliert.

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1. Introduction

Physical cohesion between cells is an essential requirement for the formation and maintenance of complex tissues and organs and is provided by adhesive protein complexes that form intercellular junctions. These intercellular junctions must be tightly regulated not only during morphogenesis but, depending on the physiological requirements and cellular functions of a tissue, also during tissue homeostasis. Different types of intercellular junctions serve distinct functions. Adherens junctions and desmosomes are primarily involved in physical cohesion whereas tight junctions serve as paracellular and membrane diffusion barriers. Gap junctions form channels between cells and thereby allowing intercellular communication of small solutes (fig. 1).

This study mainly addresses the role of adherens junctions, and more specifically its cell adhesion receptors, the classical cadherins in the regulation of not only adherens junctions but also desmosomes and tight junctions as well as their requirement in epidermal barrier formation by using loss of function approaches in primary mouse keratinocytes.

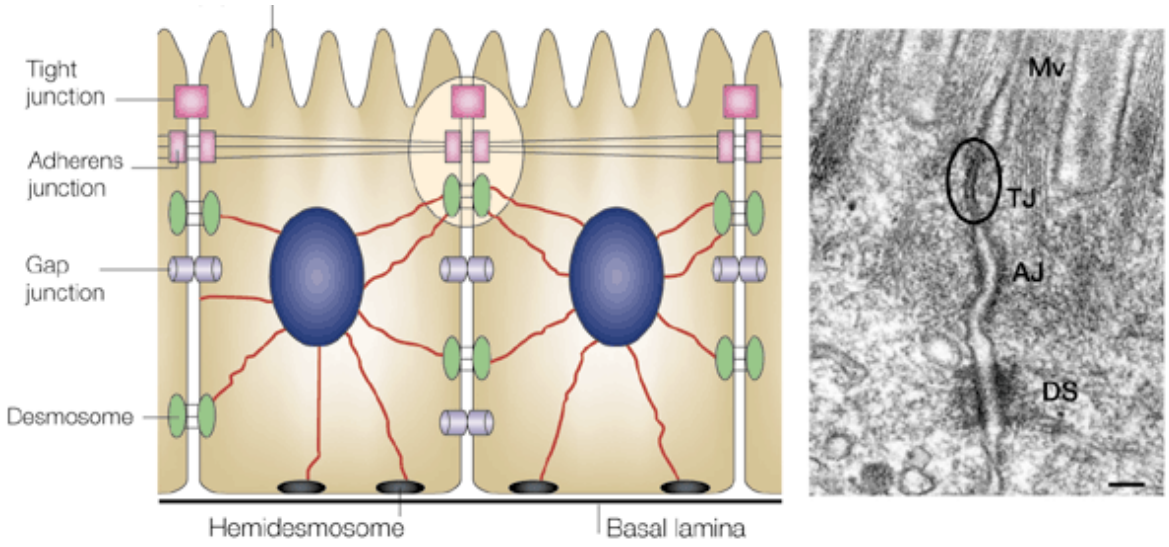


Figure 1: Intercellular junctions in simple epithelia.

Left: Schematic representation of intercellular junction complexes in polarized simple epithelia.

Right: Electron micrograph showing intercellular ultrastructures. Mv: Microvilli; TJ:tight junction; AJ: adherens junction; DS: desmosome. Taken from (Tsukita, Furuse et al. 2001)

1.1 Adherens Junctions

As the name implies, adherens junctions are intercellular adhesive structures that mediate physical cohesion between cells. First identified in 1963, the adherens junctions were characterized by electron microscopy as part of a tripartite complex in simple epithelia (Farquhar and Palade 1963) (fig. 1). On the ultrastructural level, adherens junctions appear as parallel membranes separated by an intercellular space of ~ 20 nm. Together with the apical localized tight junctions and the more basally found desmosomes, this tripartite structure was called the apical junctional complex. As predicted at the time based on their ultrastructural appearance, it is now widely accepted that adherens junctions are crucial for providing adhesion between cells and thereby establish physical integrity of tissues. They are dynamically regulated structures that serve as coordinating signaling platforms that regulate the actin cytoskeleton, signaling complexes and polarity cues, thereby regulating directly and indirectly cell shape and a variety of cellular processes. Adherens junctions consist of transmembrane adhesion receptors of the classical cadherin and nectin family which bind and recruit cytoplasmic scaffold and signaling molecules via their cytoplasmic domain. Among these recruited factors, actin binding proteins provide either direct or indirect connections to the actin cytoskeleton. In the following paragraphs, I will describe the molecular composition of the adherens junction as well as several mechanisms of their regulation.

1.1.1 Classical cadherins

Classical cadherins are type I transmembrane proteins that belong to the cadherin super family of Ca^{2+} dependent adhesion molecules, which are characterized by a Ca^{2+} binding extracellular motive, the so called cadherin repeat (EC). The number of extracellular repeats varies between family members, ranging form 4 to 34. Classical cadherins have 5 extracellular repeats which mediate homophilic interaction between cadherins of adjacent cells in a Ca^{2+} dependent manner. The EC1 domain plays a crucial role in adhesive bond formation as well as in mediating binding specificity. In addition to *trans* interaction between cells, the cadherin extracellular domain is capable to interact in *cis* laterally in the membrane resulting in cadherin dimers that are thought to represent the basic adhesive units (Brieher, Yap et al. 1996).

The most prominent members of the classical cadherin sub family are E(pithelial)-cadherin, N(euronal)-cadherin and P(lacental)-cadherin. Although initially named after the tissue where they were discovered, they show a broader expression pattern. Homophilic binding of differentially expressed cadherins was thought to be the basic driving force behind cellular sorting and segregation processes, for example in mesoderm induction, where a switch from E- to N-cadherin induces segregation from the ectoderm. Similarly, E- to N-cadherin switch is observed in many carcinomas and is this is likely to be a key step in tumor progression leading to invasiveness. However, other studies report that classical cadherins are able to interact also in a heterophilic fashion, suggesting that the molecular mechanisms that drive sorting processes are not that simple (Niessen and Gumbiner 2002).

1.1.2 Catenins

The cadherin cytoplasmic domain forms a basic complex with catenins, and this interaction is crucial for full adhesive capacity. The Ch1 domain in the juxtamembrane position interacts with p120ctn. The more distal, c-terminal Ch2 domain interacts with the armadillo repeat protein β-catenin, which in turn recruits the actin binding protein α-catenin. Thereby, dynamic, indirect association with the actin cytoskeleton is achieved. In the following, the different catenins and their role in the adherens junctions are discussed.

1.1.2.1 p120ctn

p120ctn binding to the cadherin has emerged as a critical regulation of the cadherin cell surface stability (Xiao, Oas et al. 2007). Loss of p120ctn results in increased cadherin turnover by regulating access to the endocytotic machinery (Reynolds 2007). p120 comprises 10 armadillo repeats that are crucial for the interaction with the cadherin (fig. 3). Regulation of cadherin dynamics at the cell surface occurs via intensive tyrosine and serine/threonin phosphorylation (Fukumoto, Shintani et al. 2008). In addition to its regulatory function in the cadherin core complex, p120ctn can translocate to the nucleus and regulates gene expression through binding to the transcription factor kaiso. Furthermore p120ctn turned out to mediate the regulation of Rho family GTPases upon cadherin

engagement. In particular, RhoA becomes inhibited by binding to p120ctn, whereas Rac is activated by a less understood mechanism (Anastasiadis 2007).

1.1.2.2 β-catenin

β-catenin binds to the distal region of the cadherin cytoplasmic domain and this interaction serves several functions. It protects the cadherin cytodomain from degradation, promotes cadherin trafficking from the ER to the plasma membrane, and recruits α-catenin to the core complex. In addition to these functions that all affect adhesive capacity of the adherens junction, β-catenin mediates gene regulation as a signal transducer in the wnt signaling pathway which plays crucial roles in several developmental processes. In the absence of wnts, cytosolic β-catenin that is not bound to the cadherin gets phosphorylated by the APC complex, thereby targeting it to ubiquitinylation and proteasomal degradation. Extracellular wnt signals inhibit the APC complex, allowing β-catenin to shuttle to the nucleus where it acts as a transcriptional cofactor by binding to TCF/Lef transcription factors, resulting in activation of wnt responsive genes (Nelson and Nusse 2004).

1.1.2.3 α-catenin

The actin binding protein α-catenin is indirectly associated with the cadherin via binding to β-catenin. Loss of α-catenin results in reduced adhesion, although the cadherin β-catenin complex is still present at the cell surface. Since pull down experiments showed simultaneous precipitation of β-catenin and actin together with α-catenin, it was believed for a long time and led to a textbook model that the cadherin core complex is directly connected to the actin cytoskeleton (fig 2, left). However, studies showed that α-catenin exists in a monomeric state that interacts with β-catenin, and a dimeric state that interacts with actin, and these distinct interactions were shown to be mutually exclusive. Thus, no stable connection between the actin cytoskeleton and the cadherin core complex could be demonstrated *in vitro*, suggesting that cadherin mediated actin regulation is much more dynamic than previously believed (Drees, Pokutta et al. 2005; Yamada, Pokutta et al. 2005) (fig. 2, right).

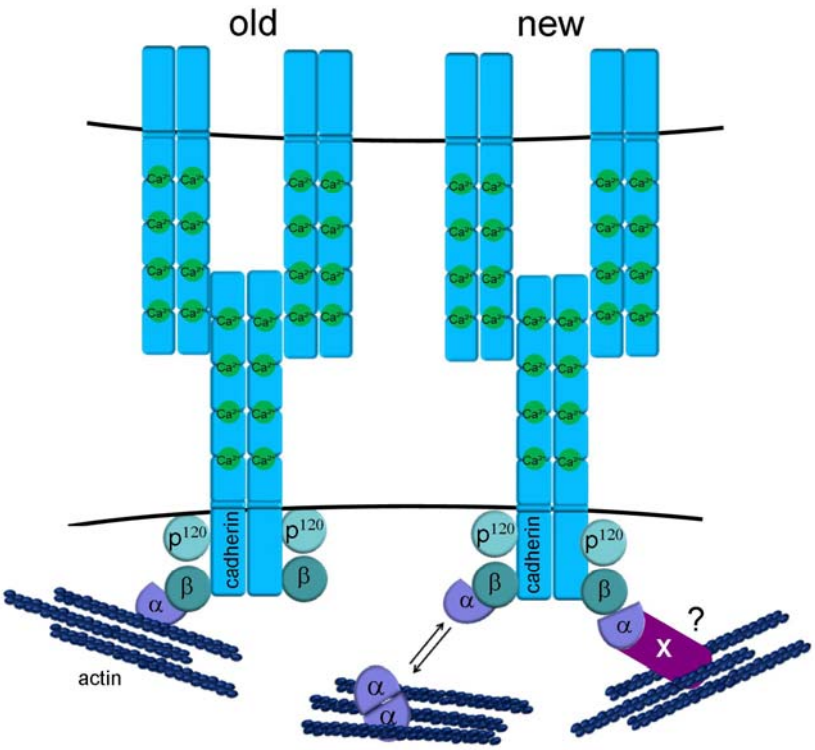


Figure 2: Schematic presentation of the cadherin core complex.

Textbook model versus newer model for how the cadherin complex interacts with actin. In the text book model (old) the cadherin complex was directly bound to actin via α-catenin. Recent data indicate that binding of α-catenin to either β-catenin or actin is mutually exclusive, resulting in a much more dynamic view on actin regulation by classical cadherins (new). In this model, actin binding either takes place in the vicinity of adherens junctions through dynamic exchange of α-catenin and binding is through other actin-binding proteins that link α-catenin to actin.

1.1.3 The nectin/afadin system

The nectin/afadin system represents another basic adhesive unit of the adherens junction. The nectin family belongs to the IgG-like super family of intercellular adhesion receptors and consists of 4 nectin variants (nectin 1-4) together with the closely related nectin like molecules (NECL1-5). Initially identified as receptors for α-herpes and polio viruses, they were later implicated in the regulation of intercellular adhesion. Nectins form lateral homo- or heterodimer and are capable to engage in either hetero or homophilic adhesion with other nectins, which is, in contrast to the cadherins, independent of Ca^{2+} (Takai, Miyoshi et al. 2008).

They consist of an extracellular domain comprising three IgG-like loops, and a transmembrane domain and a cytoplasmic domain with a c-terminal PDZ binding motif. The actin binding protein afadin interacts with its PDZ domain and this links the nectin directly to the actin cytoskeleton. Blocking of nectin functions interferes

with cadherin mediated adhesion *in vitro*, suggesting that nectins might represent a first scaffold in the formation of adherens junctions. On the other hand, adherens junction formation is Ca^{2+} dependent, showing the requirement for cadherin engagement and suggesting a more cooperative mechanism of both complexes in the formation of adherens junctions. This could be mediated by direct interaction of the complexes since afadin binds directly to α-catenin and p120 catenin. However, the exact mechanism of such a regulation is not known.

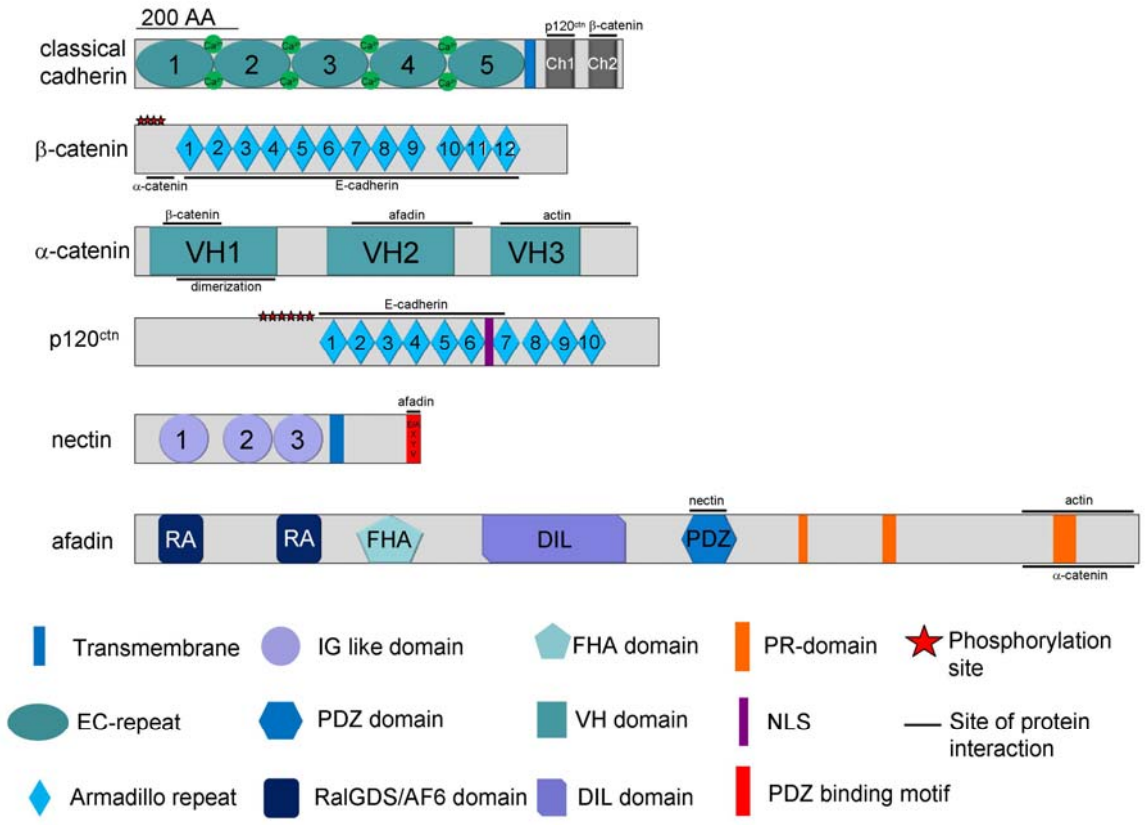


Figure 3: Overview of the domain structures and protein interaction binding sites of adherens junction components.

Domain structure of the adherens junction core components. Bars indicate sites of protein interaction. IG, immunoglobulin-like domain; Dil, dilute domain; EC, cadherin extracellular repeat; PDZ, PSD95/Dlg/ZO-1 domain; RA, Ras association domain; FHA, Forkhead associated domain; VH, vinculin homology domain and PR, proline-rich domain.

1.1.4 Regulation adherens junctions

Adherens junctions are tightly regulated during morphogenetic processes in development and tissue growth and maintenance. Especially classical cadherins

play a key role in the dynamic regulation of intercellular contacts during processes that involve cell rearrangements, like cell sorting, epithelial to mesenchymal transition (EMT) and migration (Gumbiner 2005).
Regulation of the adherens junctions occur on multiple levels ranging from gene expression via alterations of core complex components by post translational modification to regulation of cell surface expression by either trafficking or cleavage (fig. 4). A short overview of the various mechanisms is given in the following chapters.

1.1.4.1 Regulation of gene expression

Epithelial to mesenchymal transition (EMT) is a reversible developmental process in which cells lose their epithelial characteristics and gain mesenchymal properties. Hallmarks of EMT are loss of polarity and of adherens junctions concomitant with cytoskeletal rearrangements and increased cell motility. A key feature of EMT is the loss of E-cadherin. This process is also thought to reversibly occur during cancer progression when cells gain migratory and invasive properties. Signals that induce EMT upregulate transcriptional repressors of different families, for example Snail, which bind to the promoter of E-cadherin and several other key epithelial markers, thereby resulting in reversible silencing of these promoters (Peinado, Olmeda et al. 2007). Importantly, downregulation of E-cadherin is crucial to gain migratory/invasive properties. Epigenetic changes, such as promoter methylation, result in long-term silencing of gene expression. The best known adherens junction component for which this occurs is E-cadherin. The promoter of E-cadherin is silenced by CpG methylation in a range of cancers (Berx, Nollet et al. 1998).

1.1.4.2 Regulation of interactions in the core AJ complexes by phosphorylation

Adherens junction assembly/disassembly and maintenance is directly influenced by phosphorylation-dependent alterations in the interactions between the core complex components of the cadherin and nectin adhesion complexes. This may occur directly at adherens junctions since both kinases and phosphatases are associated with core components. In general, serine/threonine phosphorylation is considered to strengthen adherens junctions whereas tyrosine kinase activity is often associated with disassembly of junctions. However, several examples exist

where increased tyrosine kinase activity promotes adherens junctions (McLachlan and Yap 2007). Phosphorylation of cadherin core complex components alters the affinity of the different core complex components for each other. The cadherin binds β-catenin while passing through the endoplasmic reticulum. Serine/threonine phosphorylation of the cadherin cytoplasmic domain or the C-terminus of β-catenin further increases the affinity of this already strong interaction, thus strengthening adhesion and AJ formation/maintenance (Daugherty and Gottardi 2007). Tyrosine phosphorylation of β-catenin, however, lowers the affinity for either the cadherin or α-catenin, thus promoting disassembly of adherens junctions.

1.1.4.3 Regulation through alterations in cytoskeletal dynamics.

As the ultra structure reveals adherens junctions are closely connected to the actin cytoskeleton and regulate actin dynamics at sites of cell–cell contacts. Vice versa, actin rearrangements are crucial for the formation of adherens junctions. The Rho and Rap subfamilies of the Ras super family of small GTPases, key coordinators of cytoskeletal activity, have emerged as important regulators of dynamic cell–cell adhesion and adherens junction formation and maintenance (Braga and Yap 2005). Vice versa, cadherin or nectin engagement can control the activity of members of these small GTPase subfamilies, suggesting a close reciprocal relationship. Early AJ formation is driven by lamellipodia that are formed by localized Rac activity at sites of initial cell–cell contacts. This controls actin dynamics through actin filament nucleators such as the Arp2/3 complex. Cadherin engagement then directly activates Rac1 at sites of forming cell–cell contacts thereby establishing a positive reinforcing loop that enlarges the contact size between cells. Expansion of contacts requires Rho-regulated actomyosin contractions resulting in compaction of the intercellular contacts (Nelson 2008). Cadherin binding itself recruits different myosins and thereby further promote adherens junction stabilization. However, actomyosin contractions also drive local dissociation of adherens junctions.

1.1.4.4 The Regulation of cell surface transport and endocytosis of adherens junction components

Transport of components to and removal from the cell surface through the vesicle transport machinery are dynamic processes that cells use to regulate adherens junctions. After protein synthesis, cadherin/β-catenin complexes are delivered to different destinations at the cell surface. For example, in simple epithelia E-cadherin is sorted to the basolateral membrane, which is essential for the establishment and maintenance of basolateral polarity. Control of cadherin exocytosis occurs on three levels (Delva and Kowalczyk 2009). First, the cadherin cytodomain encodes sorting signals to direct the cadherin to the correct membrane compartment. The most consistent one is a dileucine motif in E-cadherin that targets E-cadherin to basolateral membranes. Second, the cadherin–catenin complex travels along microtubules that may target specific cell surface localizations. Third, specific protein assemblies clustered on the cell surface may specify vesicle fusion. The best example is the so-called exocyst complex. This complex regulates cell surface expression of Drosophila cadherin and is localized to the apical junctional complex.

Endocytosis of cadherins has also emerged as a crucial regulatory step in the maintenance and stability of adherens junctions. Cadherins have two main destinations upon endocytosis: the lysosomal degradation pathway and recycling to the cell surface. Although lysosomal degradation likely contributes to disassembly of adherens junctions, several lines of evidence indicate that dynamic cadherin recycling is crucial not only for dynamic cell rearrangements but also essential for maintaining adherens junction stability (Wirtz-Peitz and Zallen 2009). How the decision is made between sending cadherin to the lysosomal compartment versus recycling is at present not clear. Nevertheless, since p120ctn is crucial for cell surface stability of cadherins, mechanisms that lower the interaction of p120 with the cadherin are likely to promote endocytosis. Although it is at present unclear if endocytosis of nectins regulates adherens junctions, nectin activity appears important in the regulation of cadherin endocytosis.

1.1.4.5 Regulation by cleavage of adherens junction components

Both nectins and cadherins are subject to cleavage at defined sites by different proteases resulting in a permanent loss of full-length adhesive molecules from the cell surface. In a first step, the extracellular domains of cadherins and nectins are cleaved close to the membrane resulting in release of the extracellular domain, a process called ectodomain shedding (Reiss, Ludwig et al. 2006). Subsequently, the cytoplasmic domains can be cleaved by other proteases, such as caspases and presenilin. This cleavage also generates several novel cadherin and nectin fragments that may have biological functions by themselves. For example, the cadherin extracellular domain itself promotes cell motility in development and in cancer cells. Although at present unclear if the released cadherin cytoplasmic domain has a physiological relevant function, several reports have shown that it can inhibit the activity of certain transcription factors by retaining them in the cytoplasm. Other reports have shown that the cytoplasmic domain translocates to the nucleus where it binds deoxyribonucleic acid (DNA).

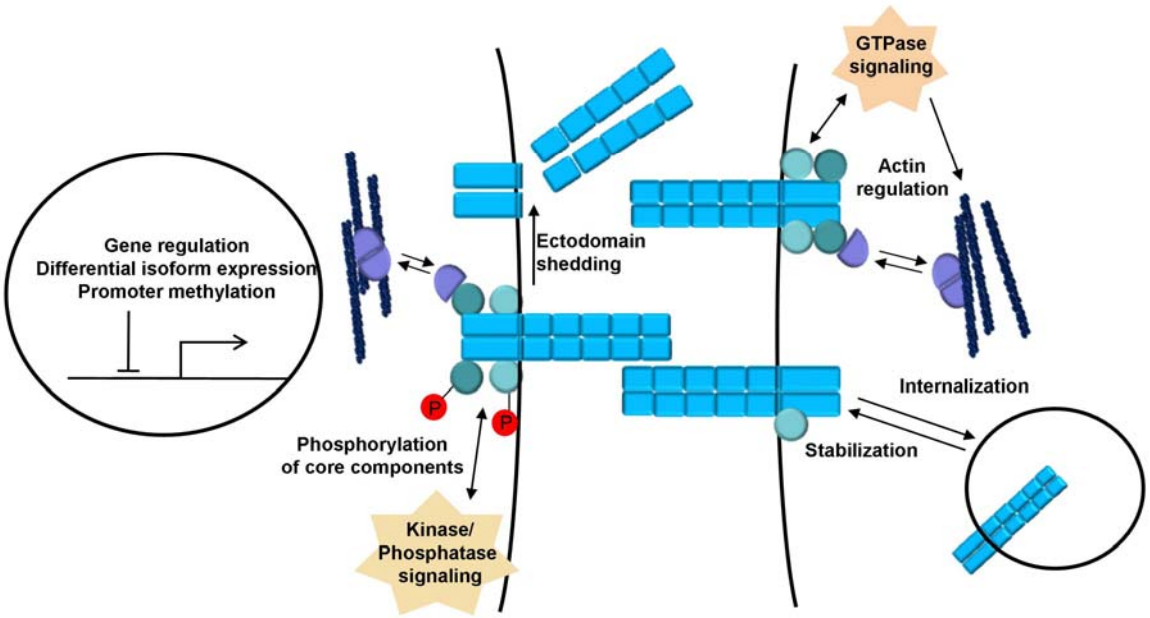


Figure 4: Cadherin regulation.

Different mechanisms regulate adherens junction formation and maintenance. Adherens junctions are regulated on multiple levels ranging from regulation of gene expression, trafficking to posttranslational modification and proteolytic cleavage.

1.2 Desmosomes

Desmosomes also form intercellular adhesive structures but unlike adherens junctions that link to the actin cytoskeleton, these junctions anchor the intermediate filament cytoskeleton to the plasmamembrane. In polarized simple epithelial cells desmosomes localize to the lateral membrane and appear ultrastructurally as electron dense plaque. On the cytoplasmic site an outer dens plaque (ODP) and an inner dense plaque (IDP) can be distinguished. Between the two plasma membranes of adjacent cells, the trans-interacting cadherin domains appear as an electron dense midline.

Like adherens junctions, they are formed by adhesion receptors belonging to the cadherin super family. Two subfamilies have been identified: desmogleins and desmocollins. Similar to classical cadherins, they have five extracellular cadherin repeats that mediate Ca^{2+} dependent adhesion between cells.

Desmogleins and Desmocollins cooperate to form an adhesive interface. The cytodomains of both family members bind to the armadillo family member plakoglobin and to plakophillins. Plakoglobin, also referred to as γ-catenin, preferentially associates with desmosomal cadherins but can also interact with classical cadherins, where it substitutes for β-catenin by occupying its binding site (Nathke, Hinck et al. 1994).

Desmoplakin, a member of the plakin protein family, binds to plakoglobin and bridges the desmosomal complex to the intermediate filament system. Plakophillins offer a more complex repertoire of interactions. Like plakoglobin, they bind to desmoplakin but can also interact with intermediate filaments directly. Lateral interactions among the armadillo proteins in the desmosome increase the stability and adhesive strength of the structure. Overall, desmosomes are tightly connected to the intermediate filaments, providing firm adhesion and physical integrity to tissues. This is highlighted by the findings that mutations in desmosomal components often result in tissue fragility, like blistering diseases in the skin (Green and Simpson 2007).

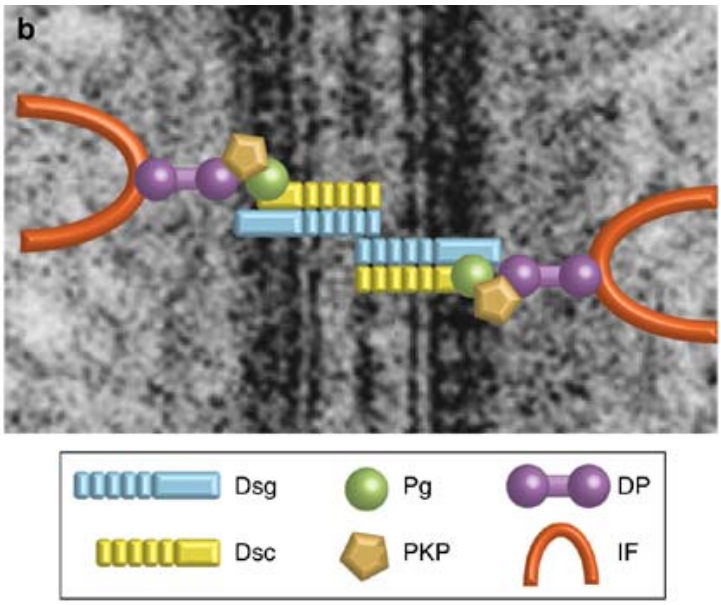


Figure 5: Schematic drawing of the molecular composition of desmosomes superimposed on a desmsosomal ultrastructure.

Dsg: Desmoglein, Dsc: Desmocollin, Pg:Plakoglobin, PKP; Plakophilin, DP: Desmoplakin, IF: intermediate filaments. Taken from (Green and Simpson 2007)

1.3 Tight Junctions

The third and most apically located intercellular junctional structure in the tripartite complex described by Farquhar and Palade are tight junctions. In thin section electron microscopy tight junctions appear as close intercellular contacts where the two opposing membranes come in such close proximity that the extracellular space almost seems obliterated. These contacts resemble the so called kissing points that have been described on the ultrastructural level for tight junctions in simple epithelia (Farquhar and Palade 1963). The formation of a network of ultrastructurally discernible interconnected strands which can be detected by freeze fracture replica electron microscopy are considered to form the basis of the seal of tight junctions (Staehelin, Mukherjee et al. 1969).

Relative to adherens junctions and desmosomes, tight junctions are most apical localized in simple epithelia (Farquhar and Palade 1963). Tight junctions form an ion and size-selective diffusion barrier and are the main regulators of paracellular permeability. In simple, polarized epithelia they are found at the border of the apical and baso-lateral membrane domains, which differ in their protein and lipid composition. In addition to restricting paracellular diffusion by having a “gate function”, tight junctions are also thought to restrict intermixing of constituents of

the two different membrane domains, which is referred to as its “fence function” (Anderson, Van Itallie et al. 2004). Like adherens junctions and desmosomes, tight junctions consist of protein complexes comprising transmembrane adhesion receptors and cytoplasmic scaffold that are linked to the cytoskeleton.

1.3.1 Transmembrane components of the tight junction

Three types of structural transmembrane components are enriched at the tight junctions and have the potential to mediate cell adhesion: the IgG like family of junctional adhesion molecules and the claudin and occludin families. Although not homologous in sequence, occludin and claudins share a topology with four transmembrane domains and two extracellular repeats (Schneeberger and Lynch 2004).

Claudins are critical for not only tight junction strand formation, but also for providing ion and size- selective permeability properties of the tight (Van Itallie, Rahner et al. 2001). The claudin family comprises at least 24 members in the human genome, where as 21 have been identified in mice. They all show tissue specific expression patterns. Claudins engage in either homophilic or heterophilic interaction across opposing membranes as well as laterally in the same cell. Thus, depending on the amount of claudin isoforms that are expressed and their relative expression levels, the claudin composition determines barrier properties of the tight junction and enables this protein family to account for the different barrier requirements of different tissues.

The function of occludin at the tight junction is less clear. Occludin was the transmembrane protein that was identified to specifically localize to the tight junction (Furuse, Hirase et al. 1993). Overexpression or mutations in occludin affected TER *in vitro* (Balda, Whitney et al. 1996). However, it appeared dispensable for the ultrastructural strand formation and for the establishment of a paracellular diffusion barrier *in vivo*. Nevertheless, occludin deficient mice revealed several phenotypes such as growth retardation, mineral deposits in the brain, male sterility and gastritis, which may implicates barrier regulation (Saitou, Furuse et al. 2000).

Tricellulin, an occludin related molecule, was shown to enrich at tricellular junctions in simple epithelia and to contribute to barrier function at these specialized sites

(Ikenouchi, Furuse et al. 2005). Both molecules share a MARVEL transmembrane domain which is thought to mediate their preferential accumulation in cholesterol rich membrane micro domains. Based on this structural feature, very recently another protein belonging to this family, MarvelD3, was discovered by bioinformatic approaches and was shown to specifically localize to the tight junctions and to have partially overlapping functions with occludin and tricellulin, thus defining the occludin family as tight junction associated MARVEL proteins (TAMP) (Raleigh, Marchiando et al.).

Junctional adhesion molecules (JAM) represent another type of adhesion receptors at the tight junction. They belong to the family of IgG like adhesion molecules and engage both in homophilic and heterophilic adhesion. Unlike claudins, they do not induce ultrastructural strand formation when expressed in fibroblasts and there expression is not confined to cell types that form tight junctions. They have been implicated in the regulation of migration and polarity (Ebnet, Suzuki et al. 2004).

1.3.2 Cytoplasmic scaffold proteins at the tight junction

An important group of tight junction scaffold molecules are the zonula occludens (ZO) proteins. These proteins belong to the membrane associated guanylate kinase-like homologs and are characterized by three N-terminal PDZ domains, an SH3 domain and a guanylate kinase domain (GUK). The first PDZ domains interact with claudins and the GUK domain with occludin. The C-terminus interacts with actin, thereby linking the tight junction transmembrane components to the actin cytoskeleton. The second PDZ domain was shown to mediate homo- and hetero dimerization of ZO proteins and the third PDZ domains interacts with Jam-1 (Ebnet, Schulz et al. 2000). ZO-1 and ZO-2, but not ZO-3, were shown to be crucial for the formation of claudin based strands and for the establishment of a tight junctional barrier, and this was dependent on the first PDZ domain. (Umeda, Ikenouchi et al. 2006). ZO-1 represents one direct link between tight junctions and adherens junctions since it can also interact with α-catenin (Rajasekaran, Hojo et al. 1996).

Several other PDZ domain containing proteins, such as MUPP1 and MAGI proteins are associated with the tight junction cytosolic plaque and can directly interact with one or more of the tight junctional transmembrane components (Schneeberger and

Lynch 2004). It is at present unclear if these molecules are directly involved in the formation of the tight junctions or serve a more regulatory function.
Cingulin, a non-PDZ tight junctional plaque protein, interacts with ZOs, JAMs and actin as well as myosin. As such, this protein may be a regulator of tight junctional dynamics during actomyosin contraction (Clayburgh, Barrett et al. 2005).

1.3.3 Signaling and polarity complexes at the tight junction

At the interface of two distinct membrane domains in simple epithelia, tight junctions serve as a spatial landmark which recruits protein complexes that are essential for the formation of apico-basal polarity. Ternary polarity complexes like the Par3/Par6/aPKC and the Cumbs/Pals/Patj complexes localize to the tight junction and are required for epithelial polarity. Many studies highlight the close interrelationship between intercellular junction formation and the formation of apico-basal polarity in simple epithelia. Adherens junctions and tight junctions might cooperate in the process of epithelial polarization, since JAM-1 and nectins have been reported to recruit Par3 to the apical junction complex which might represent an initial step in the setup of epithelial polarity (Ebnet, Suzuki et al. 2001; Takekuni, Ikeda et al. 2003). On the other hand, functional interference with the polarity complexes affects paracellular permeability, indicating a role of these complexes in the structural formation of the tight junction and pointing out a concerted action of junction formation and polarization in simple epithelia (Anderson, Van Itallie et al. 2004).

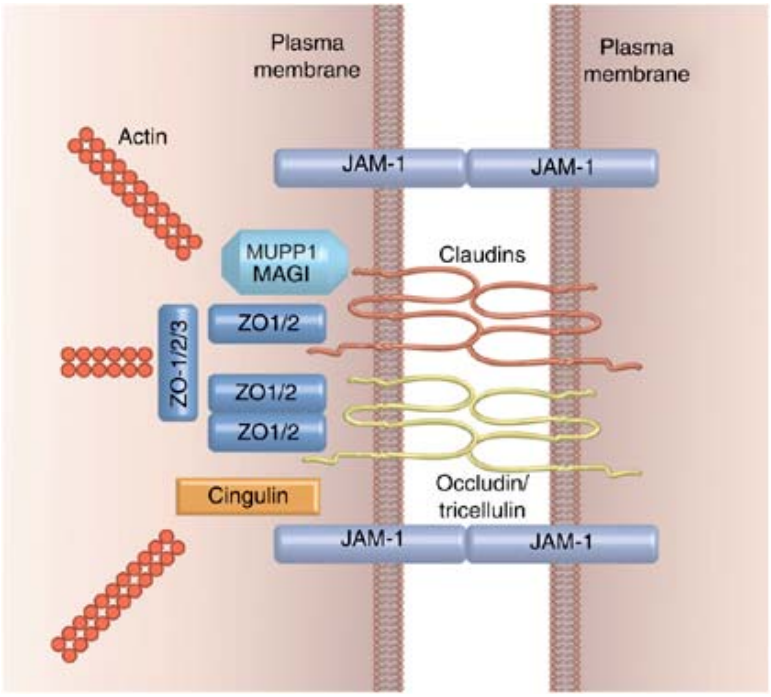


Figure 6: Schematic representation of the basic structural components of the tight junctions. ZO-1 or ZO-2 is important for clustering of claudins and occludin. The role of other scaffolding proteins (ZO-3/MAGI/MUPP1) is less clear. The ZOs and cingulin can provide a direct link to the actin cytoskeleton. Taken from (Niessen 2007).

1.4 Regulation of Rho family GTPases by intercellular junctions.

The Rho family belongs to the Ras superfamily of small GTPases. These GTPases switch between a GDP bound inactive state and a GTP bound active state. This transition is regulated by proteins that interact with the small GTPase which do either enhance its GTPase activity (GTPase activating protein, Gap), or do facilitate the exchange of GDP for GTP (Guanidine nucleotide exchange factor, Gef). In the GTP bound form the small GTPases specifically interacts with downstream effector molecules that then mediate various cellular responses by having multiple signaling outputs. In addition, small GTPases can be activated by several upstream signaling events. Thus, small GTPases serve as signal integrators that are controlled by tight spatio-temporal regulation.

RhoA family GTPases emerged as critical regulators of the actin cytoskeleton, thereby directly affecting cell shape and migration. Their most intensively studied members are RhoA, Rac and cdc42, which have been shown to organize distinct actin structures *in vitro*. Rac1 is an activator of Arp2/3 mediated actin assembly and induces lamellipodia formation, whereas RhoA is thought to act in actin

nucleation responsible for the formation of stress fibers. Cdc42 stimulates filopodia assembly (Etienne-Manneville and Hall 2002). Intense crosstalk between Rho family GTPases and intercellular junction formation has been reported in simple epithelia.

For instance, both cadherin and nectin engagement activate Rac1 and Cdc42, which in turn is required for proper organization of the cortical actin cytoskeleton. RhoA can either be activated or inhibited upon cadherin engagement depending on the cell type that was used in the study. In addition, Rac inhibits E-cadherin endocytosis, thereby affecting junction stability and remodeling. Cadherin mediated regulation of Rho GTPases was shown to be mainly mediated by p120ctn, although alternative pathways implicating PI3K have been discussed (Gavard, Lambert et al. 2004; Anastasiadis 2007).

Similarly, the formation of macromolecular complexes at the tight junction by ZO-1 and Jam localization affect Rho and cdc42 activation (Miyoshi and Takai 2008). In addition, properly compartmentalized regulation of Rho family GTPases is important for tight junction homeostasis since overexpression of either dominant active or negative variants of each Rho family GTPase disrupts tight junctions *in vitro* (Jou, Schneeberger et al. 1998). Both Rac and Cdc42 directly interact with the Par complex and thereby regulate epithelial polarity and tight junction function (Suzuki and Ohno 2006).

Conflicting results regarding activation or inhibition of specific GTPases come from studies using different cell types, thus, GTPase regulation might be highly cell context dependent (Braga and Yap 2005).

1.5 Intercellular junctions in the epidermis

The epidermis of the skin is a multilayered epithelium that serves as the outermost barrier to the environment. Its function is to establish protective barriers against invading pathogens and chemical or physical assaults as well as to prevent dehydration by trans epidermal water loss. The epidermis is a continuously self renewing tissue that is composed of mitotically active keratinocytes in the innermost basal layer that enter a program of terminal differentiation in order to form more differentiated suprabasal layers. Upon detachment from the basement membrane the cells withdraw from the cell cycle and enter the spinous layer,

thereby assembling a durable cytoskeletal framework that provides mechanical integrity. By entering the granular layer, the cells flatten and are characterized by accumulation of keratin macrofibrils and lipid containing lamellar bodies. Finally, in the outermost stratum corneum, structural proteins of the so called corneocytes are irreversibly crosslinked by transglutaminases. Lipids that extrude from the lamellar bodies seal the extracellular space around the corneocytes, thereby creating an insoluble meshwork that results in the formation of an epidermal barrier (Segre 2003).

Throughout the process of epidermal differentiation, intercellular junctions are maintained and actively remodelled to provide physical integrity to the tissue. In the following, the different types of intercellular adhesive structures in the epidermis and the localization of their structural components and functions in the epidermis will be discussed.

1.5.1 Adherens junctions in the epidermis

Two classical cadherins, E- and P-cadherin are expressed in the epidermis. While E-cadherin is expressed in all layers of the epidermis, P-cadherin expression is confined to the basal layer. P120ctn, β-catenin and α-catenin are associated with E-cadherin in all viable layers of the epidermis. Thus, functional adherens junctions are maintained during epidermal homeostasis in all viable layers. Conditional ablation of adherens junction components did not only affect mechanical stability of the epidermis and their appendages, but also signaling mechanisms that affect differentiation, proliferation and inflammation.

Loss of E-cadherin results in hair loss due to impaired adhesion, but also altered epidermal differentiation (Young, Boussadia et al. 2003; Tinkle, Lechler et al. 2004; Anastasiadis 2007). Loss of P-cadherin did not cause any obvious skin phenotype (Radice, Ferreira-Cornwell et al. 1997). However, mutations in human P-cadherin are associated with hair disorder and with ectodermal displasia (Sprecher, Bergman et al. 2001; Kjaer, Hansen et al. 2005).

Epidermal deletion of the cadherin associated catenins revealed overlapping and specific functions in mice. Knock out of α-catenin resulted in loss of adherens junctions, reduced desmosome formation, hyperproliferation and altered growth factor signaling (Vasioukhin, Bauer et al. 2001). Loss of p120ctn reduced adherens

junction but also activated an inflammatory responses, which was independent of its cadherin complex function but linked to increased NF-kB signalling in keratinocytes (Perez-Moreno, Davis et al. 2006). (Perez-Moreno, Davis et al. 2006). Ablation of β-catenin in the epidermis highlighted its importance in the Wnt signalling pathway during hair morphogenesis and stem cell regulation. No differences in intercellular adhesion were observed which is likely explained by the replacement of β-catenin by plakoglobin in the cadherin complex (Huelsken, Vogel et al. 2001). Taken together, the results indicate that adherens junction components may couple structural integrity to intracellular signalling events.

1.5.2 Desmosomes in the epidermis

Structural desmosomes can be found in all layers of the epidermis, thereby providing firm adhesion to the tissue. However, appearance and size of the desmosomes vary between the different layers. Smaller, less organized desmosomes in cells of the basal layer are replaced by larger, more electron dense desmosomes in the suprabasal layers. Also the composition of desmosomal constituents varies between the layers. Desmoglein 1 and Desmocollin 1 expression increases in the more differentiated suprabasal layers and show only little expression in the basal layer. Desmocollin 2, 3 and Desmoglein 3 show opposite expression patterns with high expression in the basal layer and decreasing expression in the suprabasal layers (Green and Simpson 2007). Desmoglein 4 is concentrated in the granular and cornified layer whereas desmoglein 2 is only observed in the basal layer. The importance of epidermal desmosomes in the maintenance of epidermal tissue stability is highlighted by findings that ablation or mutation in desmosomal components is often associated with blistering diseases (Green and Simpson 2007).

1.5.3 Tight junctions in the epidermis

Ultrastructurally discernable tight junctions can only be found in the granular layer of the epidermis and tracer penetration assays confirmed this layer as a site of a functional permeability barrier (Proksch, Brandner et al. 2008). Tight junction molecular key components like claudin-1 and ZO-1 are expressed in all viable

layers of the epidermis, whereas claudin-4 and Occludin show specific localization to the membrane of the granular layer (Brandner 2009).

For a long time it was assumed that the lipid envelop of the stratum corneum was sufficient to provide a structural barrier to the epidermis. However, conditional deletion of claudin-1 in the epidermis of mice resulted in death due to trans epidermal water loss. This revealed for the first time a requirement for functional tight junctions in the epidermis to maintain inside-out barrier function (Furuse, Hata et al. 2002).

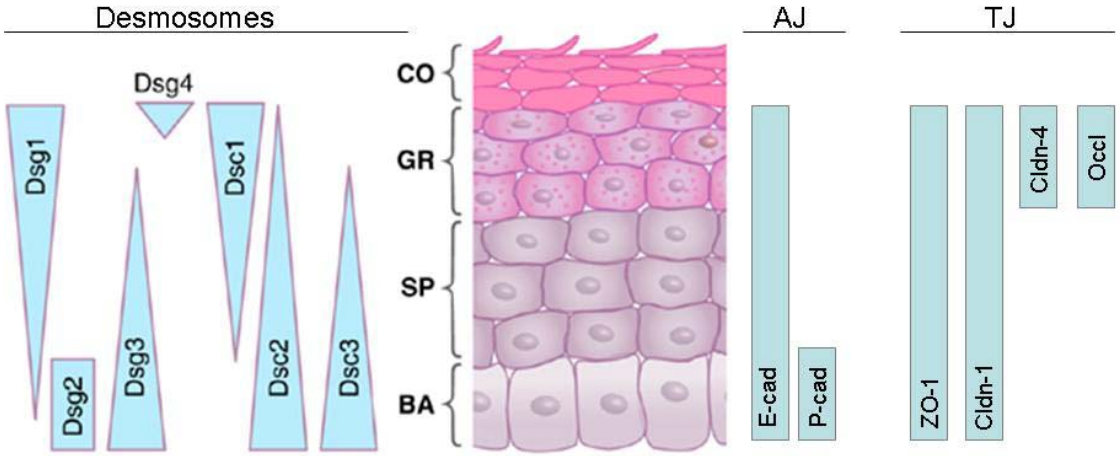


Figure 7: Expression of structural components of desmosomes, adherens junctions (AJ) and tight junctions (TJ) in the different layers of the epidermis,

Ultrastructural adherens junctions and desmosomes are found in all viable layers of the epidermis, whereas ultrastructural tight junctions are only found in the stratum granulosum.

BA: stratum basale; SP: stratum spinosum; GR: stratum granulosum; CO: stratum corneum

Dsg: Desmosglein; Dsc: Desmocollin; cad: cadherin; ZO-1: zonula occludens 1; Cldn (claudin), Occl: occludin. Taken from (Green and Simpson 2007)), modified.

1.6 Cadherin mediated regulation of intercellular junctions and barrier formation

Classical cadherins have been implicated not only in the formation and regulation of adherens junctions, but also in the formation of other types of intercellular junctions, such as desmosomes and tight junctions. E-cadherin was thought to be specifically required for the formation of intercellular junctions in simple epithelia (Gumbiner, Stevenson et al. 1988), whereas cooperative roles for E- and P-cadherin were suggested in stratifying epithelia (Lewis, Jensen et al. 1994). *In vivo* epidermal deletion of α-catenin confirmed the requirement for adherens junctions

in epidermal desmosome formation, since these structures were largely reduced in numbers (Vasioukhin, Bauer et al. 2001). Whereas P-cadherin appeared to be dispensable for epidermal junction formation *in vivo* (Radice, Ferreira-Cornwell et al. 1997), only tight junctions were affected upon epidermal deletion of E-cadherin (Vasioukhin, Bauer et al. 2001; Tunggal, Helfrich et al. 2005).

Epidermal deletion of E-cadherin in the epidermis resulted in perinatal death due to trans epidermal waterloss, which closely resembled the phenotype of the claudin-1 deficient epidermis (Tunggal, Helfrich et al. 2005). Interestingly, the structural formation of tight junction was not completely disturbed. Occludin, claudin-4 and ZO-1 still localized to the cell surface as judged by immunofluorescence, albeit with changed staining patterns for the latter two molecules. More importantly, tight junction like structures could still be detected in electron microscopy, suggesting that E-cadherin is not required for their initial structural formation in the first place, but may regulates maturation at later stages. Intercellular cohesion was unaffected in E-cadherin deficient epidermis, since no blistering or fragility could be detected. This was explained by upregulation of P-cadherin in the basal layer and the formation of ultrastructurally normal desmosomes which might have been reinforced by upregulation of desmosomal components. Taken together, E-cadherin appeared dispensable for epidermal cohesion and junction formation and turned out to be a critical regulator of epidermal tight junctions by regulating the specific incorporation of claudins in an adhesion independent manner. Indeed, the mislocalization of the tight junction regulators Rac and phosphorylated aPKC in E-cadherin deficient epidermis suggested that E-cadherin regulates epidermal tight junctions by proper recruitment or activation of tight junction regulatory molecules that then might mediate claudin incorporation and thereby barrier formation (Tunggal, Helfrich et al. 2005). The results raise the question whether classical cadherins are indeed required for epidermal desmosome and tight junction formation, and whether there are specific or overlapping functions of E- and P-cadherin. Furthermore, since conditional ablation techniques were used, it remains unclear whether classical cadherins regulate *de novo* junction formation in keratinocytes, a question which is difficult to address *in vivo.*

1.7 Regulation of growth and migration by classical cadherins

Especially E-cadherin was implicated in the regulation of various cellular processes like proliferation and migration. In simple epithelia, cadherin engagement and subsequent formation of intercellular contacts restricts proliferation, a phenomenon called contact inhibition of proliferation (Perrais, Chen et al. 2007) (Liu, Jia et al.; St Croix, Sheehan et al. 1998).

Control of cell motility is crucial for tissue integrity and morphogenesis. Epithelial cells undergoing epithelial to mesenchymal transition during organogenesis disassemble intercellular contacts and are characterized by a marked down regulation of E-cadherin, mediated by the repression of gene expression via the transcription factor snail (Cano, Perez-Moreno et al. 2000; Peinado, Olmeda et al. 2007). Similarly, E-cadherin is mutated in a variety of human cancers, and down regulation occurs during invasive tumor progression, again accompanied by acquisition of a mesenchymal phenotype and increased motility (Batlle, Sancho et al. 2000). Thus, E-cadherin is considered to act as a tumor suppressor and as a determinant of epithelial cell shape. Combined epithelial deletion of E-cadherin and p53 resulted in accelerated development of invasive mammary tumors (Derksen, Liu et al. 2006). However it remains unclear whether loss of E-cadherin is primarily causing enhanced migration or whether this occurs in cooperation with other oncogenic mutations.

1.8 Aims of this thesis

In vitro studies in simple epithelial cells indicated an essential role for E-cadherin not only in the formation of AJs but also other intercellular contacts, such as desmosomes and tight junctions. In contrast, *in vivo* tissue specific knockout studies did not reveal a necessity of E-cadherin in the formation of intercellular junctions, raising the question if classical cadherins are necessary or if other classical cadherins can compensate for the loss of E-cadherin in the formation of other junctions. Previous work in the laboratory had revealed that *in vivo* loss of E-cadherin in a stratifying epithelium, the epidermis, resulted in loss of tight junctional function but not desmosomes. Since *in vivo* P-cadherin expression but not localization was upregulated, this may potentially compensate for E-cadherin in desmosome assembly. Alternatively, these results may suggest that cadherins are

dispensable for desmosome formation. In addition, in cancer cells loss of E-cadherin alters the growth and invasive properties of cells but *in vivo* studies suggested that loss of E-cadherin in non-transformed tissues do not affect these functions.

The overall aim of this thesis was to address the roles of classical cadherins in intercellular junction and barrier formation, and migration. Isolated keratinocytes from control and epidermal specific E-cadherin knockout mice provided a unique model system to study the consequences of cadherin loss in primary epithelial cells. Switching keratinocytes from low Ca^{2+} conditions to high Ca^{2+} conditions activates cadherin-dependent intercellular adhesion and enables *de novo* intercellular junction formation. This allows one to follow the kinetics of junction formation and function. In addition, one can compare growth and migration properties under cadherin adhesion non-permissive or permissive conditions.

Specifically, the following questions were asked:

1. Is there a requirement of classical cadherins in keratinocyte *de novo* desmosome formation and are there specific or overlapping functions for E- or P-cadherin?
2. Is the regulation of tight junctions a specific function of E-cadherin?
3. How does E-cadherin regulate tight junctions and thus epidermal barrier function?
4. How does loss of E-cadherin affect growth and migration properties of primary epithelial cells?

2. Results

In order to address these questions, primary mouse keratinocytes are utilized as a model system since *de novo* junction formation is difficult to assess *in vivo* but can be controlled *in vitro.* The so called Ca^{2+} switch protocol allows for precise induction of intercellular contact formation and enables to follow their kinetics.
A combined approach of conditional gene deletion and shRNA mediated silencing is used to address the specific roles of E- and P-cadherin, respectively. Furthermore, gene delivery by lentiviral transduction of keratinocytes allows testing for the significance of potential candidate molecules or protein domains that are involved in the regulation of epidermal desmosome and barrier formation.

2.1 Classical cadherins are required for intercellular junction formation in primary mouse keratinocytes

2.1.1 Isolation of E-cadherin negative primary mouse keratinocytes

Primary mouse keratinocytes were isolated as described in materials and methods. Keratinocytes derived from mice of the genotype K14Cre-$Ecad^{fl/+}$, K14Cre-$Ecad^{-/+}$ or $Ecad^{fl/fl}$ and $Ecad^{fl/-}$ were used as control (Ctr), whereas keratinocytes isolated from K14Cre-E-$cad^{fl/fl}$ and K14Cre-$Ecad^{fl/-}$ were termed E-cadherin negative ($Ecad^{-/-}$). Western blot analysis confirmed that E-cadherin protein expression is indeed absent in $Ecad^{-/-}$ keratinocytes (fig. 8). In phase contrast microscopy, no morphological difference was observed between $Ecad^{-/-}$ and control keratinocytes when cells were cultured in low Ca^{2+} concentration (50 μM) (fig. 2-1). This is not unexpected since under these conditions cadherins cannot engage in intercellular adhesion.

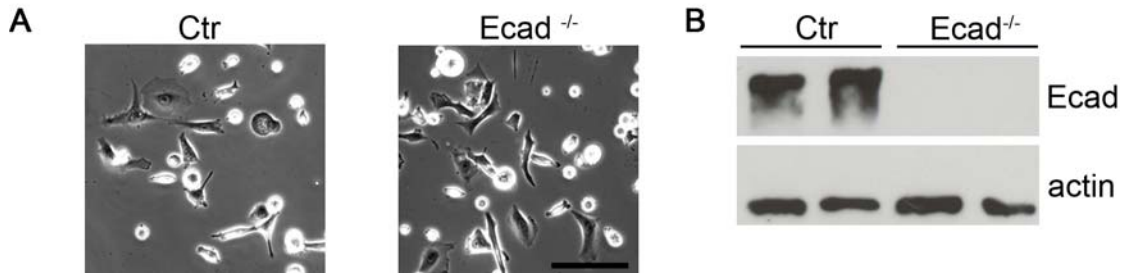


Figure 8: Keratinocytes isolated from K14Cre-Ecad fl/fl mice are deficient for E-cadherin.
(A) Phase contrast images of Control and Ecad$^{-/-}$ keratinocytes cultured under low Ca^{2+} condition (50 µM). Bar = 25 µm. (B) Western blot analysis of total cell lysates using anti-E-cadherin antibody. Actin was used as loading control.

2.1.2 Adherens junctions form in the absence of E-cadherin

To address the role of E-cadherin in intercellular epidermal junction formation, kinetics of adherens junction formation was analyzed in primary Ecad$^{-/-}$ keratinocytes. Control and Ecad$^{-/-}$ keratinocytes were subjected to a Ca^{2+}-switch by replacing the medium containing 50 µM (low Ca^{2+}) with medium containing 1.8 mM Ca^{2+} (high Ca^{2+} medium). The Ca^{2+}-switch not only induces differentiation of keratinocytes but also allows the formation of intercellular junctions. Using imunofluoresence microscopy this allows examining the subcellular localization of adherens junction and desmosomal components and examining their recruitment to sites of cell-cell contact.

In both control and Ecad$^{-/-}$ keratinocytes, membrane staining was observed for β-catenin 2 hours after Ca^{2+}-switch and membrane staining was even more intense after 48 hours in high Ca^{2+}, suggesting that initiation of adherens junction formation takes place with similar kinetics in the absence of E-cadherin (fig. 9A). Since all classical cadherins can interact with the catenins it was examined if P-cadherin was responsible for the observed recruitment of β-catenin. Even though recruitment of P-cadherin to sites of intercellular contacts was observed 2 hours after Ca^{2+}-switch in both control and Ecad$^{-/-}$ keratinocytes, staining was more intense in the absence of E-cadherin. Both control and Ecad$^{-/-}$ keratinocytes displayed similar recruitment of P-cadherin 48h after Ca^{2+}-switch.

Epidermal deletion of E-cadherin resulted in upregulation of P-cadherin in the basal layer of the epidermis. To test whether P-cadherin expression was altered in Ecad$^{-/-}$ keratinocytes, protein levels of P-cadherin as well as its associated catenins were

assessed by western blot analysis. Indeed, similar to *in vivo* situation, P-cadherin was up regulated in undifferentiated keratinocytes and 2h after Ca^{2+} switch in Ecad$^{-/-}$ when compared to controls, may reflecting an attempt to compensate for the loss of E-cadherin (fig.9B). Similar expression levels for control and Ecad$^{-/-}$ keratinocytes were observed 48h after Ca^{2+} switch, showing that P-cadherin is expressed in differentiated keratinocytes *in vitro.* In both control and Ecad$^{-/-}$ keratinocytes P-cadherin expression increased with longer Ca^{2+} induced differentiation periods, in contrast to the *in vivo* situation where expression of P-cadherin is confined to basal, undifferentiated keratinocytes (Tunggal, Helfrich et al. 2005).

α-catenin and β-catenin are known to be stabilized when associated with classical cadherins in the adherens junction complex, thus reflecting indirectly classical cadherin protein levels. Western blot analysis of control and Ecad$^{-/-}$ keratinocytes revealed a down regulation of these two proteins at all observed time points of differentiation, suggesting that despite the upregulation of P-cadherin overall classical cadherin levels are reduced in the absence of E-cadherin. Expression of p120ctn, which expression levels have been shown to be independent of classical cadherin levels, was unchanged (fig. 9B).

These results show that adherens junctions are assembled in the absence of E-cadherin, most likely because P-cadherin was upregulated.

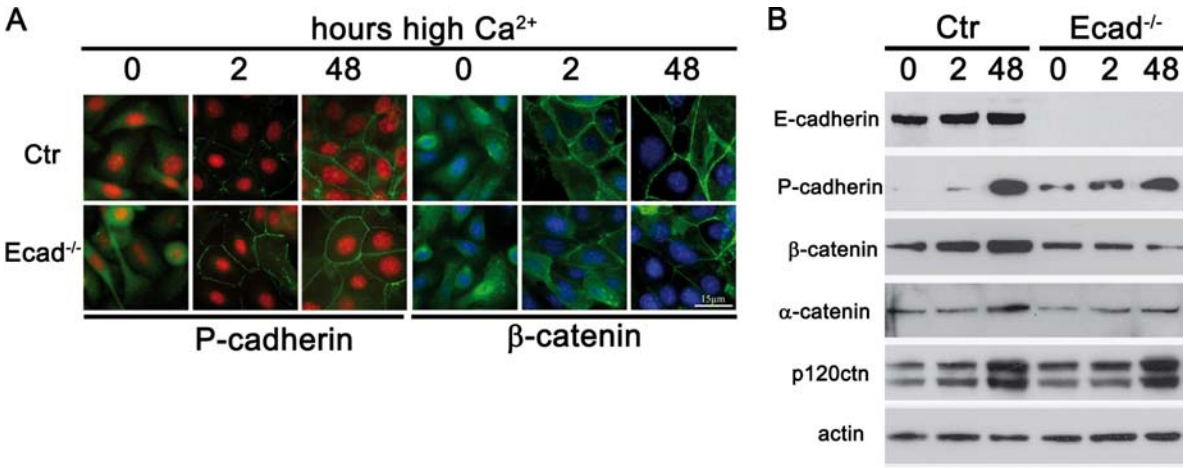


Figure 9: Adherens junction formation upon loss of E-cadherin.

(A) Immunofluorescence analysis of adherens junction components P-cadherin and β-catenin Bar=15 µm. (B) Western blot analysis of total cell lysates for adherens junction proteins. Cells were differentiated in high Ca^{2+} for the indicated time points.

2.1.3 Delay in desmosome formation in the absence of E-cadherin

In vivo loss of epidermal cadherins did not show an obvious change in number and appearance of desmosomes in newborn skin. However, it is not possible to follow the kinetics of desmosome formation *in vivo*. To examine if membrane recruitment of desmosomal components, used to assess desmosome formation, is altered by the loss of E-cadherin the localization and expression of desmoglein 3 and plakoglobin were analyzed (fig. 10A). Interestingly, whereas control keratinocytes were able to recruit these desmosomal components to the cell surface after 2 hours of Ca^{2+} stimulation, no membrane localization was observed in E-cadherin deficient keratinocytes at this time point, suggesting a delay in desmosome formation. However, membrane localization of these components was indistinguishable between E-cad$^{-/-}$ and control keratinocytes after 48 hours, indicating that desmosomes can form in E-cadherin deficient keratinocytes, albeit with a delay in kinetics. Indeed, thin section electron microscopy analysis revealed the presence of desmosomes in the absence of E-cadherin, which were ultrastructurally indistinguishable from control cells when differentiated for 48 hours, showing that E-cadherin is dispensable for desmosome formation *in vivo* (Tunggal et al., 2005) and *in vitro* in primary keratinocytes (Michels, Buchta et al. 2009)(fig. 11C).

To asses whether the observed delay in desmosomal protein recruitment was caused by alterations in desmosomal component expression, western blot analysis was performed (fig. 10B). No obvious difference was found in plakoglobin expression 2 hours after Ca^{2+} switch, suggesting that its absence form intercellular junctions at this timepoint was not caused by differences in the expression of this protein. In both control and Ecad$^{-/-}$ keratinocytes, expression of desmoglein 1 and 2 was only detectable 48 hours after Ca^{2+} switch, indicating that other desmosomal cadherins mediate desmosome formation at initial phases of junction formation.

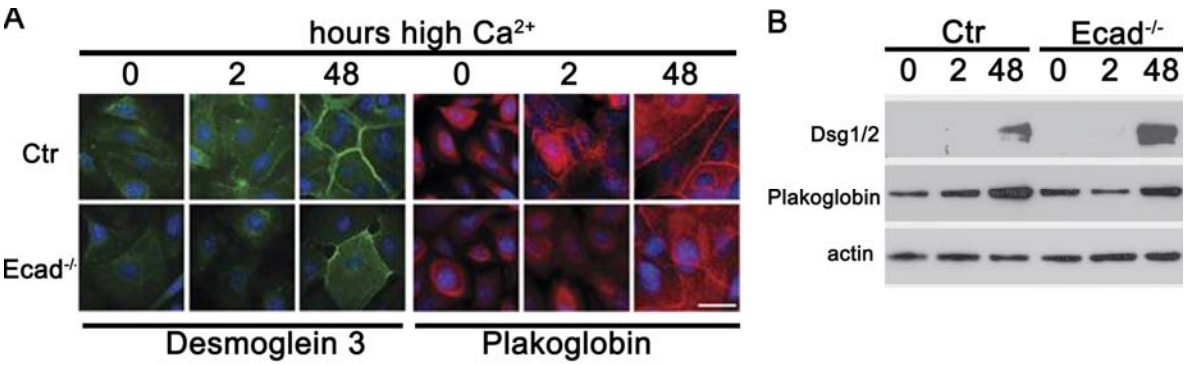


Figure 10: Desmsome formation upon loss of E-cadherin.

(A) Immuno fluorescence analysis of the desmosomal components desmoglein 3 and plakoglobin. Bar=15 µm. (B) Western blot analysis of total cell lysates for desmosomal proteins desmosglein 1/2 and plakoglobin. Cells were differentiated in high Ca^{2+} for the indicated time points.

2.1.4 Impaired adherens junction and desmosome formation in the absence of both E- and P-cadherin

Since desmosomes formed in the absence of E-cadherin, the question arises whether there is a requirement for classical cadherins in desmosome formation, or whether there is a specific role of P-cadherin in the initiation of desmosome formation.

To address the role of P-cadherin in adherens junction and desmosome formation, its expression was silenced in either control keratinocytes ($Pcad^{kd}$) to asses its specific function, or in E-cadherin deficient keratinocytes ($Ecad^{-/-}/Pcad^{kd}$) to asses classical cadherin requirements. This was achieved by using lentiviral delivery of small hairpin RNA (shRNA) which was directed against the 5´ untranslated region of the P-cadherin mRNA.

Two independent clones of $Ecad^{-/-}/Pcad^{kd}$ keratinocytes were derived which differed in their efficiency to silence P-cadherin. Those clones are referred to as $Ecad^{-/-}/Pcad^{kd\text{-}h}$, and $Ecad^{-/-}/Pcad^{kd\text{-}l}$ for the higher and lesser efficient knock downs, respectively. Efficiency of P-cadherin silencing was verified by western blot analysis and revealed more than 95% reduction of expression for $Ecad^{-/-}Pcad^{kd\text{-}h}$ and about 80% for $Ecad^{-/-}Pcad^{kd\text{-}l}$ and $Pcad^{kd}$ clones (fig. 11B).

Immunofluorescence localization of β-catenin and desmoplakin was chosen to examine the formation of adherens junctions and desmosomes, respectively. $Pcad^{kd}$ keratinocytes displayed junctional recruitment of these markers 48 hours after Ca^{2+} switch, suggesting that adherens junction and desmosomes can form

upon reduction of P-cadherin protein levels. In E-cad$^{-/-}$Pcad^{kd-h}, β-catenin and desmoplakin were lost from the membrane, indicating impairment of adherens junctions and desmosomes (fig. 11A).

In addition, in phase contrast microscopy the cells appeared to be rounded up and did not align their membranes, suggesting that intercellular contact formation is largely impaired. Interestingly, E-cad$^{-/-}$Pcad^{kd-l} keratinocytes did show some recruitment of β-catenin and desmoplakin. However the staining appeared to be less intense and more punctuate in appearance when compared to controls, suggesting impaired or delayed junctional maturation (fig. 11A).

To further characterize the presence or absence of desmosomes on the ultra structural level, thin section electron microscopy on differentiated keratinocytes was performed in collaboration with Willhelm Bloch (Deutsche Sport Hochschule, Köln). In both control and Ecad$^{-/-}$ keratinocytes ultra structurally normal desmosomes were found, whereas no desmosome like structures could be observed in E-cad$^{-/-}$Pcad^{kd-h} keratinocytes (fig. 11C). Instead, very few intercellular structures were found which lacked the typical desmosomal plaque structure. These structures showed closely aligned membranes that in morphology rather resembled tight junctions than desmosome like features.

To test whether the lack of desmosomes was caused by downregulation of desmosomal components, western blot analysis of desmoglein 3 and plakoglobin was performed. No gross differences in the expression of these proteins were found in keratinocytes that were differentiated for 48 hours.

The data shows that classical cadherins are required for keratinocyte adherens junction and desmosome formation (Michels, Buchta et al. 2009).

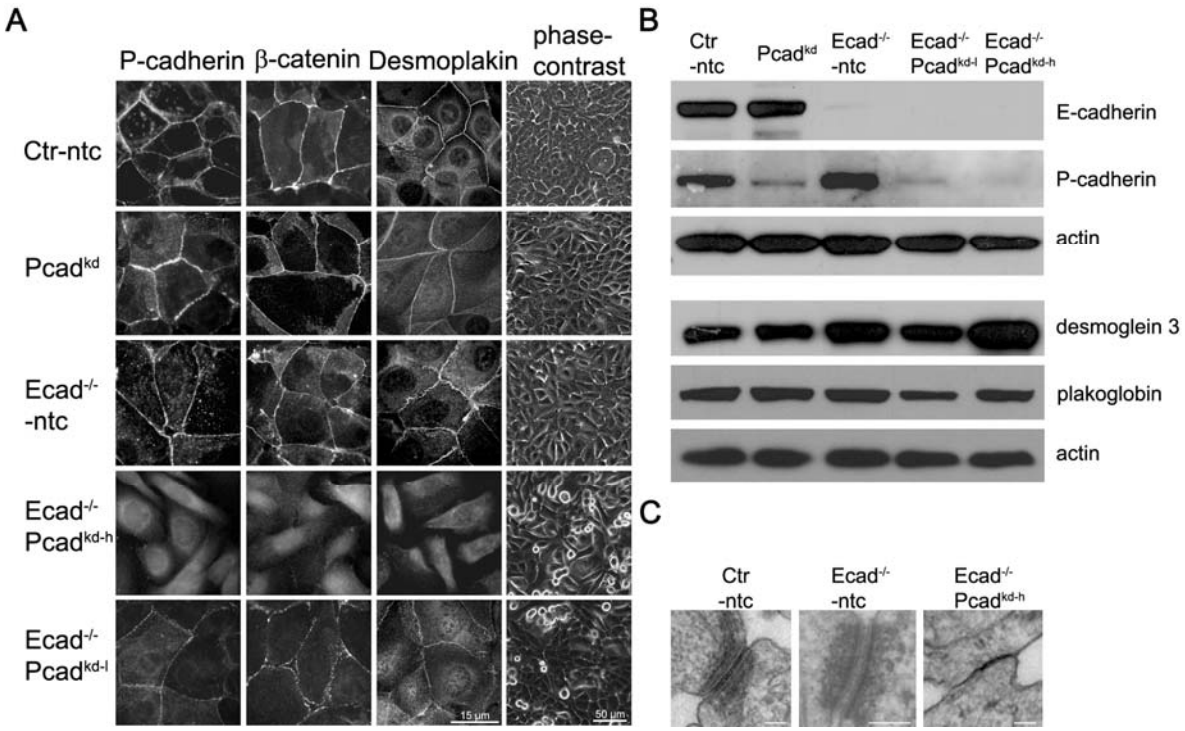


Figure 11: Impaired adherens junction and desmosome formation in the absence of classical cadherins.

(A) Immunofluorescence and phase contrast analysis of keratinocytes either deficient for E-cadherin, P-cadherin or both. Junction formation was induced 48h prior to fixation. Bars: Immunofluorescence: 15 µm, phase contrast= 50 µM. (B) Western blot analysis for the indicated proteins on keratinocytes that were differentiated for 48h. (C) Ultrastructural analysis of intercellular contacts using thin section electron microscopy. Keratinocytes were differentiated for 48h. Bar=100nm. ntc: non targeting shRNA control.

2.1.5 Desmosome formation in primary mouse keratinocytes depends on classical cadherin levels

Loss of either E-cadherin or P-cadherin is insufficient to interfere with desmosome formation. This raises the question if E- and P-cadherin have overlapping functions and thus levels of classical cadherins are crucial for desmosome formation or if desmosome assembly requires a specific function for both E- and P-cadherin. To assess this question, either E- or P-cadherin were lentivirally re expressed in the $Ecad^{-/-}Pcad^{kd-h}$ cells and asked if this could rescue the cell surface recruitment of desmosomal proteins like desmoplakin. After transduction of $Ecad^{-/-}Pcad^{kd-h}$ keratinocytes with either E-cadherin, P-cadherin or GFP, transgene expression was analyzed by western blot analysis and intercellular junction formation was assessed by Immunofluorescence microscopy. Both cadherins were expressed

and showed a specific signal at the cell surface after induction of intercellular junction formation by Ca^{2+}-switch (fig. 12A). In addition, β-catenin was stabilized at the protein level and recruited to the membrane in both E- and P-cadherin re expressing cells, showing the successful restoration of adherens junctions (fig. 12B). Furthermore, the desmosomal components desmoplakin and plakophilin 3 were recruited to sites of cell-cell contact, showing that desmosome formation was restored by the re- expression of either E- or P-cadherin, confirming the notion that both E- and P-cadherin redundantly initiate adherens junction and desmosome formation in a protein level dependant manner.

The question arose how classical cadherins regulate the formation of epidermal desmosomes. One possible mechanism could involve a requirement for catenin recruitment to sites of intercellular contacts. To test this hypothesis, a chimeric protein in which the E-cadherin extracellular repeats were replaced by the interleukin 2 receptor ectodomain (IL2R-tail) was expressed in $Ecad^{-/-}Pcad^{kd-h}$ keratinocytes. This chimeric protein has no adhesive activity and was shown to be able to recruit the cadherin cytoplasmic binding partners to the plasma membrane and reverse growth and migration/invasion properties induced by the loss of E-cadherin (Gottardi, Wong et al. 2001; Wong and Gumbiner 2003). Upon expression of Il2R-tail in $Ecad^{-/-}Pcad^{kd-h}$ keratinocytes, β-catenin expression levels were stabilized to the same levels as in the cells that re express E- or P-cadherin and, more importantly, recruited to the cell surface (fig.12). However, no restoration of desmosomes was observed, indicating that β-catenin recruitment is not sufficient to induce desmosome formation. Moreover, these results suggest a requirement for adhesive engagement of classical cadherins in the formation of desmosomes. Unfortunately, a construct encoding a chimeric protein in which the β-catenin binding domain was replaced by α-catenin, previously shown to rescue cadherin adhesive activity (Gottardi, Wong et al. 2001), was not properly expressed despite multiple attempts. This made it impossible to directly test this hypothesis.

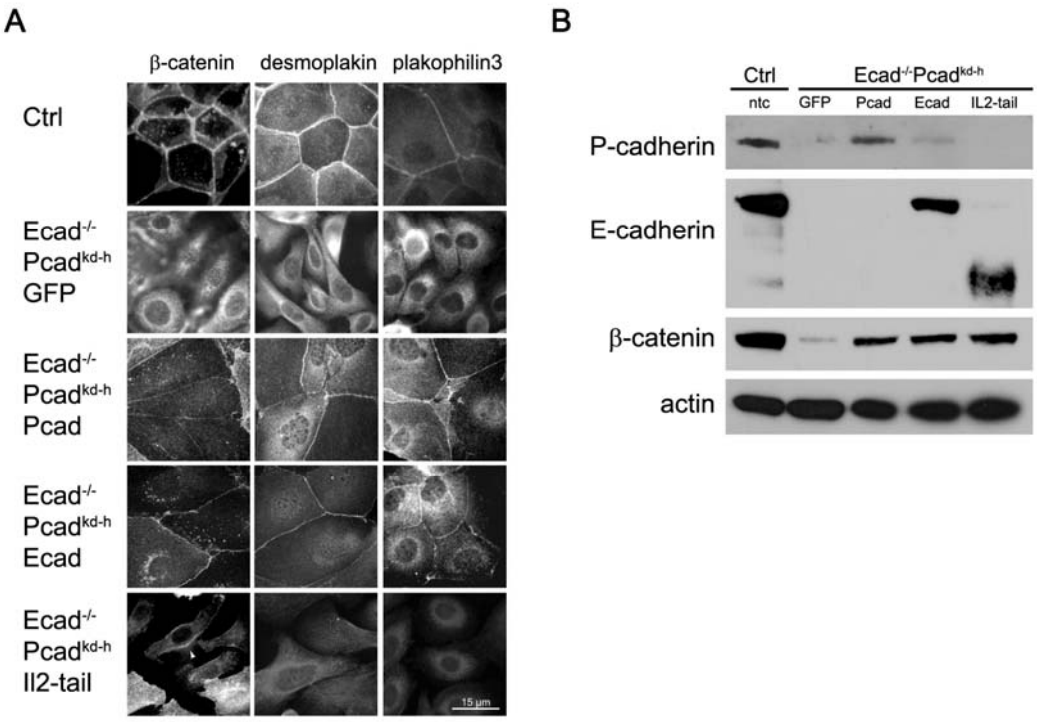


Figure 12: Both E- and P-cadherin, but not the Ecad cytodomain rescue desmosome formation in Ecad$^{-/-}$Pcad^{kd-h} keratinocytes.

(A) Immunolocalization of β-catenin, desmoplakin and plakophilin 3 on keratinocytes 48 hours after Ca^{2+} switch. Bar=15µm (B) Western blot analysis of the indicated proteins on keratinocyte lysates 48 hours after Ca^{2+} switch.

2.2 In vitro barrier formation is impaired in E-cadherin deficient primary mouse keratinocytes

Mice with an E-cadherin deficient epidermis died shortly after birth due to trans epidermal water loss, which was caused by functional impairment of epidermal tight junctions (Tunggal, Helfrich et al. 2005). This raises the question on how E-cadherin regulates epidermal tight junctions. The analysis of the molecular requirement for E-cadherin in epidermal *de novo* tight junction formation requires a system where intercellular junction formation can be controlled. Therefore, the ability of E-cadherin deficient primary keratinocytes to form tight junctions and to establish an epidermal barrier was analyzed.

2.2.1 Recruitment of TJ proteins is not affected in E-cadherin deficient keratinocytes

E-cadherin deficient epidermis displayed defects in the subcellular localization of tight junction components claudin-1 and ZO-1, may causing functional impairment of the in vivo junctions. Thus, E-cadherin might regulate tight junction formation by the proper incorporation of tight junction key components (Tunggal, Helfrich et al. 2005). To test whether E-cadherin is required for proper incorporation of tight junction key components, Immunofluorescence analysis was performed on control and $Ecad^{-/-}$ keratinocytes that were differentiated for 48 hours in high Ca^{2+}. Interestingly, the tight junction components claudin-1, claudin-4, occludin and ZO-1 were recruited to sites of intercellular contacts in control as well as $Ecad^{-/-}$ keratinocytes, suggesting that E-cadherin is dispensable for their proper localization to sites of intercellular contacts *in vitro* (fig. 13).

Tricellulin, a recently discovered TJ component, which was shown to be specifically enriched at tricellular junctions in simple epithelia, was shown to be relevant for the formation of TER in simple epithelia (Ikenouchi, Furuse et al. 2005). To test whether this tight junction component was affected in the absence of E-cadherin, its localization was analyzed. With the rabbit polyclonal serum that was used in the study, a signal at intercellular contacts was observed, albeit with no enrichment at tricellular junctions, suggesting that the specific localization to tricellular contacts might be tissue dependent. Both control and $Ecad^{-/-}$ keratinocytes showed

junctional recruitment in this staining, suggesting that tricellulin localization is not affected in the absence of E-cadherin (fig.13).
The data suggests that tight junctions form in the absence of E-cadherin.

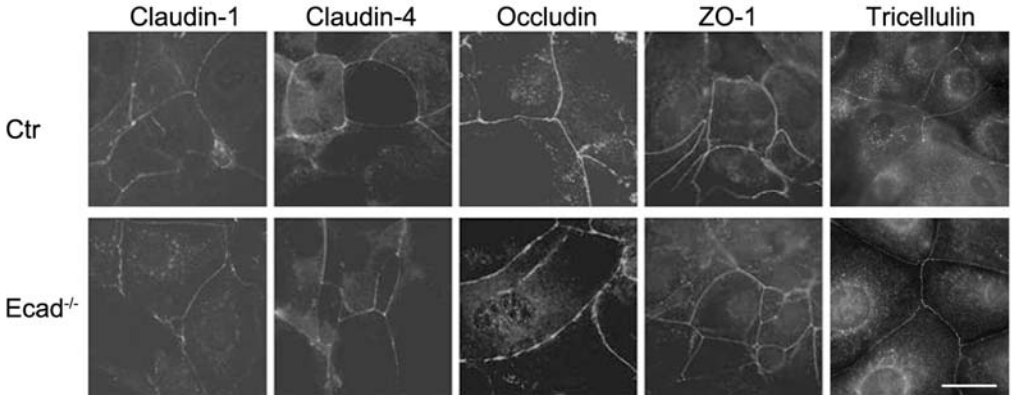


Figure 13: Tight Junction key components localize to the cell surface in Ecad$^{-/-}$ keratinocytes. Immunofluorescence analysis of tight junction key components in control and Ecad$^{-/-}$ keratinocytes. Keratinocytes were differentiated for 48h prior to fixation. Bar=15 µm.

2.2.2 Ultrastructural Tight Junctions form in the absence of E-cadherin.

Since loss of E-cadherin did not obviously alter the recruitment of tight junctional components to sites of intercellular contacts, the ultrastructural appearance was assessed in more detail. Thin section electron microscopy revealed the presence of intercellular contacts in which the inter membrane space was almost completely obliterated in control as well as Ecad$^{-/-}$ keratinocytes (fig. 14A). These contacts resemble the so called kissing points that have been described on the ultrastructural level for tight junctions in simple epithelia (Farquhar and Palade 1963). Furthermore, freeze fracture replica electron microscopy analysis revealed the presence of TJ strand networks in the absence of E-cadherin (fig.14A). More importantly, quantification of the strands did not reveal any difference in strand numbers between controls and Ecad$^{-/-}$ cells (fig. 14D). In addition, no significant differences could also be detected in strand morphology as judged by the number of continuous versus particle type of strand category (fig. 14B) or as straight versus curved type of strands (fig. 14C). The number of strand breaks can reflect ultrastructural instability. However, no significant differences were found when Ecad$^{-/-}$ keratinocytes when compared to controls (fig. 14E).

The only parameter that showed a difference was the spatial extension width of the network which reflects the overall spatial separation distance of individual parallel strands. This parameter was significantly reduced in Ecad$^{-/-}$ keratinocytes, reflecting a spatial compaction of the strand network (fig. 14F). However, the functional significance of this parameter is unclear at present.

Taken together, the data show that the ultrastructural formation of TJs as assessed by electron microscopy does take place in the absence of E-cadherin, showing that E-cadherin is dispensable for structural tight junction formation *in vitro*.

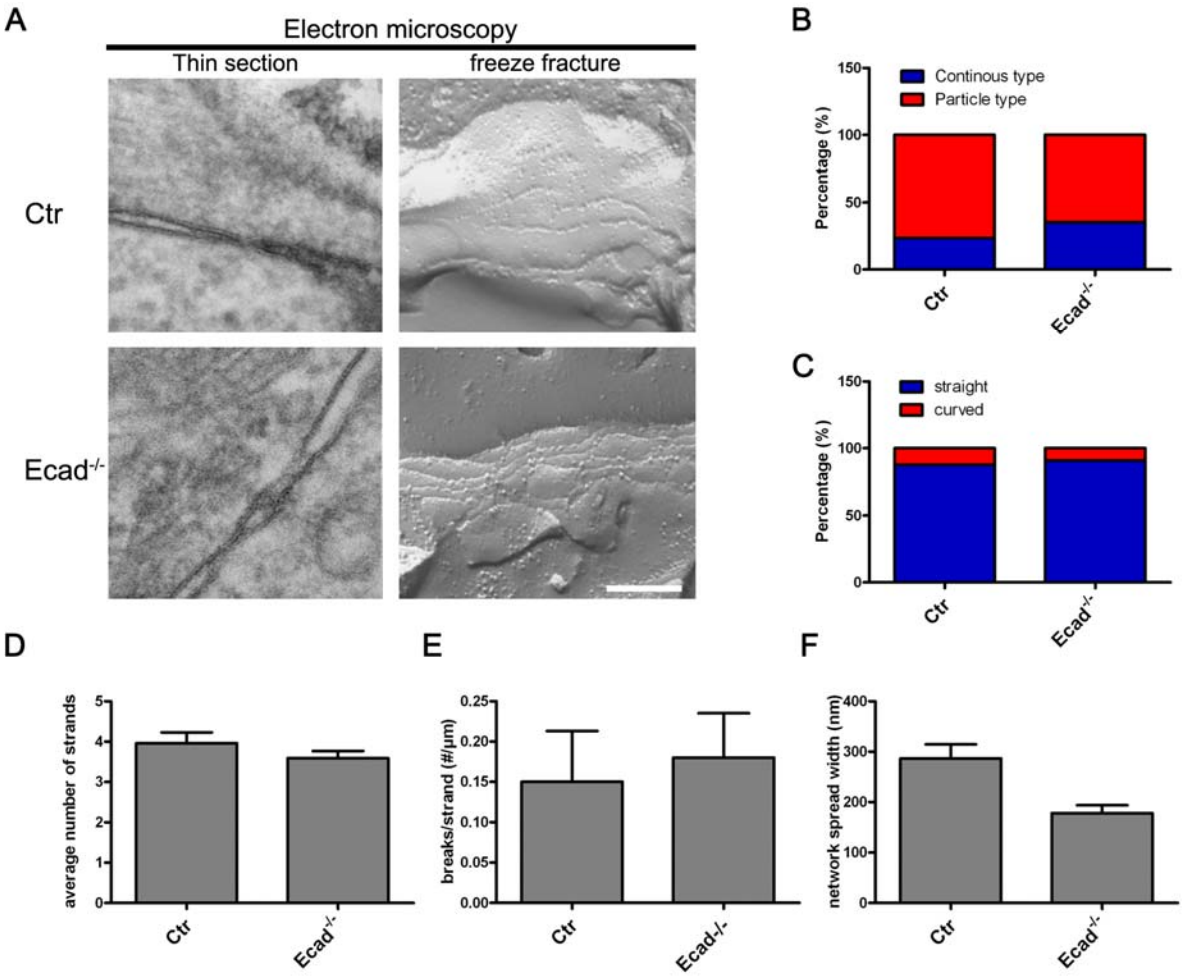


Figure 14: Tight Junction ultra structure in primary mouse keratinocytes.

(A) Thin section and freeze fracture electron micrographs of keratinocytes that were differentiated for 72h in high Ca^{2+}. (B) Quantification of continuous and particle type strands. (C) Quantification of straight and curved type strands. (D) Quantification of strand number. (E) Quantification of strand breaks. (F) Quantification of network extension width.

2.2.3 No *in vitro* barrier in E-cadherin deficient keratinocytes

The ultrastructural formation of keratinocyte tight junctions in the abscence of E-cadherin suggests that their is no requirement for E-cadherin in epidermal tight junction formation. E-cadherin negative epidermis displayed tight junction like structures despite their inability to form an epidermal barrier, showing that E-cadherin was required for tight junction function *in vivo.*

To test whether E-cadherin regulates tight junction function *in vitro* and whether the observed tight junction ultrastructures were functional, the capacity of E-cadherin negative keratinocyte to form a tight junctional barrier was analyzed.

Tight junctional barrier function was assessed using trans epithelial resistance measurement (TER). This assay measures para cellular diffusion of ions which is monitored as electrical resistance, and is thereby assessing ionic barrier properties of the tight junctions. Control and E-cadherin deficient keratinocytes were plated on filter inserts in a confluent manner. Under low Ca^{2+} condition, the TER that was measured did not differ from those of cell free filter inserts, showing that keratinocytes without intercellular contacts do not form electrical resistance. When intercellular junction formation was induced by switching to high Ca^{2+}, control keratinocytes did build up TER over time to values that ranged from 400-700 Ohm*cm² within 48 to 72 hours, showing that primary keratinocytes form a tight junctional barrier subsequent to intercellular contact formation (fig. 15B).

Interestingly, despite their ability to form structural tight junctions, E-cadherin deficient keratinocytes showed only minor increase in TER, showing impairment of their capacity to form a tight junctional ion barrier (fig. 15B).

To assess how loss of E-cadherin affects the size specific tight junctional barrier properties, paracellular diffusion of fluorescently labelled dextran of different molecular weight (3kD or 40 kD) was measured. Two hours after tracer application, the fluorescent signal in the basal compartment was measured to detect the paracellular diffusion of the tracer. The amount of tracer in the basal compartment was significantly higher in E-cadherin$^{-/-}$ keratinocytes compared to control for both the 3kD and the 40kD dextran, indicating increased paracellular leakage (fig.15 C and D). Thus, the tight junctional size barrier is leaky for both small and large molecular weight tracers in the absence of E-cadherin. Taken together, the results show that loss of E-cadherin severally impaires the tight junctional barrier in primary keratinocytes.

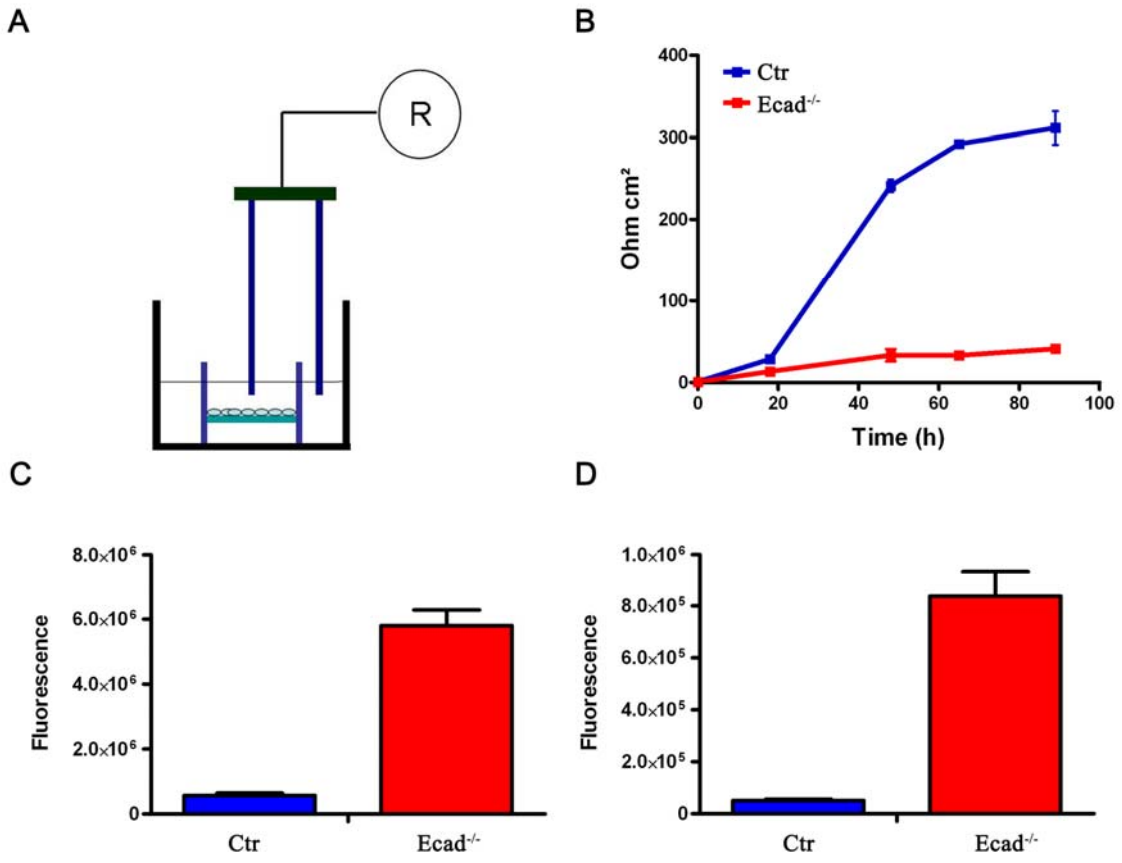


Figure 15: Impaired *in vitro* barrier formation in E-cadherin deficient keratinocytes.
(A) Schematic drawing of experimental setup. Keratinocytes were plated on porous filter inserts and trans epithelial resistance was measured using an Ohm meter. (B) Trans epithelial resistence (TER) measurement of Control and Ecad$^{-/-}$ keratinocytes. (C) Paracellular flux assay using 3 kD FITC-labelled dextran. (D) Paracellular flux assay using 40 kD FITC-labelled dextran.

2.2.4 Normal barrier formation upon knock down of P-cadherin in primary keratinocytes

Since Ecad$^{-/-}$ keratinocytes failed to form a functional barrier in *vitro*, we next asked whether there is a specific requirement of E-cadherin, or whether E-cadherin and P-cadherin cooperatively regulate barrier formation. To address a potential role for P-cadherin in TJ regulation, PCad^{kd} as well as Ecad$^{-/-}$Pcad^{kd-h} keratinocytes were analyzed in TER and paracellular diffusion experiments.

Pcad^{kd} keratinocytes were able to form TER in an extend that was comparable to control keratinocytes, suggesting normal tight junctional ion barrier function upon reduction of P-cadherin expression (fig. 16A). In addition, the ability to restrict paracellular diffusion as judged by paracellular diffusion of tracer molecules was unchanged in these cells when compared to controls (fig. 16B).

Both ionic and size barrier capacities were further reduced and similar to background levels in Ecad$^{-/-}$Pcad^{kd-h} keratinocytes when compared to Ecad$^{-/-}$ keratinocytes. This was expected since these cells only form very few intercellular junctions and thus have increased paracellular space caused by the absence of intercellular junctions (fig. 16A and B).

The result either suggests that tight junction formation is a specific function of E-cadherin that does not involve P-cadherin or that the reduction in overall classical cadherin levels by P-cadherin knock down was not sufficient to cause barrier impairment.

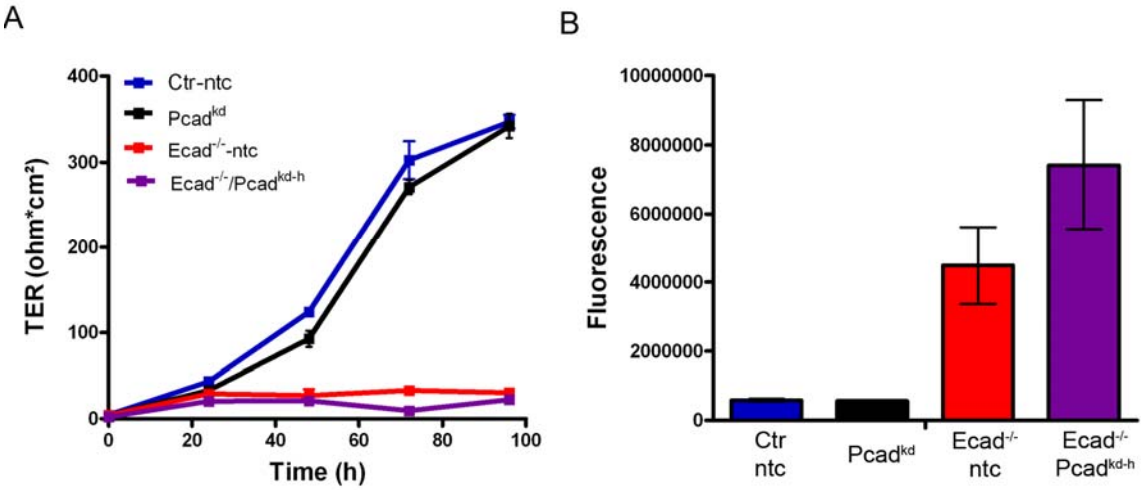


Figure 16: Trans epithelial resistance and paracellular permeability in the absence of either E-cadherin, P-cadherin or both.

(A) TER measurement of Ctrl, Pcad^{kd}, Ecad$^{-/-}$ and Ecad$^{-/-}$Pcad^{kd-h} keratinocytes. (B) Paracellular flux assay using FITC-3kD dextran as a tracer. Cells were differentiated in high Ca^{2+} for 72 hours before the tracers were added.

2.2.5 Re-expression of either E-cadherin or P-cadherin rescues barrier formation

To ask whether there is a specific regulation of tight junctions by E-cadherin or whether both classical cadherins redundantly regulate barrier formation, rescue experiments were performed by expressing either E-cadherin or P-cadherin in Ecad$^{-/-}$ keratinocytes.

Full length cDNAs encoding E- or P-cadherin were cloned into a lentiviral expression vector to allow for lentiviral transduction of primary keratinocytes.

Transgene expression for both cDNAs was verified by westernblot analysis (fig. 17G and H). Interestingly, Ecad$^{-/-}$ keratinocytes that were transduced with either E- or P-cadherin, but not GFP, restored the capacity of E-cadherin negative keratinocytes to induce TER over time (fig. 17A and B) as well as to restrict paracellular diffusion for both 3kD (fig. 17C and D) and 40kD dextran (fig. 17E and F).

The results show that there is no specific requirement for either E- or P-cadherin but instead show functional redundancy and that the level for classical cadherin expression determines the capacity to form a functional tight junctional barrier.

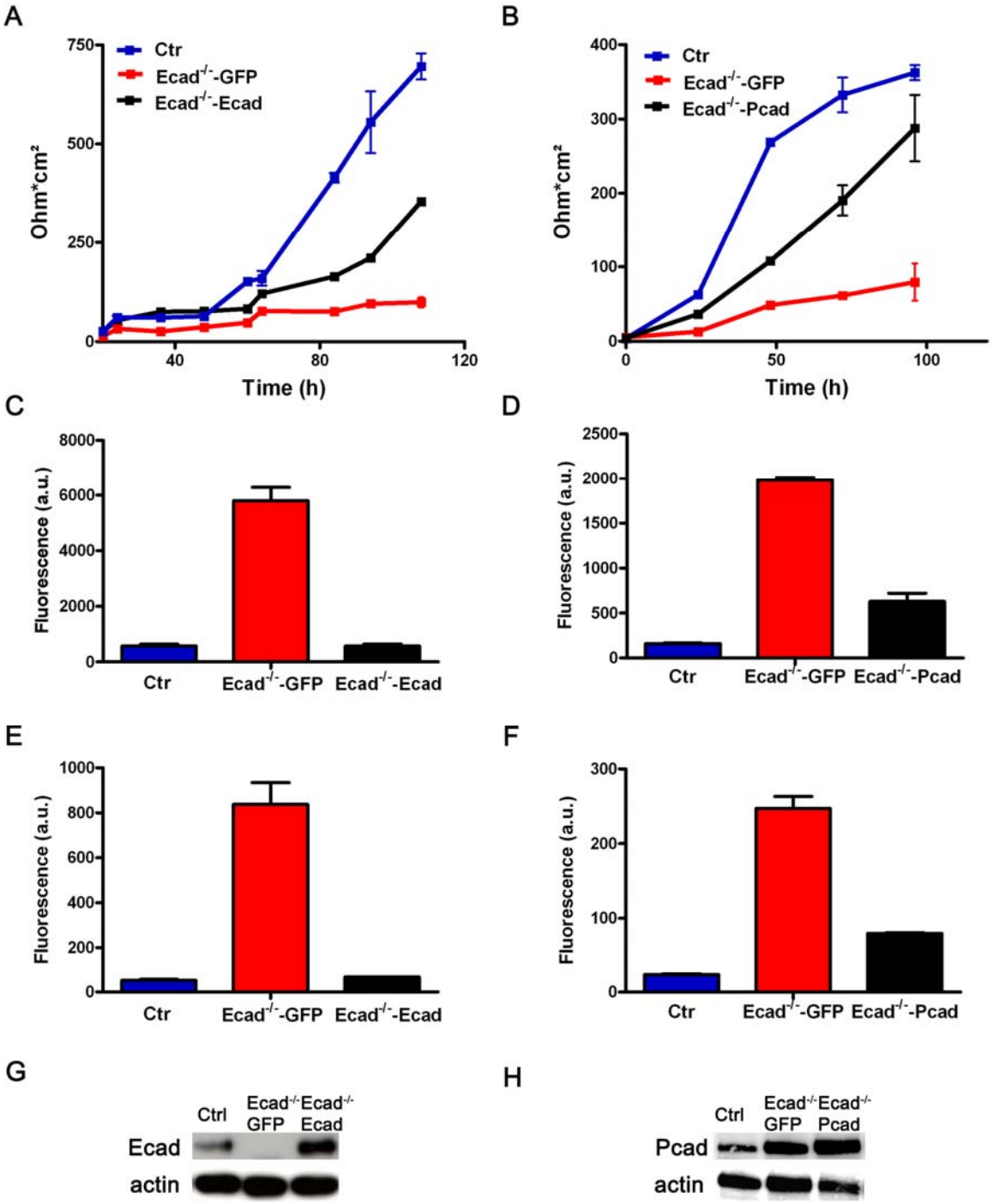


Figure 17: Both re expression of E-cadherin and over expression of P-cadherin rescued barrier formation in Ecad$^{-/-}$ keratinocytes.

(A and B) TER measurement of lentivirally transduced keratinocytes expressing either E-cadherin (A) or P-cadherin (B). (C and D) Paracellular flux assay using 3kD FITC-dextran as tracer on cells expressing either E-cadherin (C) or P-cadherin (D). (E and F) Paracellular flux assay using 40kD FITC-dextran as a tracer on cells expressing either E-cadherin (E) or P-cadherin (F). (G and H) Western blot of cells used in barrier measurements showing expression of the transgenes. GFP was used as control.

2.2.6 Expression of the E-cadherin cytoplasmic tail interferes with adhesion

To understand the molecular requirement for classical cadherins in regulation of barrier function, we asked whether cadherin dependent regulation of the epidermal barrier is mediated through recruitment of its cytoplasmic binding partners independent of its adhesive function. Therefore, the previously described IL2R-tail construct was lentivirally expressed in Ecad$^{-/-}$ keratinocytes to examine if this would restore tight junction barrier formation. No restoration of barrier formation was observed in keratinocytes expressing the IL2R-tail fusion protein. Instead, a further decrease of TER (fig. 18A) and increase in paracellular tracer diffusion (fig. 18B) were observed. Furthermore, the IL2R-tail expressing keratinocytes appeared more undifferentiated as judged by phase contrast microscopy, suggesting disturbed intercellular contact formation (fig. 18C). Thus, the IL2R-tail construct appeared to act as dominant negative towards classical cadherin mediated adhesion.

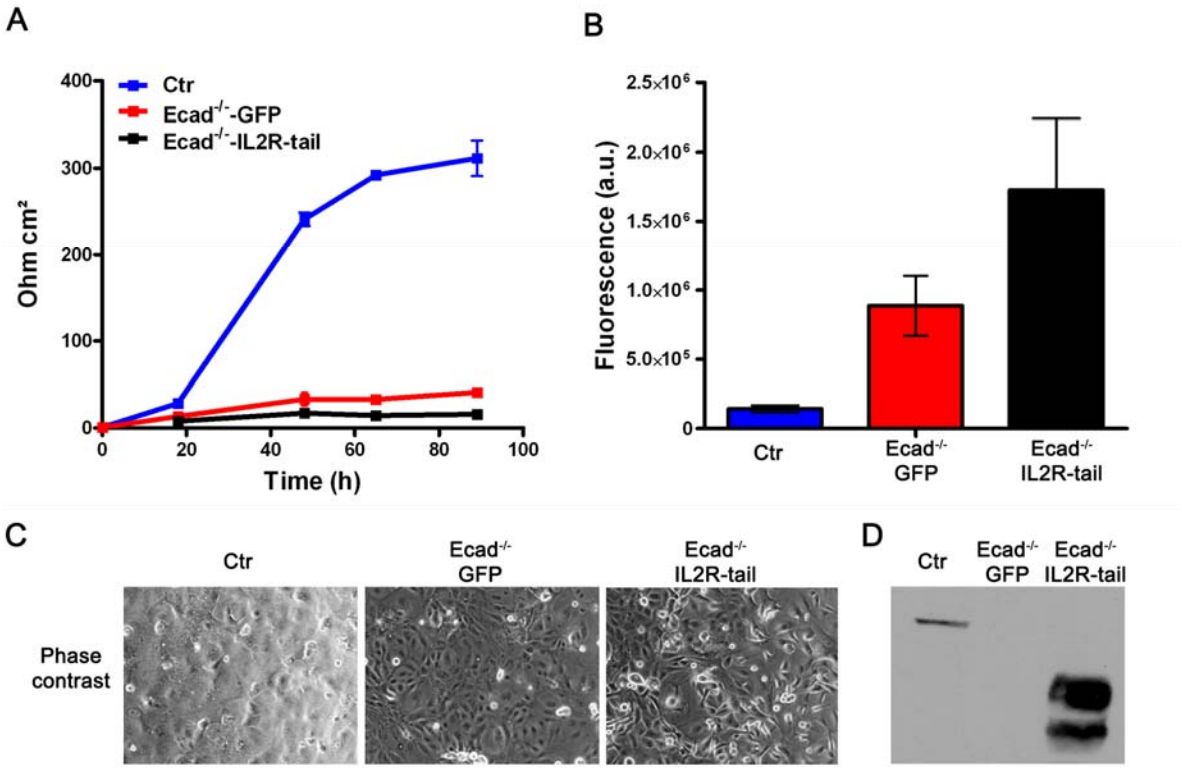


Figure 18: Impaired junction formation upon expression of the chimeric IL2R-tail protein in Ecad$^{-/-}$-keratinocytes.

(A) TER measurement of control, Ecad$^{-/-}$-GFP and Ecad$^{-/-}$-IL2R-tail keratinocytes. (B) Paracellular flux assay using 3kD FITC-dextran on keratinocytes that were differentiated for 72h. (C) Phase contrast images of keratinocytes that were differentiated for 72h. (D) Western blot using anti E-cadherin cytoplasmic tail antibody showing expression of the IL2R-tail trans gene.

2.2.7 No Rescue of barrier formation by overexpression of aPKC

Epidermal deletion of E-cadherin resulted in altered recruitment of phosphorylated aPKCλ (Tunggal, Helfrich et al. 2005). aPKCλ is, together with Par3 and Par6, part of a conserved complex which was shown to be essential for a variety of cellular polarization processes (Lin, Edwards et al. 2000). Interestingly, formation of TER in primary keratinocytes was shown to depend on aPKCλ activity as peptides acting as pseudosubstrates abolished barrier formation *in vitro.* This was not accompanied by loss of tight junction key components from intercellular junctions as judged by immunofluorescence, similar to the E-cadherin negative situation (Helfrich, Schmitz et al. 2007).

To test whether aPKCλ is involved in E-cadherin mediated regulation of tight junction function, its expression was analyzed by westernblot analysis in $Ecad^{-/-}$ keratinocytes. In addition, since aPKC might be regulated by its phosphorylation, phospho-aPKC levels were assessed by using phospho-specific antibodies directed against its phosphorylated serines 555 and 565. Using these antibodies no change in either total protein expression or phospho-aPKC levels were observed (fig. 19A).

Overexpression of aPKC increased TER in primary mouse keratinocytes (Helfrich, Schmitz et al. 2007), perhaps by enhancing its activity at the junction. To test whether over expression of aPKCλ can restore barrier formation in the absence of E-cadherin, a GFPaPKCλ fusion protein was cloned into a lentiviral expression vector. Transgene expression was confirmed by western blot (fig. 19B). However, no restoration of barrier formation was observed either in TER or paracellular flux assays (fig. 19C and D). Thus, overexpression of aPKCλ is not sufficient to restore barrier formation in primary keratinocytes that are deficient for E-cadherin.

Since membrane recruitment of aPKCλ might be required to mediate regulation of tight junctions, attempts were undertaken to express a membrane tagged version of aPKC by fusing it to a CAAX domain. Unfortunately, the construct was not expressed upon transduction of primary keratinocytes, thus no conclusion can be made about the role of aPKC membrane recruitment in epidermal barrier formation.

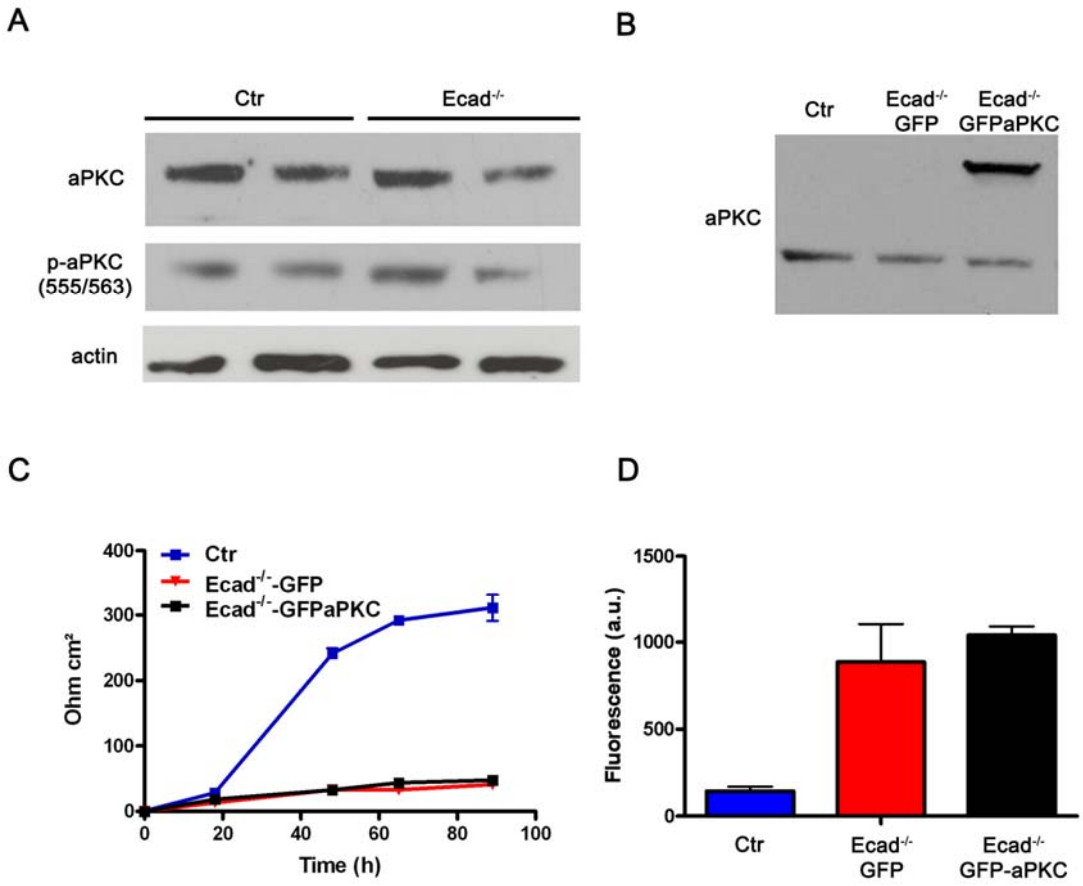


Figure 19: Overexpression of GFP-aPKC does not rescue barrier formation in Ecad$^{-/-}$ keratinocytes.

(A) Western blot analysis of aPKC expression and phosphorylation. (B) Western blot analysis of aPKC expression of lentivirally transduced keratinocytes. (C) TER measurement of lentivirally transduced keratinocytes. (D) Paracellular flux assay using 3kD FITC-dextran as a tracer.

2.2.8 Decreased Rac activity in Ecad$^{-/-}$ keratinocytes

In E-cadherin deficient epidermis, Rac1 localization was lost from the membrane (Tunggal, Helfrich et al. 2005). A critical role for Rac activity in the regulation of *in vitro* keratinocyte barrier formation was demonstrated to be mediated via its exchange factor Tiam1 (Mertens, Rygiel et al. 2005). In addition, Rac is known to be directly activated upon E-cadherin engagement in simple epithelia (Noren, Niessen et al. 2001).

To test whether E-cadherin regulates Rac1 activation levels in primary keratinocytes, pull down assays using the Pak-Crib effector domain were performed on control and E-cad$^{-/-}$ keratinocytes to assess levels of the active GTP-bound Rac1 upon induction of differentiation and the formation of intercellular

junctions. In the absence of E-cadherin a reduced Rac activation was observed at different time points after cells were allowed to form intercellular junctions. Whereas control keratinocytes showed a strong increase in Rac activation 1 hour after induction of junction formation, no increase in Rac activation was observed in $Ecad^{-/-}$ keratinocytes (fig. 20A and B). Assays performed at later stages of differentiation revealed reduced Rac activity levels 48 hours after Ca^{2+}-switch, a time point that correlates with the onset of TER formation in control keratinocytes (fig. 20A and B). Thus, E-cadherin regulates Rac activity levels in primary keratinocytes.

To test whether the decreased Rac levels are responsible for barrier dysfunction in $Ecad^{-/-}$ keratinocytes, a dominant active Rac1 construct (mycRacL61) was cloned into a lentiviral expression vector and expressed in these cells by lentiviral transduction. However, no rescue of barrier formation was observed upon expression of this construct, suggesting that constitutively activated Rac is not able to induce barrier formation in the absence of E-cadherin (fig. 20B). Previous studies reported that ectopic expression of either dominant active or dominant negative mutants of Rho GTPases impairs tight junction formation and function. Thus, specific activity levels of Rac might be required for barrier formation (Jou, Schneeberger et al. 1998).

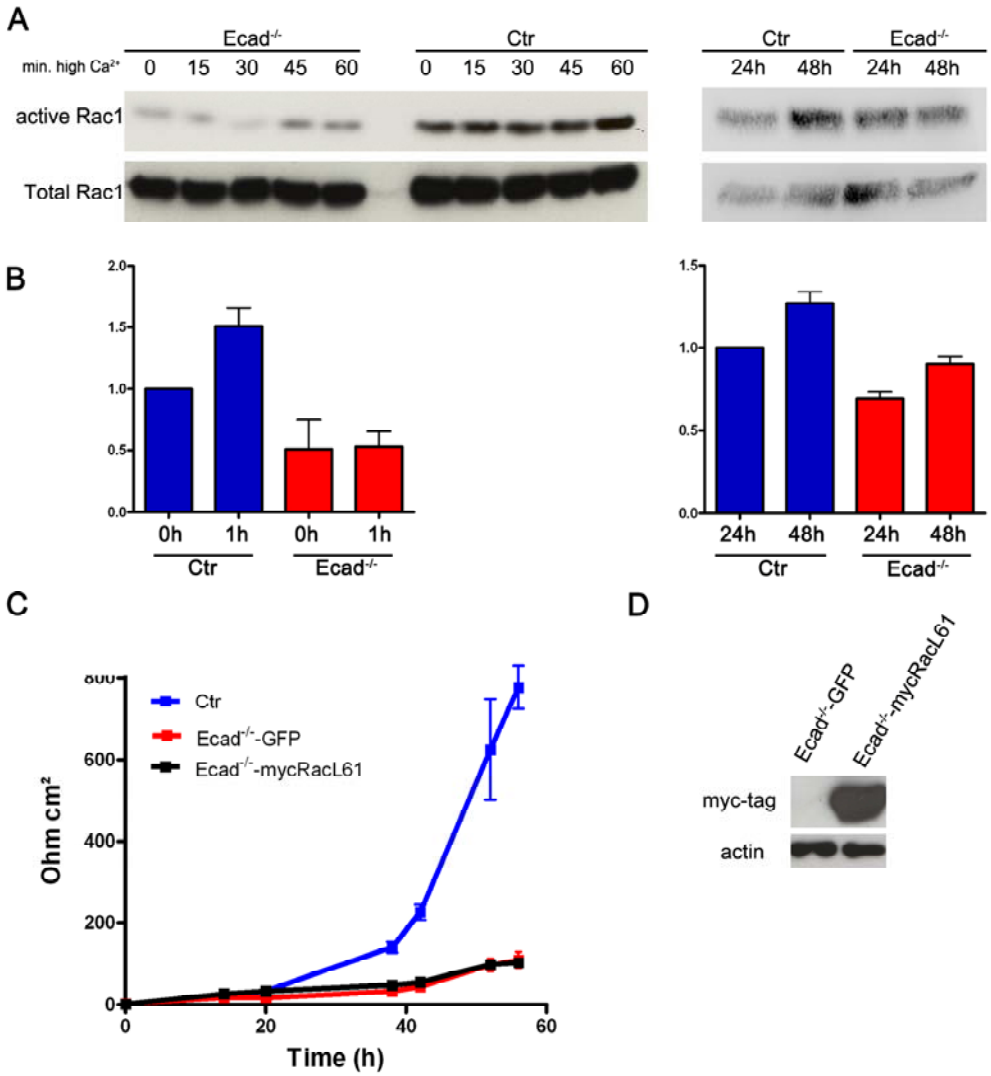


Figure 20: Decreased Rac activity in Ecad$^{-/-}$ keratinocytes.

(A) Rac activity assay of Control and Ecad$^{-/-}$ keratinocytes at different time points after induction of junction formation. (B) Densitometric quantification of Rac activity. (C) TER measurement of Control, Ecad$^{-/-}$-GFP and Ecad$^{-/-}$-mycRacL61 keratinocytes. (D) Western blot analysis using myc-tag antibody to detect transgene expression in lentivirally transduced keratinocytes.

2.2.9 No obvious alterations in actin cytoskeletal architecture in the absence of E-cadherin

Both adherens junctions and tight junctions connect to and regulate the actin cytoskeleton. Conversely, proper organization of cortical actin is crucial for junction formation and polarity (Miyoshi and Takai 2008). Rac is known to regulate actin polimerization. Since Rac activity was reduced in Ecad$^{-/-}$ keratinocytes, alterations in cortical actin structures might be responsible for barrier dysfunction in Ecad$^{-/-}$ keratinocytes. Phalloidin was used to visualize filamentous actin structures in

keratinocytes in which junction formation was initiated by Ca^{2+} switch. In low Ca^{2+} conditions control and $Ecad^{-/-}$ keratinocytes displayed stress fibres but little enrichment of cortical actin structures. Two hours after induction of junction formation, both control and E-cadherin deficient keratinocytes recruited actin to sites of intercellular contacts. 48 hours after Ca^{2+} switch, control as well as $Ecad^{-/-}$ keratinocytes showed largely reduced numbers of stress fibers and accumulation of actin at the membrane (fig. 21). The results suggest that actin organization at sites of intercellular contacts is mostly unaffected by the loss of E-cadherin in primary keratinocytes.

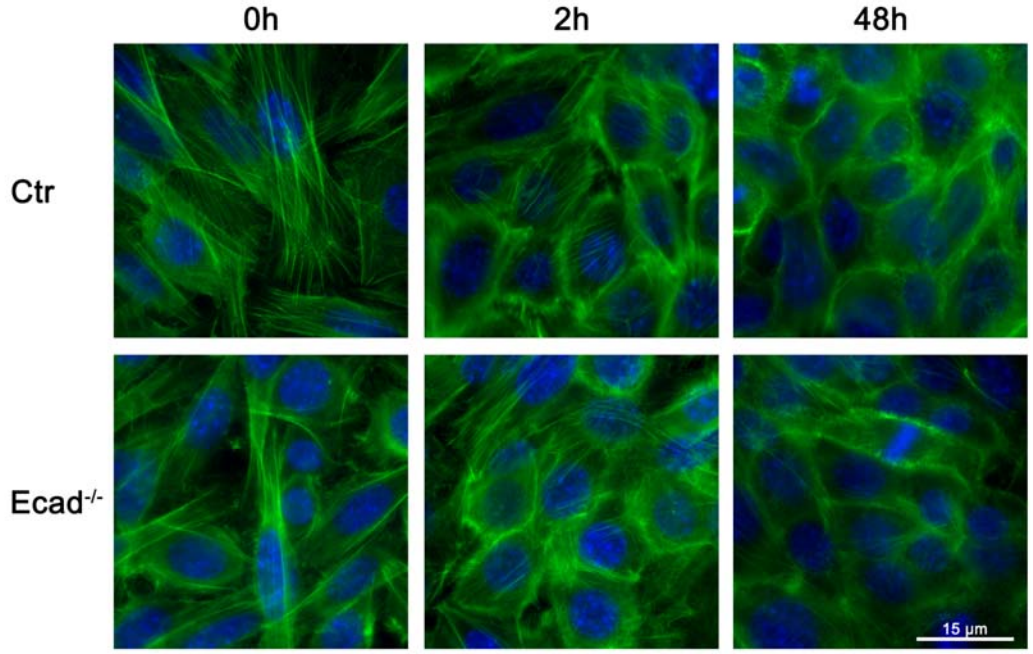


Figure 21: F-actin organization in the absence of E-cadherin.
Phalloidin-FITC staining to visualize F-actin in control and $Ecad^{-/-}$ keratinocytes that were differentiated in high Ca^{2+} medium for the indicated time points Bar=15 µm.

2.2.10 Inhibition of Myosin-ATPase, but not PI3-kinase affects barrier formation in primary keratinocytes

Phosphoinositide 3 kinases (PI3-kinase) are enzymes that phosphorylate the 3 hydroxyl group of the phosphatidylinositol (Hawkins, Anderson et al. 2006). E-cadherin mediated activation of PI3-kinase was suggested to regulate Rac activity. Furthermore, PI3-kinase can directly associate with tight junction key components (Woo, Ching et al. 1999). To test whether PI3-kinase activity is required for

epidermal tight junction barrier formation, its activity was inhibited by Wortmannin application. Surprisingly, no effect on barrier formation was observed when control keratinocytes were treated with different concentrations of Wortmannin, suggesting that E-cadherin mediated barrier formation does not occur via activation of PI3-kinase signaling (fig. 22A) (Michels, Aghdam et al. 2009).

Actomyosin contraction has been shown to be important for not only junctional remodeling but also for specific regulation of TJ permeability. Especially the phosphorylation of myosin by myosin light chain kinase (MLCK) has been shown to specifically affect paracellular permeability, since constitutively active MLCK led to increased paracellular permeability by increasing myosin light chain phosphorylation (Shen, Black et al. 2006).

Actomyosin contraction can be inhibited by using Blebbistatin, a specific inhibitor of myosin ATPase activity. To test whether actomyosin contraction is critical for keratinocyte barrier formation, control keratinocytes were treated with Blebbistatin at different concentrations. Analysis of barrier formation of the Blebbistatin treated cells revealed a dose dependent decrease in TER, suggesting that indeed actomyosin contraction is critical for barrier formation (fig. 22B).

Analysis of filamentous actin in Blebbistatin treated cells revealed a dose dependent disruption of cortical actin structures, suggesting that impairment of barrier formation was may caused by structural deregulation of the junctions, thus not resembling the $Ecad^{-/-}$ phenotype (fig. 22C).

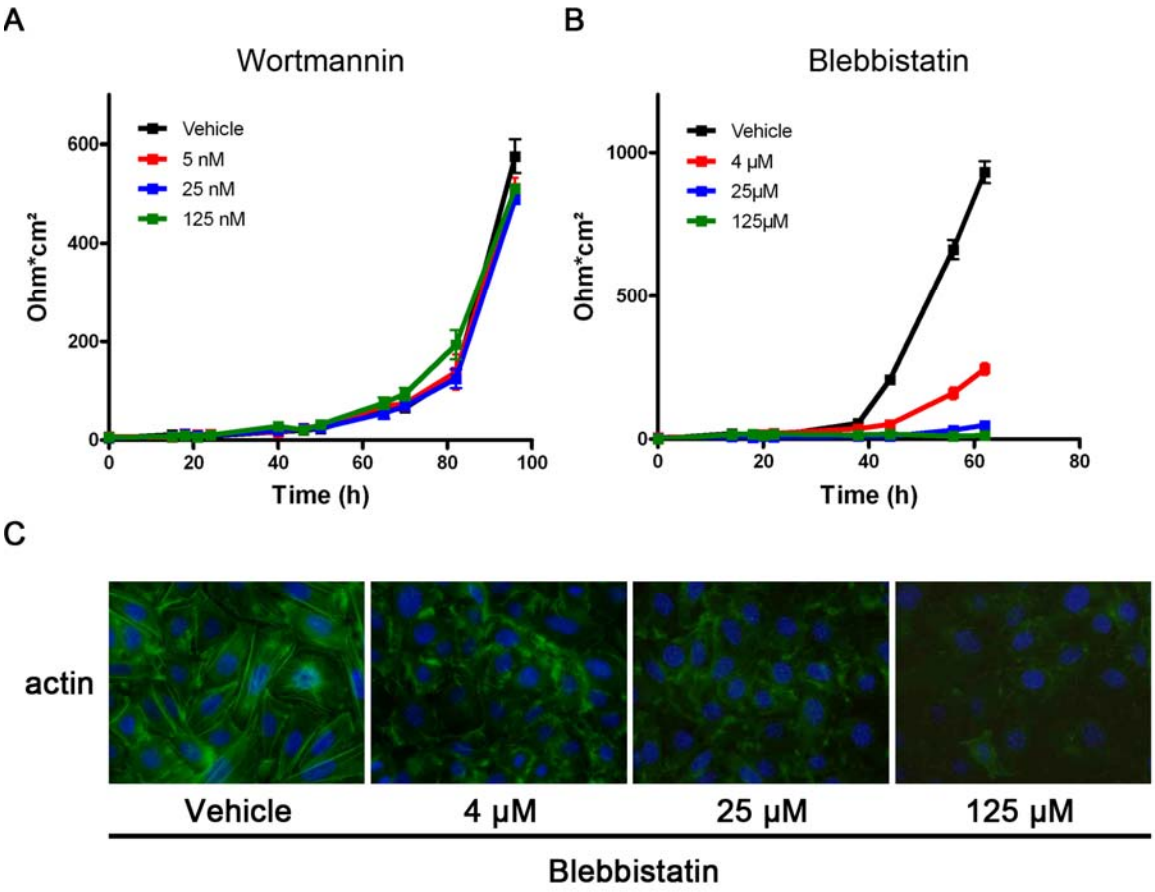


Figure 22: Impact of PI3K and myosin ATPase on keratinocyte barrier formation.

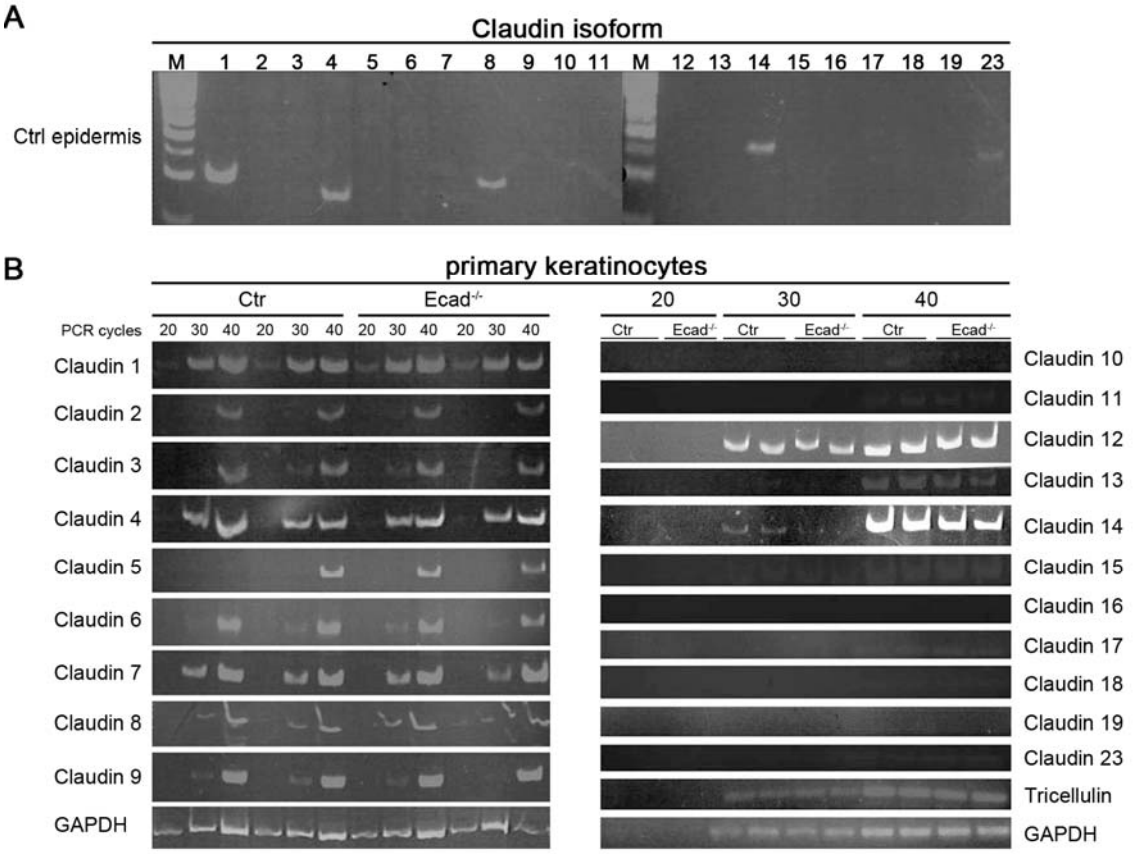
(A) Effect of Wortmannin on keratinocyte barrier formation. Inhibitor was added to the differentiation medium at the indicated concentrations. (B) Effect of Blebbistatin on keratinocyte barrier formation. (C) Actin staining on Blebbistatin treated keratinocytes. DMSO was used as vehicle control.

2.3 E-cadherin regulates claudin-14 expression in vivo and in vitro

The size and charge selectivity and permeability of tight junctions is determined by the relative expression and composition of claudin isoforms that are incorporated into the tight junction selas (Will, Fromm et al. 2008). Thus, alterations in claudin expression potentially affect tight junction barrier properties.

2.3.1 Claudin-14 is down regulated in the absence of E-cadherin

In order to test whether the tight junctional barrier impairment in E-cadherin$^{-/-}$ keratinocytes was caused by alteration in claudin isoform expression, a RT-PCR expression profiling was conducted using keratinocytes that were cultured in high Ca^{2+} medium for 48h. RT-PCR was performed on epidermal cDNA from control mice to verify their specificity. Signals were detected for claudin 1, 4, 8, 14 and 23 (fig. 23A).



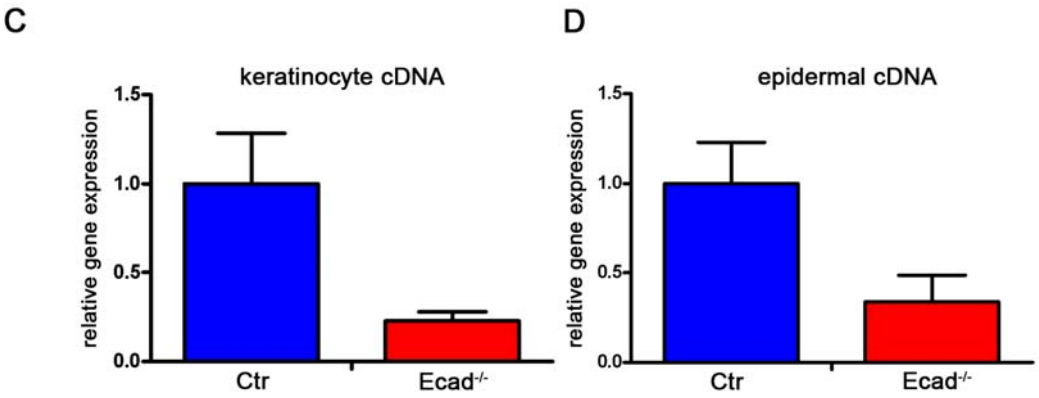


Figure 23: Claudin-14 expression is downregulated in the absence of E-cadherin.
(A) RT-PCR using primers specific for different claudin isoforms on control epidermal cDNA. GAPDH was used as input control. (B) Semi-quantitative RT-PCR on control and Ecad$^{-/-}$ keratinocyte cDNA using different amount of amplification cycles. (C and D) Quantitative Real time PCR using claudin-14 specific primers on keratinocyte cDNA (C) and epidermis cDNA (D).

Semi-quantitative RT-PCR on control keratinocyte cDNA revealed a signal after 25 rounds of amplification for claudin-1, 4 and 7, which represent the most abundant claudin isoforms in the mouse epidermis. Claudin-12, which showed an early detectable signal using keratinocyte cDNA, was not found to be expressed in epidermal cDNA, suggesting that claudin expression profiles might differ between *in vitro* and *in vivo* conditions. Late amplification signals after 40 rounds of amplification were detected for claudin-2, 3, 5, 6, 8 and 9 (fig.23B) and a similar sensitivity in transcript detection was observed in the E-cadherin$^{-/-}$ cDNA, indicating unaltered RNA expression for these claudins.

In contrast, claudin-14 showed a consistent down regulation in the absence of E-cadherin. To verify the result, quantitative real time PCR was utilized to measure claudin-14 expression in the presence or absence of E-cadherin more quantitatively. Ecad$^{-/-}$ keratinocyte cDNA again showed a significant downregulation of the claudin-14 transcript (fig.23C), indicating that E-cadherin is involved in the regulation of its gene expression. In addition, since claudin-14 promotes barrier formation, it represents a potential candidate to mediate E-cadherin dependent regulation of tight junction function (Wattenhofer, Reymond et al. 2005). In this case, downregulation of claudin-14 in the absence of E-cadherin should also occur *in vivo*. Indeed, reduced transcript levels were detected in E-cadherin deficient epidermal cDNA when compared to control epidermal cDNA, suggesting that E-cadherin regulates claudin-14 expression *in vivo* (fig. 23D).

Since antibodies against mouse claudin-14 are not commercially available, it could not be tested whether claudin-14 protein levels were indeed reduced in the absence of E-cadherin.

2.3.2 Partial knock down of claudin-14 does not affect barrier formation in mouse keratinocytes

The reduced expression of claudin-14 RNA in E-cadherin$^{-/-}$ keratinocytes raises the question whether this caused the observed barrier defect. If so, the prediction would be that inactivation of claudin-14 in keratinocytes would disturb tight junction function. To address this question, we lentivirally knocked down claudin expression in control keratinocytes and checked whether barrier formation was affected. 5 different shRNA clones were used, however, none of them achieved a knock down efficiency that exceeded 50% reduction of transcript level when compared to control shRNA transduced keratinocytes (fig. 24B). No reduction in barrier formation was observed over time in any of the keratinocytes when claudin-14 knock down keratinocytes were assayed in TER measurements, suggesting that the degree of knockdown that was achieved did not affect barrier formation in control keratinocytes (fig.24A). Since an almost complete loss of barrier formation in Ecad$^{-/-}$ keratinocytes correlates with a 70% reduction of claudin-14 expression, the result suggests that either the down regulation of claudin-14 is not causal to the barrier phenotype in the absence of E-cadherin, or the barrier requires a more extensive reduction of claudin-14 expression than the 50% that was maximally achieved in these experiments. Alternatively, loss of claudin-14 alone is insufficient to disturb epidermal barrier formation and other not yet identified factors are deregulated in the absence of E-cadherin that cause barrier dysfunction in cooperation with loss of claudin-14 expression.

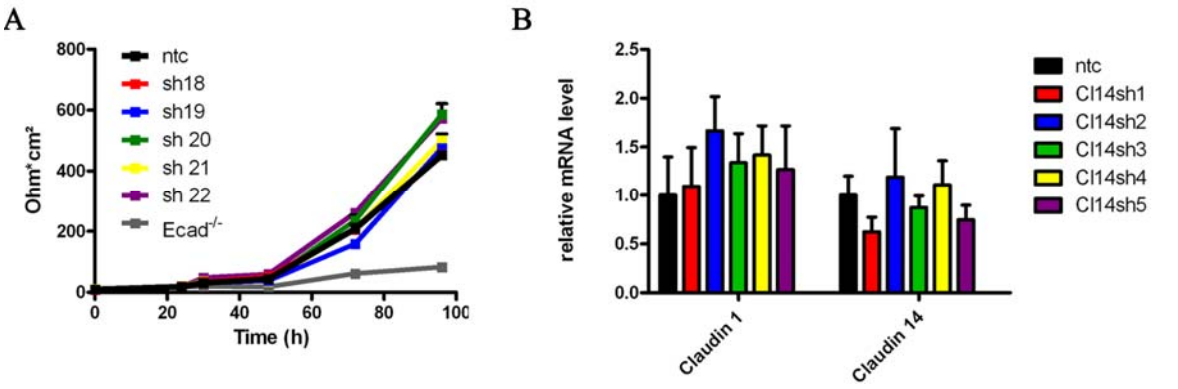


Figure 24: Knockdown of claudin-14 in primary keratinocytes.

(A) TER measurement on control keratinocytes that have been lentivirally transduced with different shRNA clones targeted against claudin-14. (B) Quantitative real time PCR to assess the efficiency of claudin-14 silencing in the respective ShRNA clones.

2.3.3 No restoration of barrier function upon expression of human claudin-14 in Ecad$^{-/-}$ keratinocytes

If reduced claudin-14 expression alone is responsible for the dysfunctional tight junctions in Ecad$^{-/-}$ keratinocytes, increased expression of claudin-14 to levels comparable to control should restore barrier formation in these cells. In order to test this hypothesis, human claudin-14 (hCl14) was cloned into a lentiviral expression vector. After transduction of Ecad$^{-/-}$ keratinocytes, transgene expression was verified by westernblot and Immunofluorescence (fig. 25B and C).

However, no restoration of TER was observed in hCl14 transduced keratinocytes (fig. 25A). Instead, a slight reduction in barrier formation was monitored. In order to characterize the proper localization of the transgene, immuno fluorescence staining using antibodies against hCl14 was performed in co staining with the TJ marker ZO-1. hCl14 was found at intercellular contacts, however, large accumulation of vesicles stained positive in the transduced cells, maybe caused by massive over expression and insufficient membrane targeting of the construct (fig. 25B). In addition, the ZO-1 staining pattern of the largely flattened, supra basal keratinocytes was lost in claudin-14 transduced keratinocytes, indicating impairment of tight junction formation. More fine tuned regulation of claudin-14 expression might be required in order to solve the problem of over expression artefacts, since massive vesicle accumulation might sequester important cytoplasmic binding partners away form the junctional complex and thereby causing impairment of the TJ barrier.

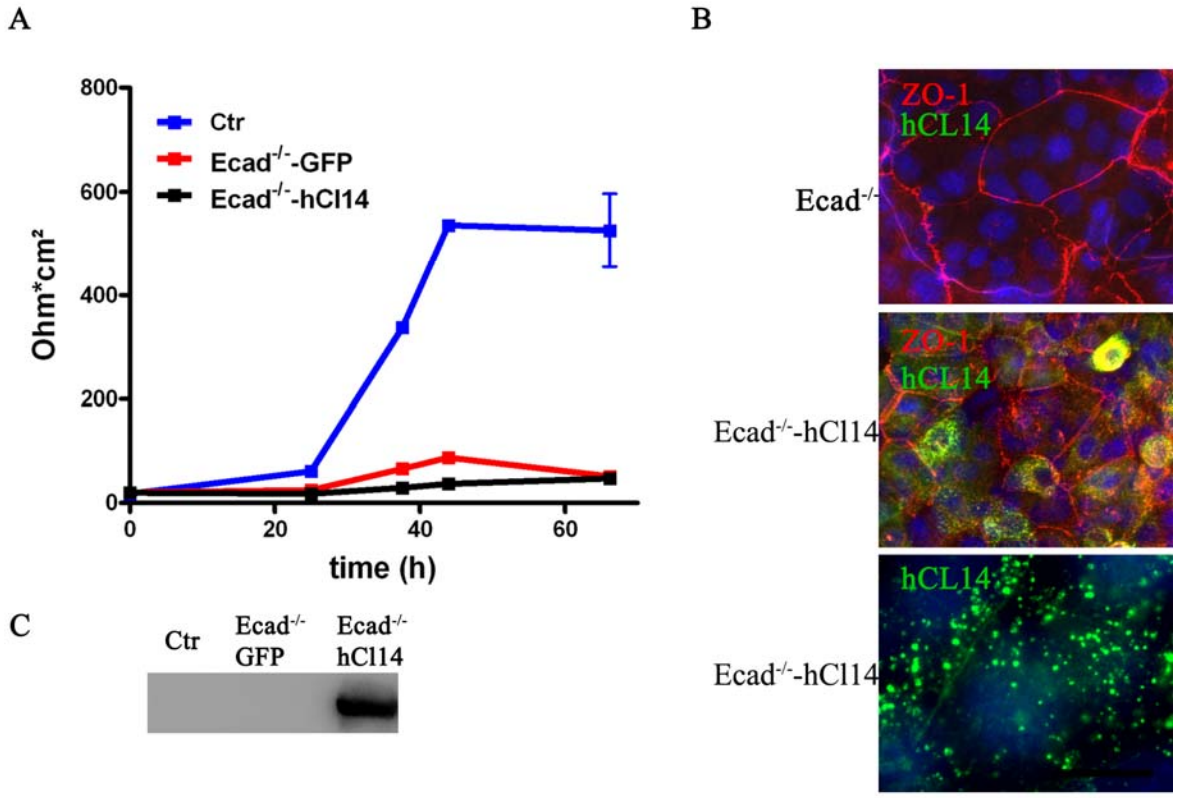


Figure 25: Over expression of human claudin-14 does not rescue barrier formation.
(A) TER measurement of Ecad$^{-/-}$ keratinocytes expressing human claudin-14. GFP was used as a control. (B) Immuno fluorescence analysis of ZO-1 and hCl14 in hCl14 transduced Ecad$^{-/-}$ keratinocytes. (C) Western blot analysis using hCl14 antibody to confirm transgene expression.

2.4 E-cadherin is dispensable for mouse keratinocyte migration and proliferation

To ask whether E-cadherin directly regulates proliferation and migration properties of primary mouse keratinocytes, proliferation and migration was analyzed in the presence or absence of E-cadherin *in vitro*.

2.4.1 Proliferation and proliferative potential not affected in the absence of E-cadherin

Primary keratinocytes were cultured under low Ca^{2+} condition to prevent terminal differentiation, thereby preserving their proliferative capacity. However, no intercellular contacts form under these conditions. Switching to high Ca^{2+} medium allows for intercellular junction formation, but also triggers terminal differentiation, which will ultimately result in growth arrest of keratinocytes.

In vitro growth of control and $Ecad^{-/-}$ keratinocytes was indirectly assessed using a viability assay which is based on ATP detection. Cells were plated and samples were taken after different time points to measure ATP as an indirect read out for cell number. No difference was observed between control and $Ecad^{-/-}$ keratinocytes (fig. 26A).

To asses the proliferative potential, colony formation assays were performed. Single cell plating and quantification of colony formation allows distinguishing between abortive colonies, which were founded by transient amplifying cells and which stop growing after a limited amount fo cell cycles, and non-abortive colonies of high proliferative potential, which display unlimited growth and therefore allow further expansion of the colony (fig. 26C). Colony size was measured and, after sorting the individual colonies for size in ascending order, blotted as a curve to reveal the size distribution profile of the entire colony population (fig. 26D). Abortive colonies appear in this diagram as a low slope phase representing similar sized colonies which cover about 85% of the overall colonies in control keratinocytes, whereas non-abortive colonies of high proliferative potential appear as a high slope phase of bigger colonies that spread over a wider size range and cover about 15% of the overall colonies in both control and $Ecad^{-/-}$ keratinocytes. Thus, colony size and relative proportion of highly proliferative clones is unaffected upon loss of E-

cadherin in primary keratinocytes, suggesting that E-cadherin does not regulate proliferative potential.

To asses proliferation more directly and to also include situations were intercellular junctions are formed, BrdU incorporation assays were performed using either undifferentiated keratinocytes, or differentiated keratinocytes that have been subjected to high Ca^{2+} for either 24 hours or 48 hours. No difference in the amount of incorporated BrdU was observed for all conditions, suggesting that loss of E-cadherin does not affect keratinocyte proliferation *in vitro* (fig. 26B).

Taken together the results show that loss of E-cadherin does not directly affect keratinocyte migration.

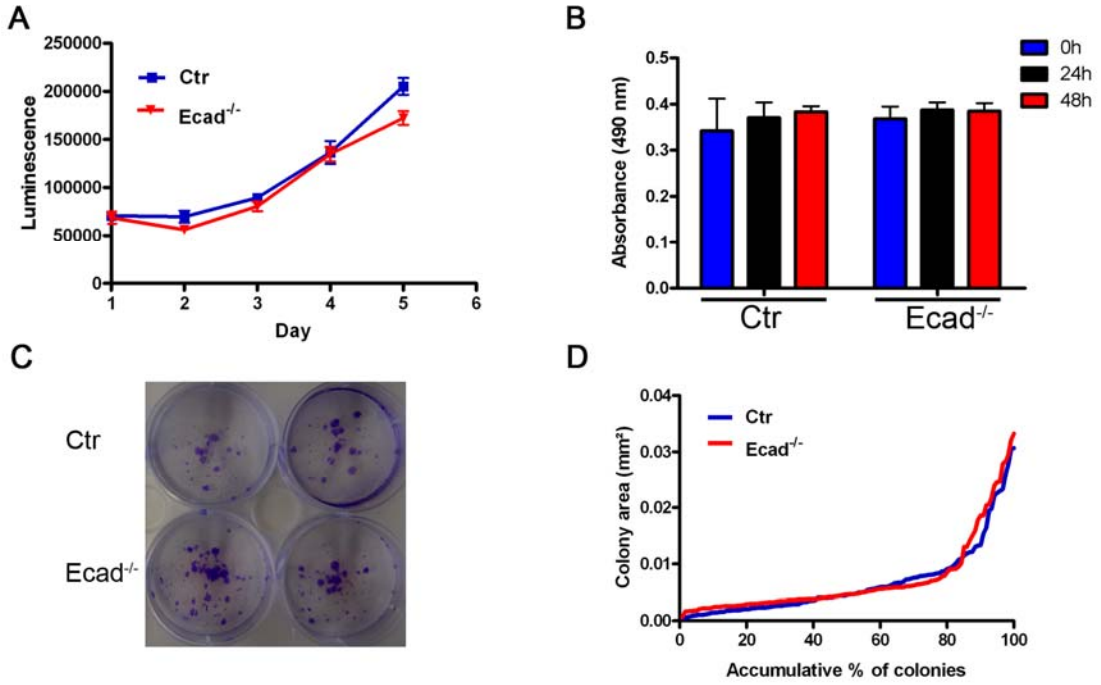


Figure 26: Growth and proliferative potential is not altered in Ecad$^{-/-}$ keratinocytes.

(A) ATP detection based viability assay to indirectly assess growth of control and Ecad$^{-/-}$ keratinocytes. (B) BrdU incorporation assay. Cells were either undifferentiated, or pre differentiated for the indicated time points. (C) Colony forming assay, plates were stained with crystal violet to visualize colonies. (D) Quantification of colony sizes and presentation in size distribution plot.

2.4.2 Keratinocyte migration is not affected upon loss of E-cadherin

To analyze the impact of E-cadherin in keratinocyte migration, control and Ecad$^{-/-}$ keratinocytes were monitored using time lapse microscopy, and migration was quantified using the tracking function of the Olympus CellR software. Trajectory was measured as the length of the migration pathway for each individual cell, and

the overall distance between start point and endpoint of migration was measured which reads out directionality of movement. Experiments using single cells in low Ca^{2+} conditions showed that E-cadherin deficient keratinocytes have the capacity to migrate in a random fashion which is comparable to control keratinocytes in both trajectory and distance (fig. 27A).

To test whether E-cadherin regulates keratinocyte migration when intercellular junctions are established, junction formation was induced by prior incubation in high Ca^{2+} medium. Migration was then analyzed in differentiating keratinocytes by performing *in vitro* wound healing experiments using the scratch method. Under these conditions, the cells appeared to migrate in a directed manner to close the gap. The closure rates of E-cadherin deficient keratinocytes showed no difference when compared to controls, indicating that the loss of E-cadherin is insufficient to induce alterations in keratinocyte migration, even in the presence of intercellular junctions. Although $Ecad^{-/-}$ keratinocytes appeared less differentiated than controls in high Ca^{2+} condition, they assembled into a stratified sheet and closed the scratch in a directed manner.

Taken together, the data do not implicate E-cadherin as a major regulator of keratinocyte migration.

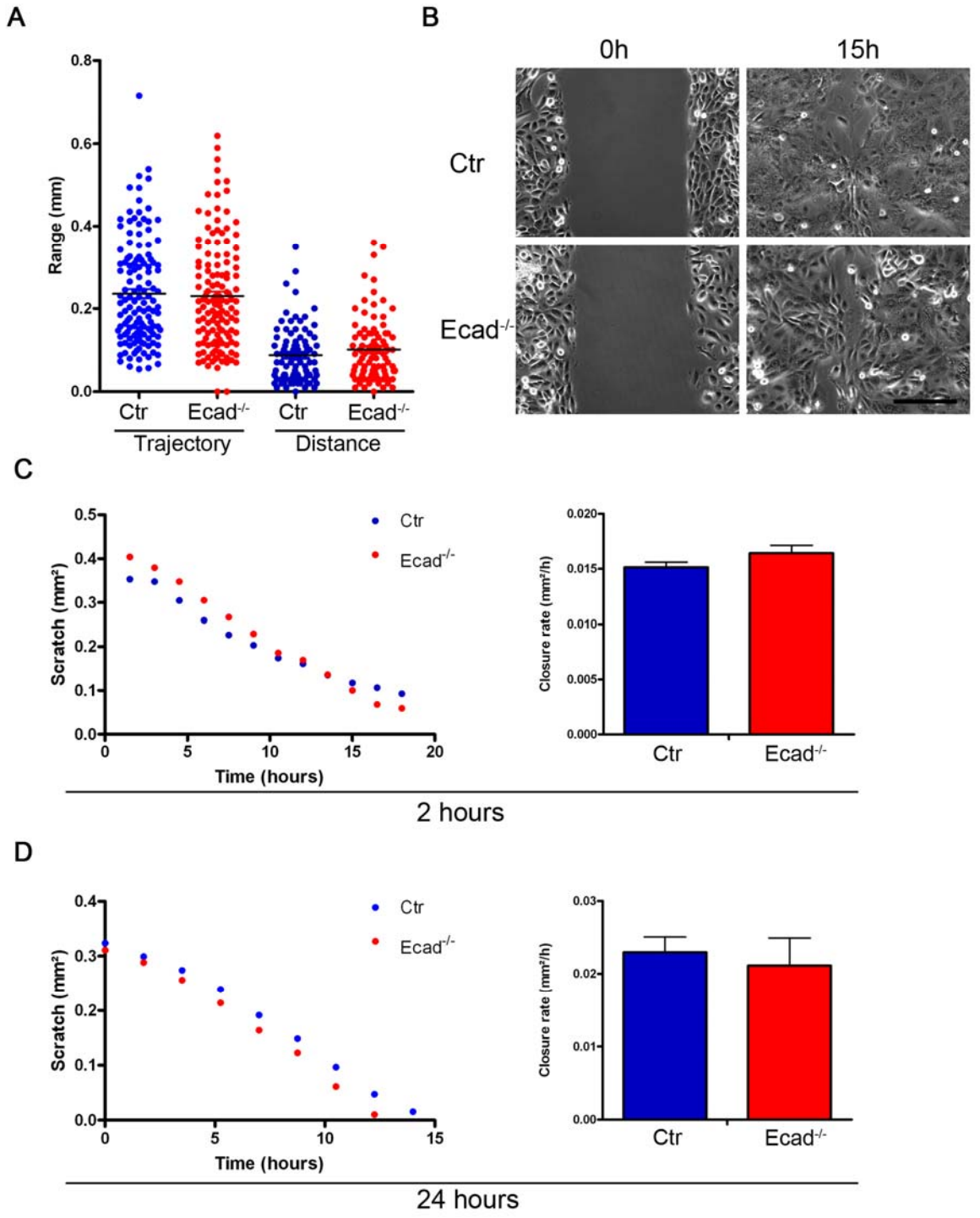


Figure 27: Keratinocyte migration is not affected by the loss of E-cadherin.

(A) Trajectory length and start to end point distance of single cells that were migrating in low Ca^{2+} conditions for 18 hours. (B) Phase contrast images of keratinocytes that were pre differentiated for 2h before scratch assay. Time points indicate hours after scratch. Bar=50 µm. (C and D) Quantification of scratch assays which were differentiated for either 2 hours (C) or 24 hours (D) prior to scratch. Cell free area was measured at indicated time points and closure rates were calculated from independent experiments.

3. Discussion

Classical cadherins have been implicated not only in the formation and regulation of adherens junctions, but also in the formation of other types of intercellular junctions, such as desmosomes and tight junctions. *In vitro* studies in simple epithelial cells indicated a specific role for E-cadherin was thought to be specifically in the formation of intercellular junctions (Gumbiner, Stevenson et al. 1988), whereas cooperative roles for E- and P-cadherin were suggested in keratinocytes (Lewis, Jensen et al. 1994). *In vivo* epidermal deletion of α-catenin resulted in loss of adherens junctions and a reduction in desmosomes, thus partially confirming *in vitro* results. (Vasioukhin, Bauer et al. 2001). However, junctions appeared normal upon *in vivo* deletion of E-cadherin either in the thyroid gland (Cali, Zannini et al. 2007) or mammary gland (Boussadia, Kutsch et al. 2002) or P-cadherin deletion (Radice, Ferreira-Cornwell et al. 1997), whereasonly tight junctions were affected upon epidermal deletion of E-cadherin (Vasioukhin, Bauer et al. 2001; Tunggal, Helfrich et al. 2005). These results raised the question whether classical cadherins are required for desmosome and tight junction formation, and whether there are specific or overlapping functions of E- and P-cadherin in epidermal junction formation. Primary keratinocytes allow one to ask if classical cadherins are required for *de novo* intercellular junction formation, a process, a question that is impossible to address *in vivo.* The results in this thesis show unequivocally that classical cadherins are crucial not only for adherens junction but also desmosome and tight junction formation. This is regulated not by a specific function of either E- or P-cadherin but, instead, is directly dependent on classical cadherin levels. Interestingly, the results indicate that classical cadherins regulate desmosomes and tight junctions on different levels. Whereas loss of E-cadherin alone already impairs tight junction function but not assembly, perhaps by regulating specific claudin expression levels, an almost complete loss of cadherins is required to interfere with desmosome formation and membrane recruitment.

3.1 Desmsome formation in primary keratinocytes depends on classical cadherin expression levels

Upon Ca^{2+} induced junction formation in primary keratinocytes, E-cadherin was not required for *de novo* formation of adherens junctions and desmosomes. However, analysis of initial kinetics of desmosomal protein recruitment revealed that desmosome formation was delayed in the absence of E-cadherin (Michels, Buchta et al. 2009). Interestingly, adherens junctions formed with similar kinetics in $Ecad^{-/-}$ keratinocytes when compared to controls, showing that initial regulation of desmosomes, but not adherens junctions was affected. Consistent with *in vivo* findings, P-cadherin was upregulated at initial, but not later stages of differentiation, suggesting that compensation for the loss of E-cadherin takes place at early time points of differentiation, allowing for adherens junction formation. Since desmosomal protein expression of plakoglobin was unaffected at early time points of differentiation in the absence of E-cadherin, a delay of membrane recruitment of these components is likely to cause the delay in desmosome formation. However, other desmosomal components like desmocollins need to be analyzed to test whether delayed desmosome formation might be explained by altered expression of desmosomal components.

The question remains, whether increased P-cadherin expression compensates for the loss of E-cadherin, or whether P-cadherin has a specific role in epidermal *de novo* desmosome formation. When P-cadherin was knocked down in E-cadherin negative keratinocytes ($Ecad^{-/-}Pcad^{kd-h}$), adherens junction and desmosome formation was impaired, showing that P-cadherin was sufficient to mediate the formation of these junctions. No desmosomes, but very few intercellular adhesive structures were found in these cells, which closely aligned membranes resembled tight junctions rather then desmosomes. Further ultrastructural analysis is required, like immuno-gold labeling approaches, to determine the molecular basis of these structures. Silencing of P-cadherin expression in control keratinocytes ($Pcad^{kd}$) did not abolish desmosome formation in primary keratinocytes, indicating that desmosome formation does not specifically require P-cadherin. Indeed, both re expression of E-cadherin and P-cadherin rescued desmosome formation in double deficient keratinocytes, showing that both classical cadherins initiate adherens junction and desmosome formation in a redundant manner and that either E- or P-cadherin is required (Tinkle, Pasolli et al. 2008; Michels, Buchta et al. 2009).

This raises the question on how classical cadherins regulate desmosome formation. Double deficient keratinocytes were able to express the desmosomal proteins desmoglein 1, 2 and 3 as well as plakoglobin and plakophilin (Michels, Buchta et al. 2009) (Diploma thesis Thomas Buchta, 2009), suggesting that impaired desmosome formation was not caused by a downregulation of these components. The possibility remains that expression or stabilization of other desmosomal components was affected, e.g. desmocollins. Alternatively, classical cadherins could potentially mediate membrane recruitment of structural desmosomal components via its cytoplasmic domain, thereby facilitating the formation of desmosomes. Plakoglobin, which associates with both desmosomal and classical cadherins has been proposed to play a key role in cadherin mediated initiation of desmosome formation (Lewis, Wahl et al. 1997). In this study, a chimeric molecule bearing the E-cadherin extra cellular domain fused to plakoglobin was able to rescue desmosome formation in a cadherin deficient epidermoid carcinoma cell line. On the contrary, plakoglobin deficient keratinocytes displayed substantial amounts of desmosomal structures that were defective in their intermediate filament anchorage, suggesting that plakoglobin is not required for initial desmosomal cadherin trans interaction (Acehan, Petzold et al. 2008).

Recruitment of the cadherin cytoplasmic binding partners was not sufficient to induce desmosome formation since expression of a chimeric molecule where the adhesive extracellular domain was replaced by the interleukin 2 receptor was not able to rescue junction formation in the double deficient cells. The expression of the classical cadherin extracellular domain lacking the cytoplasmic tail could give an answer to the question whether cadherin adhesive engagement would be sufficient for desmosome formation.

The question remains why desmosomal cadherins require classical cadherins to form desmosomes despite their intrinsic capacity to bind in a Ca^{2+} dependent manner. One requirement for adhesive trans-interaction is stable expression on the cell surface. Thus, regulation of cell surface stability of desmosomal components remains a potential mechanism by which classical cadherins regulate desmosome formation, eventually by modulating signaling pathways. E-cadherin was found to interact with EGF-receptor and other receptor tyrosine kinases via its extracellular domain, thereby decreasing receptor mobility and ligand affinity (Acehan, Petzold et al. 2008). In addition, cell surface recruitment of desmoglein 2 was shown to be

promoted by pharmaceutical EGF-receptor inhibition (Klessner, Desai et al. 2009). Indeed, pharmaceutical inhibition of the EGF-receptor rescued desmosome formation in $Ecad^{-/-}/Pcadkd^{-h}$ keratinocytes as assessed by desmoglein 3 localization (Thomas Buchta, Diploma thesis), suggesting that classical cadherins mediate desmosomal protein trafficking by regulating EGF-R activity. Further analysis of adherens junction and desmosomal component phosphorylation is required to test whether desmosomal protein trafficking is directly regulated by classical cadherin mediated regulation of EGF-R activity.

3.2 The Role of classical cadherins in epidermal barrier formation

Upon deletion of E-cadherin in the epidermis, tight junction barrier function was impaired, resulting in death of mice due to trans epidermal water loss (Tunggal, Helfrich et al. 2005), raising the question how E-cadherin regulates tight junctions.

3.2.1 E-cadherin is not required for structural tight junction formation in primary keratinocytes

In vivo epidermal deletion of E-cadherin resulted in mislocalization of claudin-1 in the granular layer of the epidermis, eventually causing barrier impairment. However, epidermal tight junction like structures were still present in these mice, raising the question whether E-cadherin is dispensable for the structural formation of tight junctions, or whether it regulates its function by mediating incorporation of specific tight junction key components (Tunggal, Helfrich et al. 2005).

In contrast to the *in vivo* situation, localization of claudin-1, 4, ZO-1 and occludin was detected at sites of intercellular contacts that did not differ from control cells, suggesting that E-cadherin is dispensable for the recruitment of these components *in vitro*. Furthermore, the ultrastructural analysis using thin section electron microscopy and freeze fracture analysis indeed revealed the presence of structural tight junctions in the absence of E-cadherin, showing that E-cadherin is dispensable for their structural formation. Importantly, quantification of tight junction strands did not reveal any differences in strand numbers or strand fragility. Instead, spatial compaction of the strand network was affected, a phenomenon which has not been described so far to our knowledge and which biological significance remains unclear. Potentially, since tight junction proteins preferentially assemble in

cholesterol and sphingolipid rich membrane micro domains (Nusrat, Parkos et al. 2000), the compaction might reflect alterations in membrane micro domain architecture.

The finding that structural tight junction formation is not affected in E-cadherin negative keratinocytes contrasts findings in simple epithelia, where E-cadherin was shown to be required for the recruitment of tight junction key components during intercellular contact formation (Gumbiner, Stevenson et al. 1988; Capaldo and Macara 2007). A likely explanation for these differences might be that E-cadherin is the only type I classical cadherin in MDCK cells, whereas two type I classical cadherins, E- and P-cadherin, are expressed in keratinocytes.

This might also account for the discrepancy regarding claudin-1 localization between the *in vivo* and *in vitro* situation, which might be explained by the finding that P-cadherin is expressed *in vitro* throughout differentiation, whereas it is confined to the basal layer *in vivo*, leading to a loss of adherens junctions in suprabasal layers. This would imply that P-cadherin can compensate for the loss of E-cadherin regarding the structural formation of tight junctions *in vitro*. However, tight junction like structures were found in $Ecad^{-/-}Pcad^{kd-h}$ keratinocytes, the possibility remains that in keratinocytes tight junctions can structurally form independently of classical cadherins.

3.2.2 E-cadherin is required for epidermal barrier formation

Analysis of tight junction function by TER revealed barrier impairment of the tight junctions in the absence of E-cadherin, showing increased ion permeability. Furthermore, size specific tracer diffusion assays showed increased permeability for both 3kD and 40kD non-ionic dextran tracers in the absence of E-cadherin. Thus, increased permeability appears to involve non ionic molecules and is not restricted to certain molecular weights.

In conclusion, the ultrastructural tight junctions that were present in $Ecad^{-/-}$ keratinocytes failed to function as an epidermal barrier, showing that E-cadherin regulates tight junction function not only *in vivo*, but also *in vitro* and that the latter regulation occurs independently of structural tight junction formation. Furthermore, it shows that the formation of a structural tight junction network is not sufficient for

epidermal tight junctional sealing and that an E-cadherin dependent regulation is required to trigger barrier formation.

Several molecular mechanisms could mediate this regulation. Post translational modification of tight junction proteins could be involved, e.g. phosphorylation. Many tight junction proteins are subject to phosphorylation which can generally affect paracellular permeability. For instance, tyrosine phosphorylation of ZO proteins and occludin can occur under conditions that both favour tight junction formation (Van Itallie, Balda et al. 1995; Tsukamoto and Nigam 1999) or occur during loss of barrier formation in conditions like oxidative stress (Oldstone, Nerenberg et al. 1991; Rao, Basuroy et al. 2002; Basuroy, Sheth et al. 2003). Likewise, claudin phosphorylation can result in either increase or decrease of permeability. Claudin-1 phosphorylation was implicated in barrier function since mutation of the potential MAPK phosphorylation site Threonin 203 resulted in decreased TER in endothelial cells (Fujibe, Chiba et al. 2004). Phosphorylation of claudin-4 at tyrosin 208 increased paracellular permeability by attenuating its association with ZO-1 (Tanaka, Kamata et al. 2005). In addition, serine 195 of claudin-4 was identified as a substrate for aPKC and this regulated barrier formation in human keratinocytes (Aono and Hirai 2008). Indeed, inhibition of aPKC by pseudo substrate application did abolish TER but did not affect tight junction component localization, reminiscent of E-cadherin deficient keratinocytes (Helfrich, Schmitz et al. 2007). Considering the mislocalization of phosphorylated aPKC in E-cadherin negative epidermis, it is tempting to speculate that E-cadherin mediates barrier formation by regulating aPKC at the tight junction. However, whereas overexpression of aPKC enhanced barrier formation in the study by Helfrich et. al, no rescue was obstained by overexpressing aPKC in $Ecad^{-/-}$ keratinocytes. If E-cadherin acts upstream by either recruiting or activating aPKC, increase of aPKCλ protein levels might not be sufficient. Attempts to test the requirement of aPKC membrane recruitment by expressing a membrane tagged aPKCcaax fusion protein failed due to lack of expression in keratinocytes.

Rac1, an upstream activator of aPKC, was implicated in epidermal tight junction formation by regulating the Par complex (Mertens, Rygiel et al. 2005) and was found to be mislocalized in E-cadherin negative epidermis. Since E-cadherin is known to activate Rac1 in simple epithelia, these results favour the hypothesis that E-cadherin regulates tight junction via a Rac1-aPKC activation pathway. Indeed,

Ecad$^{-/-}$ keratinocytes displayed reduced Rac activity, showing that E-cadherin activates Rac1 in primary keratinocytes. However, expression of a constitutively active mutant RacL61 did not rescue barrier formation. This might be due to the fact that Rho family GTPases need fine tuned regulation at intercellular junctions, since either constitutively active or negative mutants interfere with tight junction formation (Jou, Schneeberger et al. 1998).

PI3-kinase was proposed as a candidate to mediate cadherin dependent regulation of Rac1 (Kovacs, Ali et al. 2002), since it directly associates with E-cadherin and can by itself activate Rac1 (Reif, Nobes et al. 1996). In addition, PI3-kinase can associate with ZO-1 and affect tight junctional sealing (Woo, Ching et al. 1999). Potential influence of PI3-kinase on epidermal barrier formation was assessed by inhibiting its activity with Wortmannin. Interestingly, no impairment of barrier formation was observed, suggesting that, at least in stratifying keratinocytes, PI3-kinase is not involved in epidermal barrier regulation.

Rac1 is a regulator of the actin cytoskeleton and tight junction function depends on proper actin organization (Miyoshi and Takai 2008). However, phalloidin staining did not show any alterations in cortical actin recruitment in the absence of E-cadherin, suggesting that tight junctions might be properly linked to the actin cytoskeleton. However, this method does not provide insights into possible changes in actin dynamics which could also influence tight junction function. Dynamic regulation of actinomyosin contraction has been implicated in the physiological regulation of tight junction permeability (Turner 2006). Especially phosphorylation of non muscle myosin II by myosin light chain kinase has been proposed to affect tight junction function via regulation of actinomyosin contractility (Shen, Black et al. 2006). To address the relevance of actinomyosin contraction, myosin ATPase activity was inhibited by Blebbistatin application. A dose dependent impairment of barrier function was observed, which was accompanied by severe disruption of the actin cytoskeleton. Interestingly, when Blebbistatin was applied at similar concentrations to wild type MDCK cells, no effect on TER was observed, whereas Blebbistatin treatment of ZO-1 knock down MDCK cells resulted in an increase in TER (Van Itallie, Fanning et al. 2009). This suggests tissue specific differences in the role of myosin in the regulation of tight junctions and indicates that proper actinomyosin contractility is critical during epidermal barrier formation in keratinocytes.

Taken together the data shows that E-cadherin is dispensable for structural tight junction formation, but required for the regulation of epidermal barrier formation by a not yet defined mechanism.

3.2.3 Keratinocyte barrier formation depends on classical cadherin levels

An important question remains whether there is a specific requirement for E-cadherin in tight junctional barrier formation. Despite the upregulation of P-cadherin no barrier formed in the absence of E-cadherin, suggesting a specific function for E-cadherin. Alternatively, a reduction in overall classical cadherin levels, as judged by β-catenin expression levels, might be responsible if barrier formation is dependent on classical cadherin levels. Silencing of P-cadherin in control keratinocytes did not result in major impairment of tight junction function, showing that E-cadherin, which did not upregulate upon P-cadherin silencing, is sufficient for barrier formation and arguing for a specific role of E-cadherin. However, re-expression of E-cadherin as well as over expression of P-cadherin in $Ecad^{-/-}$ keratinocytes rescued barrier function, showing that both molecules have the capacity to initiate barrier formation in a redundant manner. In addition, it suggests a requirement for classical cadherin levels. However, the possibility remains that regulation of tight junction function is specific function for both E- and P-cadherin. Other classical cadherins like N-cadherin need to be tested to verify whether functional regulation of tight junctions is indeed a common function of the classical cadherin family.

E-cadherin negative epidermis displayed normal intercellular adhesion and $Ecad^{-/-}$ keratinocytes are able to form desmosomes as well as structural tight junctions, suggesting that E-cadherin mediates barrier regulation via signalling, rather than providing physical cohesion. Thus, E-cadherin mediated tight junction regulation might be independent of its adhesive activity. The requirement for E-cadherin extracellular domain and whether recruitment of cytoplasmic binding partners is sufficient for barrier regulation was addressed by expression of the previously desribed IL2R-tail chimeric molecule. However, further increase of paracellular permeability was observed. In addition, phase contrast microscopy suggested impairment of intercellular contact formation, hinting at dominant negative effects of the construct towards cadherin mediated adhesion.

Taken together, either E- or P-cadherin is required for epidermal barrier formation, and cadherin levels that exceed the requirement for adherens junction and desmosome formation are needed for functional initiation of barrier formation, suggesting an overall requirement for classical cadherin levels in epidermal tight junction barrier formation.

3.2.4 E-cadherin regulates claudin-14 expression

Claudins mediate size and charge selectivity of the tight junction and several claudin isoforms can associate in a homophilic and heterophilic manner within a tight junction strand, thus determining permeability properties (Hewitt, Agarwal et al. 2006). Therefore, regulation of claudin gene expression might be a potential mechanism by which tight junctions are regulated. Indeed, a recent paper demonstrated that claudin-5 expression, critical for endothelial barrier formation, was directly dependent on VE-cadherin expression, showing that classical cadherins can directly take part in gene regulation (Taddei, Giampietro et al. 2008). The question whether E-cadherin participates in claudin regulation in an epidermal system was addressed by RT-PCR profiling of the different claudin variants. Only claudin-14 was consistently down regulated in the absence of E-cadherin *in vivo* and *in vitro*, showing that E-cadherin can take part in regulation of tight junction protein expression and presenting a potential mechanism how E-cadherin might regulate tight junctions.

Mutations in the claudin-14 gene result in the nonsyndromic deafness DFNB29 in human, highlighting the importance of tight junctions in the hearing process (Wilcox, Burton et al. 2001). Claudin-14 knock out mice suffer from hearing loss and serve as a model for studies on DFNB29 related diseases (Ben-Yosef, Belyantseva et al. 2003). However, these mice are viable and don't show any obvious skin phenotype, suggestive for an intact epidermal barrier. Since a complete knock out was analyzed in the study, compensation by other claudins could have taken place in these mice, since altered claudin isoform expression has been demonstrated upon deletion of claudin-9 and claudin-14 (Elkouby-Naor, Abassi et al. 2008). Importantly, claudin-14 was shown to assemble into functional strands when expressed in fibroblasts, resulting in formation of a paracellular barrier (Wattenhofer, Reymond et al. 2005). Thus, down regulation of claudin-14

might contribute to increased paracellular permeability in Ecad$^{-/-}$ epidermis and keratinocytes. The question of the physiological relevance of claudin-14 in epidermal barrier formation was addressed by silencing its expression in control keratinocytes. ShRNA mediated silencing of claudin-14 up to 50% did not affect barrier formation, suggesting that either claudin-14 is dispensable for barrier formation, or that reminiscent claudin-14 protein was still sufficient to induce barrier formation. Claudin-1 expression was used as a control and was not affected in the different knock down clones. However, other claudins must be profiled to test whether compensation occurs via upregulation of other claudins.

A causal role of claudin-14 down regulation in the barrier dysfunction Ecad$^{-/-}$ keratinocytes would predict that ectopic expression of claudin-14 in these cells would restore the tight junction barrier. However, no rescue of barrier formation was observed upon overexpression of human claudin-14. Instead, cell shape, level of stratification and ZO-1 localization was affected in hCl-14 expressing cells, which perhaps was caused by overexpression and thereby resulted in disturbance of tight junction homeostasis.

Taken together, the downregulation of claudin-14 messenger RNA in the absence of E-cadherin suggests that E-cadherin can exhibit gene regulatory function in the epidermis. The precise relevance of claudin-14 in barrier function remains elusive, and better knock down efficiencies are needed in order to clarify its function. In addition, selection for lower claudin-14 expression levels might avoid overexpression artefacts in claudin-14 rescue experiments and might clarify whether claudin-14 downregulation is causing barrier dysfunction in the absence of E-cadherin.

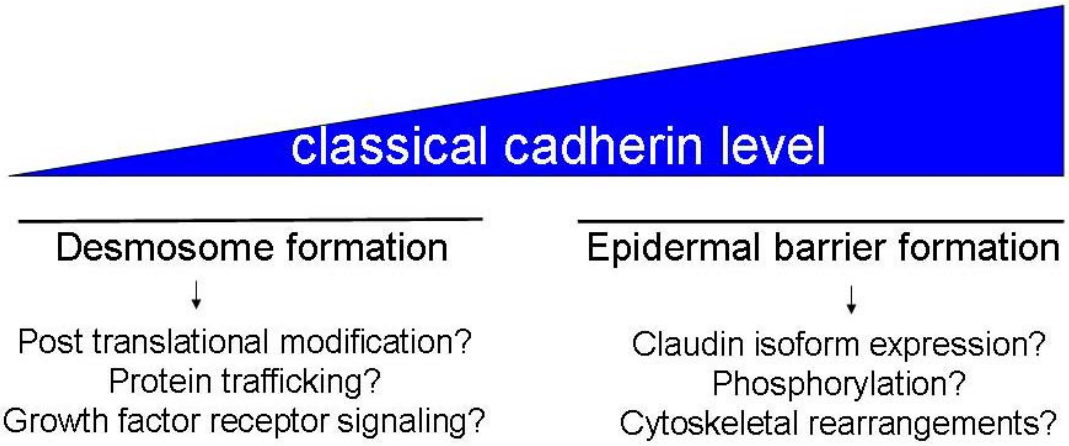


Figure 28: Model and working hypotheses.

Classical cadherin might regulate desmosome and tight junctional barrier formation on different levels.

3.3 E-cadherin does not regulate keratinocyte proliferation and migration

Growth and proliferation analysis as well as the analysis of the proliferative potential did not show any impairment of these processes in the absence of E-cadherin. This was true for both low and high Ca^{2+} conditions, suggesting that E-cadherin is not a critical regulator of keratinocyte proliferation.

Migration analysis revealed that E-cadherin deficient keratinocytes do not show any changes in their capacity to migrate. The results show that loss of E-cadherin is not sufficient to cause any changes in the migratory properties of the keratinocytes, suggesting that it is not critically involved in their regulation.

The analysis of P-cadherin knock down keratinocytes is required to show whether compensation by P-cadherin upregulation occurs, or whether classical cadherins are dispensable for keratinocyte growth regulation.

The results show that loss of E-cadherin is not sufficient to induce an invasive phenotype in primary keratinocytes, suggesting that other mechanisms participate upon loss of E-cadherin in cancer cells.

4. Material and Methods

4.1 Cell Culture and lentiviral transduction

4.1.1 Isolation and culture of primary keratinocytes

Epidermis of newborn pubs was separated from dermis by overnight trypsin digestion and subsequent mechanical stripping. Epidermis was chopped into peaces and agitated in low Ca^{2+} (50 µM) keratinocyte medium at 37 degree for 1 hour. Cell suspension was plated on collagen type 1 coated dishes (0.03 mg/ml) in co-culture with a J2 3T3 fibroblast feeder layer. All experiments were performed on collagen type 1 coated dishes.

Keratinocyte cell culture medium

DMEM (FAD)-medium (Growth medium for keratinocytes)

- 10 %	FCS, chelated
- 0.4 µg/ml	hydrocortisone
- 5 µg/ml	insulin
- 10 ng/ml	epidermal growth factor (EGF)
- 10^{-10} M	cholera toxin
- 100 units/ml	penicillin and 100 µg/ml streptomycin
- 2 mM	L-glutamine

PBS: Dulbecco´s phosphate buffered saline without calcium and magnesium

Trypsin: 1x trypsin/ EDTA 0.05 % (w/v)

4.1.2 Splitting

In order to split the cells growth medium was removed and the cells were washed twice with PBS and trypsinized for approximately 10 min at 37 °C. Trypsin activity was disabled by adding 3 fold amount of DMEM medium. The suspension was transferred into a 15 ml falcon and centrifuged at 800 rpm for 5 min. The supernatant was discarded and cells were resuspended in required volume of growth medium and plated on newly coated 6 cm dishes.

4.1.3 Differentiation and induction of intercellular contact formation by Ca^{2+} switch

Confluent keratinocytes were differentiated by raising calcium level from 50 µM to 1.8 mM and subsequent incubation for indicated time points.

4.1.4 Production of lentivirus and lentiviral transduction

Lentivirus for transgene expression was produced by lipofectamin 2000 (Invitrogen) mediated co-transfection of TLA-HEK293T (Invitrogen) packaging cells with pCMVlenti lentiviral expression vector (gift from AG peifer, Bonn) and ViraPower packaging plasmid mix (Invitrogen). Transfection was performed according to the manufactures instructions. Packaging cells were cultured in keratinocyte growth medium and virus containing supernatant was collected for 3 subsequent days after transfection. Transduction was achieved by overnight incubation of primary keratinocytes in virus containing supernatant. Polybrene (5µg/ml) was added to enhance transduction efficiency.

4.1.5 Lentiviral gene silencing by shRNA transduction

Lentiviral Particles containing either specific shRNA sequences or non targeting control sequence (ntc) were purchased from sigma. Virus containing solution was added to keratinocytes medium. 48 hours after transduction, cells were selected by culturing in puromycin containing growth medium (1µg/ml).

shRNA sequences:

Silencing of mouse P-cadherin (NM_007665.1-3014s1c1):

CCGGCCTGGTACATTTCTCTGACATCTCGAGATGTCAGAGAAATGTACCAGGTTTTG

Silencing of mouse claudin-14 (NM_019500.3-1295s1c1):

Sh1:

CCGGGACCAATGATGGATGTGGGAACTCGAGTTCCCACATCCATCATTGGTCTTTTTG

Sh2:
CCGGACGAATGACGTGGTGCAGAATCTCGAGATTCTGCACCACGTCATTCGT
TTTTTG
Sh3:
CCGGACAGGCTGAATGACTACGTGTCTCGAGACACGTAGTCATTCAGCCTGT
TTTTTG
Sh4:
CCGGCCCAGTGGCATGAAGTTTGAACTCGAGTTCAAACTTCATGCCACTGGG
TTTTTG
Sh5:
CCGGCCGGAGCTACCACCACGGCTACTCGAGTAGCCGTGGTGGTAGCTCCG
GTTTTTG

4.2 Protein Analysis

4.2.1 Immunoblot analysis of primary keratinocytes

Cultured keratinocytes were lysed in NP40 buffer (1% NP40, 0.1% SDS, 0.5% deoxycholate, 150 mM NaCL, 50 mM Tris pH7.4). After 10 min of incubation on ice the cells were harvested with a cell scraper and transferred to a reagent tube. Lysates were cleared by centrifugation at 13000 rpm for 10 min at 4°C. Protein concentrations were determined using Bradford assay (Biorad). Lysates were diluted in Laemmli buffer and proteins were separated by SDS PAGE on either 7% or 4-12% precast gels (NuPage system) and transferred to nitrocellulose according standard blotting procedures. Membranes were blocked with 5% non fat dry milk (Haerschle) in TBS-T (0.1% Tween 20, 137 mM NaCl, 20 mM Tris pH 7.5) and incubated with the primary antibody diluted in blocking solution over night at 4°C. After 3 times washing in blocking solution, the membranes were incubated with the appropriate horseradish peroxidase coupled secondary antibody. Immunoreactive proteins were detected by enhanced chemiluminescence using either the SuperSignal West Pico or SuperSignal West Femto kit (Pierce).

4.2.2 Immunofluorescence analysis of keratinocytes

Keratinocytes were plated in 24 well plates on glass collagen coated coverslips. Differentiation was induced by switching from low calcium medium (50 µM) to medium containing 1.8 mM calcium for the indicated timepoints. Cells were washed with PBS and fixed for 10 min with ice cold methanol, 4 % PFA/PBS or acetone, depending on the antibody used in the experiment. 4% PFA/PBS fixed cells were permeabilized by 5 min incubation with 0.5% Triton X-100/PBS. After 3 rinses in PBS, unspecific binding sites were blocked with 1%BSA/PBS for 30 min. All antibodies were diluted in blocking solution and incubated for 1 hour at room temperature, followed by 3 washes in blocking solution for each 5 minutes. After washing, coverslips were incubated with approbriate secondary antibodies coupled to either Alexa 488 or Alexa 594. Nuclei were counterstained with either DAPI or propidium iodide. Coverslips were mounted with gelvatol on microscope slides (VWR). An Olympus IX71 microscope in a Deltavision system was used for analysis (Precision Instruments). Images were taken using a Coolsnap HQ2 Camera and processed with Adobe Photoshop.

4.2.3 Rac GTPase activity assay

Keratinocytes were lysed in NP40 buffer containing biotinylated PAK-CRIB peptide (0.025 mg/ml). All steps were performed at 4°C. Lysates were rotated for 45 minutes and cleared by centrifugation at 13000 rpm for 10 minutes. After spinning, total lysate samples were taken, and 30 ml of streptavidin agarose was added to bind biotinylated PAK-CRIB/RacGTP complexes. After 30 minutes incubation, beads were pelleted by centrifugation at 6000 rpm for 30 seconds. The supernatant was removed and pellet was washed 3 times in lysisbuffer (w/o NP40). Pellet was collected in Laemmli buffer and heated to 95°C for 5 min followed by SDS-PAGE and immunoblot analysis. Blots were densitometrically quantified by using ImageJ software.

4.2.4 Antibodies and antisera

4.2.4.1 Primary antibodies

Antigen	host	Workin dilution	clone number	Company/Lab
β-catenin monoclonal	mouse	WB 1:500 IF 1:100	14	BD Transduction labs
Desmoglein ½ monoclonal	mouse	WB 1:500 IF 1:100		progen
Desmoglein 3 monoclonal	mouse	WB 1:500	AK18	MBL
Desmoplakin polyclonal	rabbit	WB 1:5000 IF 1:50		Gift from Kathy green lab
E-cadherin monoclonal	mouse	WB 1:2000	C20820	BD Transduction labs
p120ctn monoclonal	mouse	WB 1:1000 IF 1:100	Clone 98	BD Transduction labs
Plakoglobin monoclonal	mouse	WB 1:100 IF 1:5	PG 5.1	Progen
Plakophilin 3 monoclonal	mouse	WB 1:1000 IF undiluted	310.9.1	Progen
Claudin-1 polyclonal	rabbit	IF 1:200		Zymed
Claudin-4 polyclonal	rabbit	IF 1:200		Zymed
Occludin polyclonal	rabbit	IF 1:25		Zymed
ZO-1 polyclonal	rabbit	IF 1:100		Zymed
Rac1 monoclonal	mouse	WB: 1:1000	23A8	Sigma
Actin monoclonal	mouse	WB: 1:20000	C4	MP Biomedicals
Human Claudin 14 polyclonal	goat	IF undiluted WB 1:10		Abcam

P-cadherin monoclonal	rat	WB 1:10 IF undiluted	PCD-1	Hybridoma supernatant
Tricellulin polyclonal	rabbit	IF undiluted		
aPKCλ monoclonal	mouse	WB 1:250		BD Transduction labs
p-aPKCλ polyclonal	rabbit	WB 1:250		Cell signaling

4.2.4.2 Secondary antibodies

Antigen	**host**	**Workin dilution**	**Company/Lab**
Alexa fluor 488 anti-mouse	goat	IF 1:500	Molecular probes
Alexa fluor 488 anti-rabbit	goat	IF 1:500	Invitrogen
Alexa fluor 594 anti- rabbit	donkey	IF 1:500	Molecular probes
Alexa fluor 594 anti- rat	donkey	IF 1:500	Molecular probes
Alexa fluor 594 anti- mouse	goat	IF 1:500	Molecular probes
IgG-HRP anti-rabbit	goat	WB: 1:5000	BioRad
IgG-HRP anti-mouse	goat	WB: 1:5000	BioRad
IgG-HRP anti-rat	goat	WB: 1:1000	DaKo

4.3 Barrier assays

4.3.1 Trans epithelial resistance measurement (TER)

500 000 Keratinocytes were plated on collagen 1 coated polycarbonate filter inserts (pore size 0.4 µm, Millipore) in a 24 well format. On day 1 after plating, junction and barrier formation was induced by Ca^{2+} switch and electrical resistance was measured at indicated time points by using the Millicell TER measurement system (Millipore).

4.3.2 Paracellular diffusion of nonionic tracers

500 000 Keratinocytes were plated on collagen 1 coated polycarbonate filter inserts (pore size 0.4 µm, Millipore) in a 24 well format. 72 hour after Ca^{2+} switch, the medium in the apical compartment was replaced by medium containing either FITC-Dextran 3kD (0.2 mg/ml) or FITC-Dextran 40kD (0.2 mg/ml) (Molecular Probes). After 2 hours incubation, 100 µl samples were taken from the basal compartment and fluorescence intensity was measured by using a Victor^3 1420 multilabel counter (Perkin Elmer).

4.4 Electron microscopy

4.4.1 Thin section transmission electron microscopy

Thin section electron microscopy was performed by the laboratory of Willhelm Bloch (Deutsche Sporthochschule Köln). In brief, keratinocytes were plated on collagen coated foil and were cultured for 48h in medium containing 1.8 mM Ca^{2+} to allow for the establishment of intercellular junctions and incubated with biotin-dextran (0.2 mg/ml) for 10 minutes. Cells were fixed in 0.1M PBS containing 4 % PFA and subsequently rinsed in 0.1 M PBS. Afterwards preparations were postfixed with 2% osmium tetraoxide in 0.1 M PBS for 2 hours at 4 °C. Before embedding in araldite (Ciba-Geigy, Basel, Switzerland) the cells were dehydrated in a graded series of ethanol. Ultrathin sections (60 nm) were mounted on formvar-

coated copper grids, stained with 0.2% uranyl acetate and lead citrate, and then examined using a Zeiss (Jena, Germany) EM 902 A electron microscope.

4.4.2 Freeze fracture electron microscopy

Freeze fracture analysis of tight junction strands was performed by Susanne Krug in the laboratory of Michael Fromm (Charité, Berlin). In brief, keratinocytes were plated on filter inserts in a confluent manner and differentiated for 72 hours in high Ca^{2+}. Samples were fixed in Glutaraldehye/PBS for 1 hour. Prior to freeze fracture, samples were stepwise dehydrated with first 10% glycerol and then 30% glycerol for each 30 minutes. After detachment of the cells from the filter with a scalpel, samples were transferred into the closable freeze fracture container, which was then frozen by Freon 22. The frozen container was then inserted into the pre-cooled freeze fracture apparatus, where the container was opened under vacuum conditions ($2*10^{-7}$ Torr, -100°C), leading to fracture of the epithelium. For contrast enhancement, fractured samples were evaporated with platinum and carbon and transferred to a copper grid for electron microscopy anaylsis.
Images were taken with a Zeiss 902 electron microscope and pictures were analysed using the iTEM software(Olympus).

4.5 Molecular cloning

4.5.1 Bacterial Transformation

The *E.coli* strain DH5α was cultured as described in Sambrook et al,, 1989. For transformation and production of chemically competent DH5α, the method of Hanahan was applied (Hanahan 1983).

4.5.2 Recombinant DNA techniques

The following recombinant standard DNA techniques were performed as described in Sambrook et al., 1989, or according to the manufacturers instructions: restriction digestion, T4 DNA ligation, dephosphorylation of DNA fragments, agarose gel

electrophoresis and elution of DNA fragments, phenol/chloroform extraction and ethanol precipitation. All DNA modifying enzymes (i.e. restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, Klenow fragment polymerase) were purchased from New England Biolabs. DNA sequencing was performed by the service laboratory of the CMMC (Center for Molecular Medicine Cologne) and sequences were analysed using the gENTLE sequence analysis software (Magnus Manske, Cologne).

4.5.3 Polymerase Chain Reaction (PCR)

For several cloning strategies polymerase chain reactions were performed to amplify DNA fragments. The Pfu DNA polymerase (Stratagene) was used at a concentration of 0.1 U/µl in combination with 0.25 µM of both sense and antisense primers (MWG-Eurofin), 0.25 mM dNTP mix (Roche), 1x reaction buffer (stratagene) and 10 ng plasmid DNA as template in 50 µl reaction volume. The template DNA was denatured at 95°C for 2 min followed by 35 rounds of amplification each consisting of 1 min at 95°C, 1 min at the appropriate annealing temperature, and 2 min per kb length of the amplified DNA fragment at 72°C for elongation. Based on the number of specific nucleotides in the primer, the following formula was used to estimate the melting temperature of primers: $T_m=2(A+T)+4(G+C)$.

4.5.4 Constructs and cloning strategies

All constructs were cloned into pCMVlenti vector which was kindly provided by Alexander Pfeifer (Bonn) and described in (Pfeifer, Kessler et al. 2001). The vector was provided as pCMVlenti-GFP. For all cloning strategies, GFP insert was removed by BamH I/Sal I digestion.

pCMVlenti-Ecad

mouse E-cadherin cDNA was sub-cloned from pBatem-E-cadherin by Bgl II/NrU I digestion. Both pCMVlenti vector backbone and E-cadherin insert were subjected to 5`overhang fill in reaction by using klenow polymerase and subjected to blunt end ligation. The correctness of insert orientation and sequence was confirmed by DNA sequencing.

pCMVIenti-Pcad

P-cadherin cDNA was subcloned from pCDNA3 by Hind III/Xba I digestion, followed by Klenow polymerase fill in reaction to generate blunt ends. The purified insert was ligated into the pCMVIenti vector backbone. Correctness of sequence and orientation was confirmed by sequencing.

pCMVIenti-IL2Rtail

IL2Rtail was subcloned from pCMV-IL2Rtail by Nhe I/Xba I digestion, followed by klenow fill in reaction to generate blunt ends. The purified insert was ligated into the pCMVIenti vector backbone. Correctness of sequence and orientation was confirmed by sequencing.

pCMVIenti-eGFP-aPKCλ

eGFP-aPKCiota was amplified by PCR thereby introducing BamH I and Sal I restriction sites at the 5` and 3` termini, respectively. After purification of the PCR product and digestion with BamH I and Sal I, the insert was ligated into the BamH I/Sal I digested pCMVIenti. The correctness of the sequence was confirmed by sequencing.

pCMVIenti-mycRacL61

The mycRacL61 was subcloned from a pCS2+ vector by BamH I/Xho I digestion. After insert purification, mycRacL61 was directly ligated into the BamH I/Sal I digested pCMVIenti vector by compatible end ligation. The correctness of the sequence was confirmed by sequencing.

pCMVIenti-hCl14

Human claudin-14 cDNA was amplified by PCR from pLOX-Myc-GFP-claudin-14 thereby introducing BamH I and Sal I restriction sites at the 5` and 3` termini, respectively. After purification of the PCR product and digestion with BamH I and Sal I, the insert was ligated into the BamH I/Sal I digested pCMVIenti. The correctness of the sequence was confirmed by sequencing.

4.6. Reverse transcription and Real time PCR

4.6.1 RNA isolation from tissues and cells

Isolation of RNA from epidermis and cultured keratinocytes was achieved by using RNeasy mini kit (Quiagen) according to the manufacturers instructions.

4.6.2 cDNA synthesis

For first strand cDNA synthesis of RNA, random primers in combination with Superscript II Reverse transcriptase (Invitrogen) was used according to the manufacturer's protocol.

4.6.3 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed using REDTaq Readymix. 1µl of cDNA synthesis per reaction was used as Template and either 20, 25 or 30 cycles of amplification were chosen.

4.6.4 Quantitative real time PCR

Quantitative Real time PCR was performed in 96 well format using Power SYBRGREEN PCR mastermix (Applied Biosystems) with 1µl of cDNA synthesis reaction as template in 50µl reaction volumes. Measurements were performed in a StepOne plus Real time PCR cycler system and results were analysed using the the StepOne analysis software (Applied Biosystems).

4.6.5 RT-PCR primer list

Gene	Accession	Sense primer	Antisense Primer
Cldn1	NM_016674	TCTACGAGGGACTGTGGATG	TCAGATTCAGCAAGGAGTCG
Cldn2	NM_016675	GGCTGTTAGGCACATCCAT	TGGCACCAACATAGGAACTC
Cldn3	NM_009902	AAGCCGAATGGACAAAGAA	CTGGCAAGTAGCTGCAGTG
Cldn4	NM_009903	CGCTACTCTTGCCATTACG	ACTCAGCACACCATGACTTG

Cldn5	NM_013805	GTGGAACGCTCAGATTTCAT	TGGACATTAAGGCAGCATCT
Cldn6	NM_018777	CATTACATGGCCTGCTATTC	CACATAATTCTTGGTGGGATATT
Cldn7	NM_016887	AGGGTCTGCTCTGGTCCTT	GTACGCAGCTTTGCTTTCA
Cldn8	NM_018778	GCCGGAATCATCTTCTTCAT	CATCCACCAGTGGGTTGTAG
Cldn9	NM_020293	GTCACACTTTGAGCGTCCC	CCTCTTATCCAGTCCCGAAG
Cldn10	NM_021386	CCCAGAATGGGCTACACATA	CCTTCTCCGCCTTGATACTT
Cldn11	NM_008770	TCTGGTTTCCTGTATGTGCC	CGTACAGCGAGTAGCCAAAG
Cldn12	NM_022890	GTCCTCTCCTTTCTGGCAAC	ATGTCGATTTCAATGGCAGA
Cldn13	NM_020504	TAGTGTTGGCCTTCTGATGC	AGCCAAGCAATGGGTTAAAG
Cldn14	NM_019500	GCTCCTAGGCTTCCTGCTTA	CTGGTAGATGCCTGTGCTGT
Cldn15	NM_021719	CAGCTTCGGTAAATATGCCA	CAGTGGGACAAGAAATGGTG
Cldn16	NM_053241	GCCATATTCTCCACTGGGTT	AGTCATCAGCGTTCACCATC
Cldn17	NM_181490	TCGTTCTGATTCCAGTGTCC	TCCTCCAAGTTCTCGCTTCT
Cldn18	NM_019815	GACCGTTCAGACCAGGTACA	GCGATGCACATCATCACTC
Cldn19	NM_153105	ACCAGAATGAGGACCAGGAT	TCCTTCAGCAAATACGTTGG
Cldn23	NM_027998	TGTGCTTGAGGGAGAAGAAA	TGGCAGAAGTTCAAGTCACC
Tricellulin	NM_019500	CTCCTTTTTTTCCAGAAACG	ACATCATTCTGAAAACCGGC
GAPDH	NM_008084	TGCCCCCATGTTTGTGATG	TGTGGTCATGAGCCCTTCC

4.7 Cell migration analysis

4.7.1 Single cell random migration

30 000 keratinocytes were plated on collagen coated six well plate and monitored over time using phase contrast time lapse microscopy. Images were taken every 15 min in standard tissue culture conditions (5% Co2, 32°C, 60% humidity) using an Olympus OBS CCD FV2T camera on an IX81 Olympus microscope. Single cell migration was analyzed over an 18 hour period using the tracking function of the CellR software package (Olympus).

4.7.2 Scratch assay

Keratinocytes were plated in a confluent manner on collagen coated six well plates and differentiated for the indicated time points. Scratching was performed by a gentle stroke with a 200 µl pipette tip. Two hours before scratch, keratinocytes were treated with Mitomycin C (4µg/ml) to induce proliferation arrest. Images were taken every 15 min in standard tissue culture conditions (5% Co2, 32°C, 60% humidity) using an Olympus OBS CCD FV2T camera on an IX81 Olympus microscope. Scratch closure was quantified by measuring the decrease of cell free scratch area over time using the CellR software measurement tools (Olympus).

4.8 Analysis of growth and proliferative potential

4.8.1 Cell viability assay

To indirectly assess growth, ATP detection based cell viability was measured in subsequent days after plating. 10 000 keratinocytes were plated in 96 well format. Up to 5 days after plating, ATP content was measured using the Cell titer Glo (Promega) luminescent viability assay according to the manufacturers instructions. Readings were taken by colorimetric measurement using a Victor^3 1420 multilabel counter (Perkin Elmer).

4.8.2 BrdU incorporation assay

BrdU incorporation was measured by using the colorimetric Cell Proliferation ELISA, BrdU from Roche. 5000 Keratinocytes were plated in a 96 well format. 1 Day after plating, cells were pulse labelled with BrdU for 3 hours. Measurements were taken according to the manufacturers instructions.

4.8.3 Colony forming assay

4000 keratinocytes were plated on collagen coated six wells in co culture with a J23T3 feeder layer. Cells were fixed with 1% PFA for 15 min and subsequently stained for 1 h with 0.05% crystal violet in PBS. Images of the plates were taken and colony size was measured using ImageJ software.

5. Abrreviations

AJ	adherens junction / zonula adherens
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary DNA
DAPI	4′,6-Diamidino-2-phenylindol
DNA	deoxyribonucleic acid
Dsc	desmocollin
Dsg	desmoglein
EC	classical cadherin extracellular repeat
E-Cad	E-cadherin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
EMT	epithelial-mesenchymal transition
Fig	figure

GFP	green fluorescent protein
h	hours
HRP	horse radish peroxidase
IDP	inner dense plaque
IL2R-tail	interleukin 2 receptor-E-cadherin-tail
IF	intermediate filament
JAM	junctional adhesion molecule
kD	kilo Dalton
KO	knock-out
mg	milligram
min	minutes
µl	microliter
ml	milliliter
µM	micromolar
mM	millimolar
ntc	non targeting shRNA control
ODP	outer dense plaque

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
P-cad	P-cadherin
PCR	polymerase chain reaction
PFA	paraformaldehyde
PMSF	phenylmethylsulphonylfluorid
RNA	ribonucleic acid
rpm	rounds per minute
SDS	sodium dodecyl sulphate
sec	seconds
TBS	tris buffered saline
TER	trans epithelial resistance
TJ	tight junction
VH	vinculin homolog
v/v	volume by volume
w/v	weight by volume

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