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# Dissecting the Functions of Classical Cadherins in Skin

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# DISSECTING THE FUNCTIONS OF CLASSICAL CADHERINS IN SKIN

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# DISSECTING THE FUNCTIONS OF CLASSICAL CADHERINS IN SKIN

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The spectacular cellular rearrangements of keratinocytes as epidermal appendages form and the epidermis turns over rely on the rapid and precise modulation of intercellular adhesion. While genetic studies in vertebrates have revealed a pivotal role for classical cadherin-mediated cell adhesion in the regulation of coordinated cell movements in the embryo, little is know about the contribution of classical cadherins to epidermal morphogenesis. Genetic dissection of cadherin function in skin is complicated by overlapping expression of E- and P-cadherin and the fact that their genes are tightly linked, precluding conventional conditional knockout strategies. I have combined conditional gene ablation, *in vitro* analysis of *de novo* epithelial sheet formation, and transgenic RNA interference to overcome these issues in order to define the contribution of classical cadherins in epidermal physiology. Loss of E-cadherin in the skin epithelium revealed striking differences by which epidermis and hair follicles handle the loss of this critical protein, a feature I have traced to the compensatory upregulation of P-cadherin in the *E-cadherin* null epidermal basal layer but not the hair follicle. To formally test this premise, I have examined the *in vitro* and *in vivo* consequences of E- and P-cadherin inhibition. Suppression of classical cadherins blocked epidermal sheet formation *in vitro*, while overexpression of

either E- or P-cadherin rescued these defects, defining cadherin level, rather than subtype, as a critical factor in this process. Classical cadherin inhibition *in vivo* was not compatible with survival and resulted in perturbations in tissue integrity, increased apoptosis, and defects in epidermal barrier. This analysis has uncovered striking defects not observed previously in single loss of function mutations for E- and P-cadherin and has revealed a critical role of classical cadherins in skin biology. Finally, by contrasting loss-of-function mutants of epidermal catenins and cadherins, I have further defined a number of cadherin-dependent and independent roles of adherens junction components in the epidermis. The ability to clearly dissociate cadherin and catenin functions and to identify regulatory processes governing their interaction should prove useful in the context of both development and disease.

*To Kimberley, your support and understanding have made this journey possible*

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## TABLE OF CONTENTS

## LIST OF ILLUSTRATIONS

## LIST OF FIGURES

# Chapter 1: General Introduction

During development heterogeneous cell types within an organism are physically grouped into distinct compartments, forming sharply demarcated tissues and organs in which integrity must be maintained. The concept that intercellular adhesion plays a fundamental role in tissue formation and maintenance emerged at the beginning of the 20$^{th}$ century, in which single cells dissociated from sponges were shown to re-aggregate and form small sponges in a species-dependent manner (Wilson, 1907). Subsequent work by Holtfreter, Moscona, Steinberg and Takeichi (reviewed in Grunwald, 1991) established that the specificity and extent of cell-cell adhesion is a principle driving force in this "cell sorting" and that in most animal cells, the process is critically dependent on extracellular calcium.

Ultrastructural analysis has revealed that cells of most simple epithelia are connected by a tripartite complex, located just below the apical surface of apposing cells (Farquhar and Palade, 1963). This epithelial junctional complex consists of the most apical tight junction (zonula occludens), the intermediate adherens junction (zonula adherens), and the more basally localized desmosome. The molecular basis of these intercellular adhesive contacts has been largely elucidated, and while clear compositional and functional distinctions exist, they each share a common organization consisting of transmembrane adhesion receptors, cytoskeletal plaque proteins, and the cortical cytoskeletal

network. Through homophilic and heterophilic interactions on adjacent cells these proteins mediate the calcium-dependent intercellular adhesion essential for the formation and maintenance of multicellular structures (Takeichi, 1990).

## Intercellular Adhesion Complexes

### Tight Junctions

In simple epithelia, tight junctions reside at apical sites of intercellular contact, where they function not only in paracellular diffusion (gate function) but also in partitioning membrane components between apical and basolateral membrane domains (fence function) (Matter et al., 2005). In stratified epithelial tissues, tight junctions reside in the upper differentiated layers, where their primary role is surmised to prevent paracellular diffusion and maintain an epithelial barrier (Furuse et al., 2002). Tight junctions also appear to function as important landmarks of polarized epithelial cells, spatially restricting a number of protein complexes that regulate apico-basal polarity (Niessen, 2007).

The transmembrane core of tight junctions is comprised of the tetraspan proteins occludin and claudin and the single-span IgG-like junctional adhesion molecule (JAM) (Fig. 1.1). The role of claudin as the functional backbone of tight junctions is most firmly established, as exogenous expression in fibroblasts stimulates *de novo* tight junction strand formation (Furuse et al., 1998), and loss of function of several claudin family members in vertebrates functionally disrupts tight junctions (reviewed in (Furuse and Tsukita, 2006).

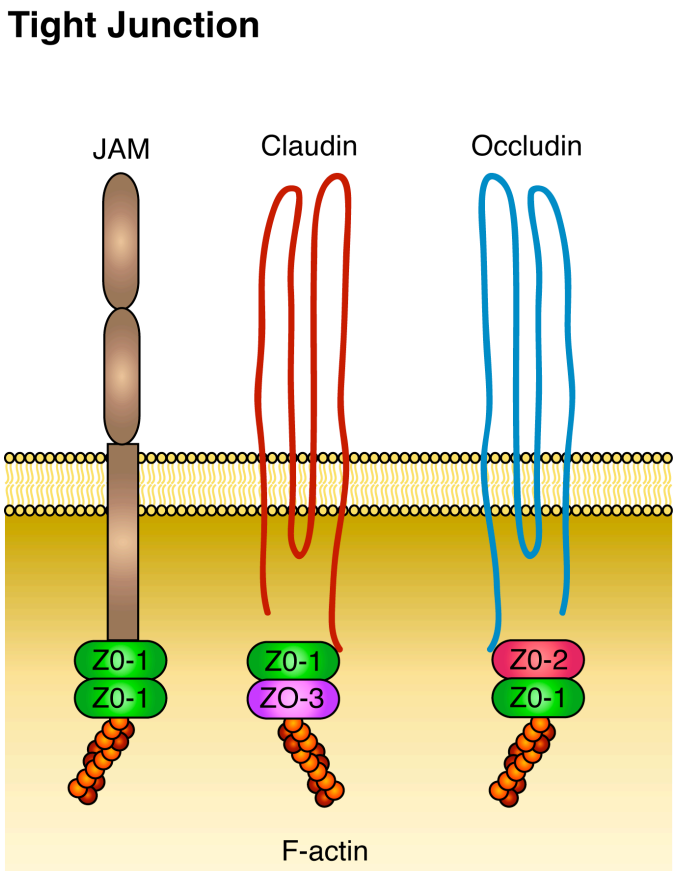



**Figure 1.1** *Schematic representation of the basic components of tight junctions.* Adhesion receptors of the tight junction consist of the single pass transmembrane IgG-like JAM protein and the tetraspan proteins, occludin and claudin. The two extracellular loops of occludin and claudin contribute to the formation of tight junction strands, a structure observed by electron microscopy and thought to provide a physical barrier between the apical and basolateral membranes of contacting cells. These adhesion receptors are linked to the actin cytoskeleton via interactions with the PDZ-domain containing proteins, ZO-1, 2 and 3. These proteins in turn interact with components of adherens junctions and polarity complexes, and proteins that regulate gene transcription.

Each of these transmembrane proteins can directly interact with the cytoplasmic, PDZ domain-containing zonula occuldens (ZO) proteins. Z0 scaffold proteins can in turn interact with the underlying actin cytoskeleton, and have thus been proposed to provide a direct link between tight junctions and the cortical actin network (Fanning et al., 1998). Additionally, ZO proteins may serve to link tight junctions and adherens junctions through their ability to interact with catenins, cytoplasmic components of adherens junctions (Itoh et al., 1997; Wittchen et al., 2003).

**Adherens Junctions**

The intermediate structure of the epithelial junctional complex is the adherens junction, the core of which consists of transmembrane glycoproteins of the classical cadherin family and the catenin family members p120-catenin, β-catenin, and α-catenin. Beyond mediating mechanical adhesion between cells, adherens junctions provide multiple functions, including the regulation of tissue morphogenesis (Godt and Tepass, 1998; Zhong et al., 1999), growth control (Perrais et al., 2007), regulation of apoptosis (Lien et al., 2006; Birdsey et al., 2008), plasticity and regulation of neuronal synapses (Togashi et al., 2002), and stem cell maintenance and activation (Song and Xie, 2002; Haug et al., 2008).

E-cadherin, the major epithelial cadherin, is a single-pass transmembrane protein of the classical cadherin family of calcium-dependent, homophilic adhesion receptors. Classical cadherins contains five extracellular cadherin (EC)

repeats domains, which represent an independently folding sequence of ~110 amino acids (Wheelock and Johnson, 2003) (Fig 1.2). Upon binding calcium, the extracellular domain is stabilized and can form both *cis* and *trans* dimers (Patel et al., 2003). The most membrane distal EC1 repeat domain appears to be critical for both the trans cadherin binding between neighboring cells and the specificity of homophilic adhesion (Nose et al., 1988; Chen et al., 2005; Patel et al., 2006).

The cytoplasmic domain represents the most highly conserved region of classical cadherins and contains distinct binding domains for two catenins, which are part of the larger armadillo repeat family of proteins. p120-catenin binds to the juxtamembrane domain (JBD), a 94-amino acid stretch just carboxy-terminal to the plasma membrane (Yap et al., 1998). The catenin binding domain (CBD), an extended region to the carboxy-terminal of the JBD, links β-catenin to the most distal region of the cadherin tail (Aberle et al., 1994). Plakoglobin (γ-catenin), a close relative of β-catenin can also interact with classical cadherins via binding to the CBD, however, classical cadherins exhibit a preference for β-catenin (Aberle et al., 1994).

Until recently, β-catenin was thought to serve as a molecular link between cadherins and the local actin cytoskeleton via it's interaction with the actin binding and bundling protein α-catenin (Rimm et al., 1995), unrelated in sequence and structure to other catenin family members. However, two recent *in vitro* studies were unable to demonstrate a quaternary complex of E-cadherin-β-

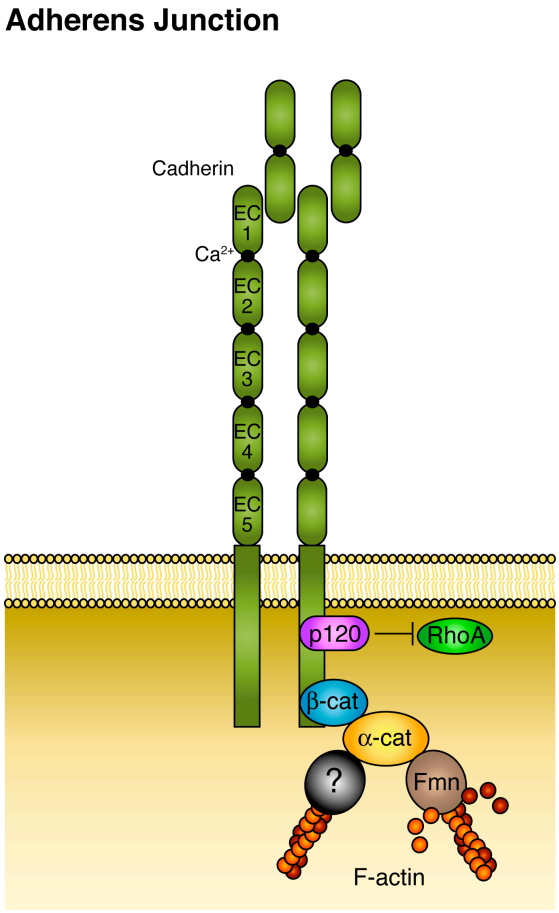



**Figure 1.2** *Schematic representation of the basic components of adherens junctions.* The transmembrane core of the adherens junction consists of a calcium-dependent transmembrane protein of the classical cadherin family. Classical cadherins initiate intercellular contacts through homophilic trans-pairing between cadherins on neighboring cells, a process thought to be mediated by the first extracellular cadherin (EC) repeat domain. Effective adhesion is mediated by direct and indirect binding of members of the catenin family of proteins, which bind to distinct regions of the cadherin intracellular domain. Catenins regulate the associated cortical actin cytoskeleton, cadherin stability and adhesivity, and intracellular signaling pathways that modulate gene transcription. Recent data suggests $\alpha$-catenin does not bind $\beta$-catenin and F-actin simultaneously, however, an indirect association may result from $\alpha$-catenin's ability to bind other actin-associated proteins, including Formin (Fmn).

catenin-α-catenin-actin (Drees et al., 2005; Yamada et al., 2005). In fact, these studies have suggested that α-catenin acts as an allosteric protein in which heterodimers of α-catenin and β-catenin interact exclusively with E-cadherin, while homodimers of α-catenin preferentially interact with F-actin. Furthermore, α-catenin homodimers appear to directly regulate cortical actin by suppressing actin-related protein 2/3 (Arp2/3), a multiprotein complex that polymerizes branched actin filaments from the side of pre-existing filaments. Arp2/3-complex-mediated actin polymerization is crucial for the reorganization of the actin cytoskeleton into the dendritic array found in lamellipodia (Pollard, 2007).

Based on these findings, a new model for adherens junctions connection to the local actin cytoskeleton has been proposed in which clustering of cadherin-catenin complexes at sites of cell-cell adhesion may result in a sufficient increase in the local concentration of α-catenin to promote homodimerization. This in turn results in α-catenin-F-actin complexes and cortical suppression of Arp2/3, both of which may promote the transition of branched actin networks found in early stages of adherens junction formation to bundled arrays associated with mature junctions. While compelling, this model is based largely on purified proteins *in vitro* and is as yet untested *in vivo*. Thus, it remains formally possible that cellular modifications of α-catenin could allow mutual binding of β-catenin and actin, or α-catenin's interaction with distinct actin binding proteins may ensure indirect cadherin-catenin linkage to the actin cytoskeleton. Indeed, α-catenin binds to multiple actin binding and bundling proteins, including the recently

identified eplin protein, which has been suggested to serve that link (Abe and Takeichi, 2008).

p120-catenin does not interact with α-catenin and it's binding to E-cadherin is independent of β-catenin binding (Marrs and Nelson, 1996). Instead, p120-catenin appears to positively regulate cadherin stability at the membrane by antagonizing endocytosis (Davis et al., 2003; Xiao et al., 2005). Additionally, p120-catenin appears to promote the conversion of weak cadherin interactions into strong, more robust adhesions independently of it's ability to regulate cadherin level, as p120-catenin-cadherin interaction strongly influences clustering of cadherins in maturing contacts (Yap et al., 1998; Anastasiadis et al., 2000). Finally, p120-catenin can also regulate the organization of the cadherin-associated actin cytoskeleton. In contrast to α-catenin, p120-catenin does not bind actin directly, but rather regulates actin dynamics through the regulation of small GTPases, most notably via inhibition of RhoA and activation of Rac1 (Anastasiadis et al., 2000; Noren et al., 2000; Wildenberg et al., 2006).

**Desmosomes**

Desmosomes are intercellular junctions composed of a transmembrane core of desmosomal cadherins, which include the desmoglein and desmocollin proteins (reviewed in Getsios et al., 2004) (Fig. 1.3). Desmosomal cadherins are also calcium-regulated and contain five cadherin repeat domains, but they differ from classical cadherins in at least three important ways. Desmosomal

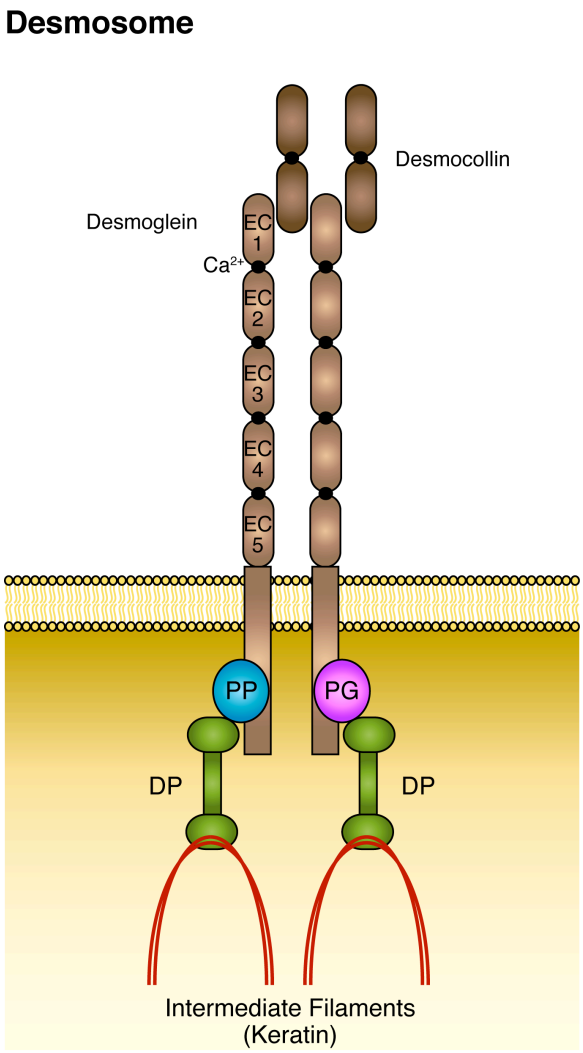



**Figure 1.3** *Schematic representation of the basic components of desmosomes.* The desmosomal cadherins, desmoglein and desmocollin, constitute the transmembrane adhesion receptors of desmosomes. While the overall structure is similar to that of classical cadherins, desmosomal cadherins form heterotypic interactions, interact with different cytoplasmic regulatory proteins, and are linked to the intermediate filament cytoskeleton. This interaction may occur through a number of ways, all of which appear to rely on desmoplakin, a member of the plakin family of cytoskeletal linker proteins. The interaction with the resilient intermediate filament ensures a robust calcium-induced adhesive interaction between cells.

cadherins typically display heterotypic, as well as homotypic, interactions between other desmosomal cadherin molecules. Members of this cadherin subtype predominantly interact with the armadillo proteins plakoglobin and plakophilin, rather than β-catenin and p120-catenin. Finally, desmosomal cadherins are linked to the intermediate filament-based cytoskeleton, through their direct and indirect interaction with the plakin family member desmoplakin.

The interaction with the resilient intermediate filament network ensures robust intercellular connections essential for mechanical integrity of tissues. Desmosomes are particularly important in tissues that are subjected to substantial physical forces, including epidermis and cardiac muscle (Bierkamp et al., 1999; Vasioukhin et al., 2001b). In the skin, desmosomes are especially abundant in the suprabasal layers, were their critical role in keratinocyte adhesion is underscored by the frequent attack of a number of autoimmune antibodies and bacterial toxins (Cheng and Koch, 2004). While desmosome function in intercellular adhesion is clear, it has also become evident that, similar to adherens junction components, desmosomal components play important roles in growth control (Eshkind et al., 2002), apoptosis (Nava et al., 2007) and differentiation (Kljuic et al., 2003).

**Spatiotemporal Regulation of Intercellular Adhesion *In Vitro***

Analysis of *de novo* intercellular adhesion in cultured cells has revealed a hierarchical organization within the epithelial junctional complex. Initial studies in

which cadherin function was inhibited through the use of function blocking antibodies or expression of dominant-negative cadherin mutants have revealed a dependence on adherens junctions for the formation of both tight junctions and desmosomes (Gumbiner et al., 1988; Wheelock and Jensen, 1992; Zhu and Watt, 1996; Troxell et al., 1999). Examination of endogenous proteins following initiation of intercellular adhesion in cells or in cultured vertebrate embryos has supported these findings, as membrane localization of components of adherens junctions typically precedes that of desmosomes and tight junctions (Green et al., 1987; O'Keefe et al., 1987; Fleming et al., 1994).

Loss of function studies and RNAi targeted depletion have further highlighted this hierarchical organization. For example, genetic loss of $\alpha$-catenin in an epithelial cell line impairs the establishment of tight junctions and desmosomes (Watabe et al., 1994), while short hairpin RNA-mediated depletion of E-cadherin results in altered tight junction formation (Capaldo and Macara, 2007). Importantly, loss of function of either desmoplakin (Vasioukhin et al., 2001b) or ZO proteins (Umeda et al., 2006), however, does not prevent the formation of adherens junctions. While the mechanism(s) by which adherens junctions promote downstream junctional assembly is unclear, plakoglobin, which can interact with components of both adherens junctions and desmosomes, and ZO-1, which can interact with components of both tight junctions and adherens junctions, have been implicated in the formation of desmosomes and tight junctions, respectively (Lewis et al., 1997; Ikenouchi et al., 2007).

Intercellular adhesion can be stimulated in a variety of cultured cells by the elevation of extracellular calcium. Initial studies in the simple epithelial MDCK cell line using immunofluorescence microscopy of either endogenous proteins or exogenously expressed E-cadherin-GFP have detailed a highly ordered, consistent process of cadherin-mediated adherens junction formation (McNeill et al., 1993; Yonemura et al., 1995; Adams et al., 1996; Angres et al., 1996; Adams et al., 1998). Based on these studies the formation of intercellular adhesion has been divided into two sequential phases in which specific changes in E-cadherin and the cortical actin network are observed. Cell contact is initiated by lamallipodial extensions between cells resulting in the rapid formation of spot-like clusters, or puncta, of cadherin at sites of contact. Concomitant reorganization of cortical actin occurs in which thin "radial" actin fibers branch from the circumferential actin belt and become oriented perpendicular to the membrane and are closely associated with E-cadherin puncta. While these puncta do appear to be associated with the actin cytoskeleton as assessed by their resistance to Triton X, initial contacts are typically dynamic and labile.

The second stage of contact formation begins as individual puncta cluster laterally along the site of contact, eventually forming larger clusters, or plaques, that typically are organized at either ends of the developing junction. The circumferential actin belt underlying the free edges of the contacting cells appears to embed into the large cadherin plaques, while the cortical actin network along the site of contact becomes oriented parallel and closely apposed

to the contacting membranes. The clustering of cadherins along the length of the contact and the reorganization of actin results in a strong, stable intercellular connection. While these studies have provided a framework of the spatiotemporal organization of cadherin-based adhesion, they are not fully representative of intercellular contact formation in other epithelial cells.

In primary keratinocytes, initiation of cell adhesion results in extensive filopodial extensions between contacting cells (Vasioukhin et al., 2000; Vaezi et al., 2002; Kobielak et al., 2004) (Fig. 1.4). After a period of dynamic and transient interactions, these filopodia eventually embed into neighboring cell, forming a recognizable intermediate stage of intercellular adhesion referred to as the adhesion zipper. This structure consists of a double row of E-cadherin-catenin puncta, organized at the tips of the embedded filopodia. Ultrastructural analysis has revealed that each puncta is associated with a prominent bundle of radial actin within the host cell, and pulse labeling of rhodamine-labeled actin has demonstrated actin polymerization at these sites.

As junction formation proceeds, puncta close into a single row, lateral puncta coalesce, and cortical actin reorganizes into parallel bundles underlying the sealed membrane. These observations suggest a model in which filopodial extensions anchored by adherens junctions bridge and mechanically link cells together, while actin polymerization in the host cell provides a “reverse” force that promotes sealing of the opposing membranes and drives epithelial sheet formation. Additionally, the ordered alignment of actin-based filopodia may

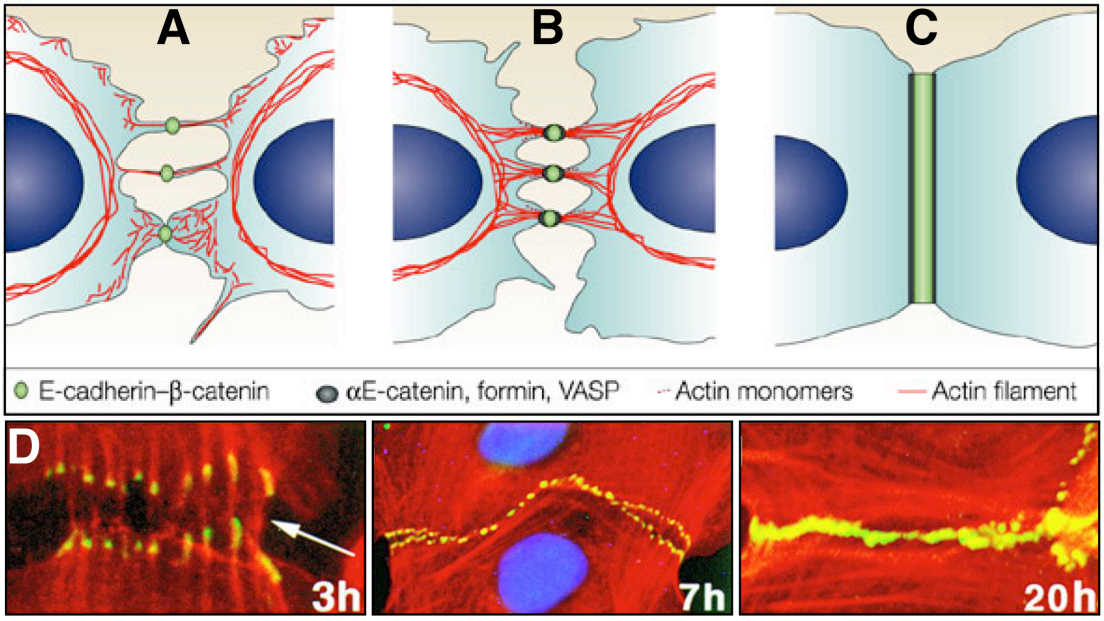



**Figure 1.4** *A model of adherens junction formation in keratinocytes.* **(A)** Initial cell-cell contacts result from extensive filopodial and lamallipodial extensions that physically embed into neighboring cells. E-cadherin-catenin complexes localize to the tips of these cellular protrusion and cluster in discrete aggregates termed puncta. **(B)** In a process dependent on α-catenin, proteins that bind, bundle and/or polymerize F-actin localize to cadherin-catenin puncta. Concomitant with this, small bundles of F-actin, termed radial cables, branch from the underlying cortical ring and become oriented perpendicular to the membrane and closely associated with cadherin-catenin complexes of the host cell. **(C)** Maturation of the intercellular junctions occurs as lateral puncta coalesce and ultimately form a linear band of cadherin-catenin complexes along the site of contact. Additionally, actin becomes aligned parallel and closely apposed to the sealed membranes. (adapted from Kobielak and Fuchs, 2004). **(D)** Cell adhesion was induced in primary mouse keratinocytes following addition of calcium for the indicated number of hours. Cells were fixed and processed for immunofluorescence with an antibody against E-cadherin (green) and labeling of F-actin with TRITC-phalloidin (red). (adapted from Vasioukhin et al., 2000).

provide an explanation for the alternating pattern of adherens junctions and desmosomes commonly observed in stratified epithelial tissues (Vasioukhin et al., 2001a; Vasioukhin et al., 2001b).

*In vitro* studies of keratinocytes from mice conditionally null for α-catenin has revealed an essential role for this protein in the regulation of cortical actin organization during initiation and maturation of intercellular adhesion. Thus, keratinocytes lacking α-catenin fail to actively bring themselves together, stabilize cell junctions, and integrate actin networks across an epithelial sheet (Vasioukhin et al., 2001a; Vaezi et al., 2002; Kobielak et al., 2004). This appears to stem from the fact that the loss of α-catenin results in the failure to form radial actin cables that promote stable adherens junction formation. While the ability of α-catenin to directly link cadherin-catenin complexes to the actin cytoskeleton is in question, these studies have emphasized a broader function of α-catenin to recruit and organize a multiprotein complex at the cell cortex with actin binding (eg, Z0-1 and afadin) (Imamura et al., 1999; Tachibana et al., 2000), actin-bundling (eg, α-actinin and vinculin) (Nieset et al., 1997; Watabe-Uchida et al., 1998), and actin polymerizing (eg, VASP/Mena, Arp2/3 and Formin) (Vasioukhin et al., 2000; Kovacs et al., 2002b; Kobielak et al., 2004) function.

The Rho family GTPases, through both p120-dependent and independent mechanism, are also important regulators of actin cytoskeleton dynamics associated with cadherin contacts. Ligation of E-cadherin homodimers results in the rapid, yet transient, activation and recruitment of Rac1, Cdc42 and

PI(3)kinase (Braga et al., 1997; Noren et al., 2001; Kovacs et al., 2002a; Perez et al., 2008). Conversely, inhibition of Cdc42, Rac 1 or Tiam 1, a guanine nucleotide exchange factor for Rac1, results in defects in adherens junction formation and cortical actin organization (Takaishi et al., 1997; Kodama et al., 1999; Malliri et al., 2004). RhoA-dependent actomyosin contractility also plays an important function in productive adherens junction-mediated adhesion. Conventional nonmuscle myosin II has been observed to be locally recruited and activated at cell-cell contacts (Shewan et al., 2005; Zhang et al., 2005; Yamada and Nelson, 2007). Inhibition of either ROCK, the Rho-activated kinase, or its downstream target myosin II perturbs the formation of radial actin cables and E-cadherin puncta formation and results in diminished intercellular adhesion (Vaezi et al., 2002; Shewan et al., 2005; Yamada and Nelson, 2007). These data suggest radial actin cables associated with puncta may function with myosin II to generate the necessary tension required to generate a functional epithelial sheet.

**Adherens Junction Function *in vivo***

While in vitro studies have provided highly detailed information regarding the mechanisms by which adherens junctions form among cultured cells, genetic studies in animal models have demonstrated the fundamental importance of adherens junction-mediated adhesion in tissue formation and maintenance. Equally as important, these studies have revealed novel signaling functions that regulate a number of major developmental pathways. Given the variety of

functions ascribed to cadherin-catenin complexes, it is perhaps then not surprising that dysregulation of adherens junction components is frequently implicated in disease states, including tumor initiation and metastasis, autoimmune inflammatory disease, and developmental anomalies of a number of ectodermal appendages (Guilford et al., 1998; Kjaer et al., 2005; Bruewer et al., 2006).

**Classical Cadherin Function *In Vivo***

Classical cadherins are expressed in a variety of vertebrate and invertebrate organisms, ranging from humans to sea urchins. Each subtype of classical cadherins typically displays a highly specific spatiotemporal pattern of localization. For example, E-cadherin is expressed in all epithelia as well as the morula stage of development; N-cadherin is expressed in neural and muscle tissue; P-cadherin is enriched in the placenta in mice and the basal layer of the epidermis; R cadherin is expressed in the retina, forebrain and bone; and, vascular endothelial (VE) cadherin is largely restricted to endothelial cells (reviewed in Nollet et al., 2000). However, it is important to keep in mind that while many tissues show restricted cadherin expression, a number of tissues express multiple cadherin family members.

Genetic analysis in the fruit fly, frog, fish and mouse has demonstrated that E-cadherin is required for epithelium formation and the integrity of a number of epithelial tissues (Larue et al., 1994; Levine et al., 1994; Tepass et al., 1996; Babb and Marrs, 2004). For example, maternal and zygotic loss of DE-cadherin,

the functional homolog of E-cadherin in flies, results in collapse of embryonic epithelia (Tepass et al., 1996), while zygotic loss of E-cadherin in mice disrupts blastomere adhesion and prevents the formation of the first polarized epithelium, the trophectoderm (Larue et al., 1994). Defects in tissue formation appear to be primarily due to alterations in cell adhesion/morphogenesis rather than cell fate specification (Larue et al., 1994; Babb and Marrs, 2004).

Given the conserved function of E-cadherin in initial embryonic epithelial biogenesis and integrity, different approaches have been employed to address E-cadherin function in other epithelial tissues and at later points in development. This has included the use of moderate DE-cadherin mutant alleles and mosaic mutant clones in *Drosophila*, activation of the closely related C-cadherin in *Xenopus* by treatment with an activating antibody, and tissue specific ablation and overexpression in mice. Additionally, zygotic loss of function mutations in murine N, P, R, and VE-cadherin have been generated and analyzed to address cadherin function in a variety of other tissues. These *in vivo* analyses have defined functions that extend to multiple aspects of tissue morphogenesis, including formation and maintenance of tissue boundaries, coordinated cell movements, and planar cell division (Fig. 1.5) (reviewed in Gumbiner, 2005; Halbleib and Nelson, 2006).

Three prominent examples of cadherin-mediated cell sorting and tissue boundary formation have been documented and provide direct *in vivo* evidence of the qualitative and quantitative differences in cadherins that mediate

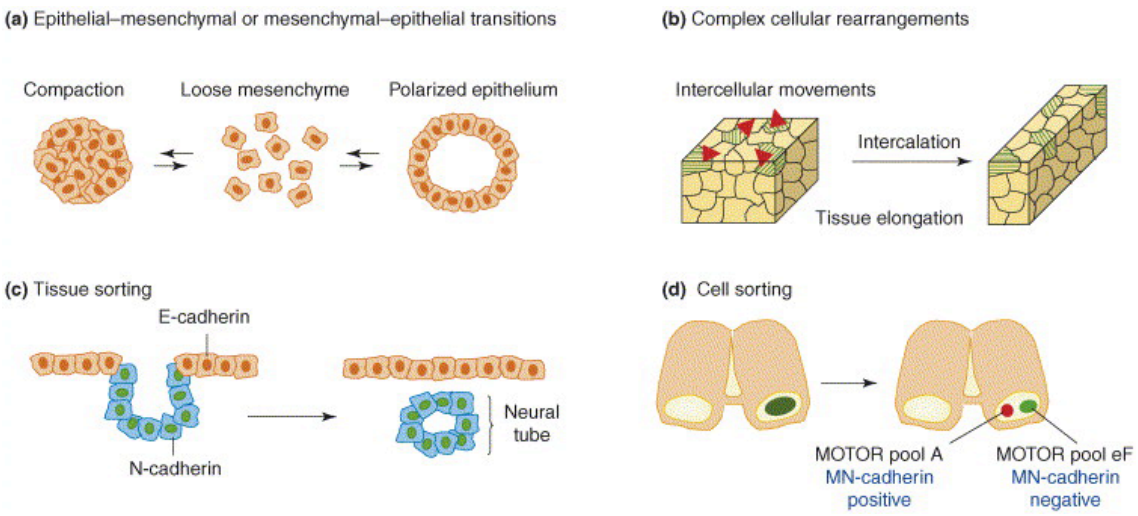



**Figure 1.5** *Roles of classical cadherins in tissue morphogenesis.* **(A)** The epithelial-mesenchymal transition and it's reverse, the mesenchymal-epithelial transition, represent morphogenetic processes that occur throughout development and are frequently co-opted during disease. E-cadherin-mediated adhesion is required for the formation of a polarized epithelium from loose mesenchymal cells and it's loss and/or dysfunction influences tumor invasion and metastasis. **(B)** Cadherin adhesiveness must be modulated during complex cellular rearrangements that occur for instance in the process of convergence and extension during gastrulation and somatic cell migration in the *Drosophila* ovary. **(C and D)** Tissue boundary formation occurs as groups of cells physically sort themselves from their neighbors to form tubular structures (C) or discrete pools of neurons (D). Quantitative and qualitative differences in cadherins between groups of cells are the primary mechanism that drives this process. (adapted from Lien et al., 2006).

cell sorting *in vitro* (Friedlander et al., 1989). Mosaic analysis of DE-cadherin mutations in the follicular epithelium covering the fly ovary has demonstrated that differential expression, and presumably the strength of adhesion, of DE-cadherin determines oocyte positioning, such that the oocyte preferentially associates with the surrounding follicular cells expressing the highest level of DE-cadherin (Godt and Tepass, 1998). Complementary expression of cadherin 6 and R-cadherin between the primordia of the cerebral cortex and the striatum within the mouse telencephalon appears to regulate boundary maintenance between these two regions, as overexpression of either cadherin in cells near the vicinity of the boundary results in a strong preferential sorting of cells to the region expressing the respective endogenous cadherin (Inoue et al., 2001). Finally, studies in the chick spinal cord have demonstrated the in vivo significance of the EC1 domain of cadherins in mediating homophilic adhesion and tissue boundary formation. Expression of a chimeric cadherin 6 mutant containing the EC1 domain of motor neuron (MN) cadherin in cadherin 6-postive neurons results in missorting of these cells into motor pools comprised of neurons expressing MN-cadherin (Price et al., 2002; Patel et al., 2006).

Coordinated cell movements are essential for many morphogenetic processes including oogenesis, gastrulation, and epithelial placode formation. In many of these cellular rearrangements, modification of cadherin level and/or subtype at the onset of cellular rearrangements is critical. For example, manipulation of DE-cadherin expression in various cells within the *Drosophila*

ovary has revealed that DE-cadherin is required for somatic cell migration and that the level of expression determines the rate of migration (Niewiadomska et al., 1999). The inability to downregulate C-cadherin's adhesive function in *Xenopus* explants through treatment with an activating antibody prevents the induction of the same convergence and extension movements observed during gastrulation (Zhong et al., 1999). Similarly, epithelial-mesenchymal transition during gastrulation in the mouse appears to require downregulation of E-cadherin through transcriptional repression via the transcription factor snail and/or protein regulation via p38 MAP kinase (Ciruna and Rossant, 2001; Zohn et al., 2006). In embryonic mouse epidermis, forced expression of E-cadherin prevents downregulation of E-cadherin at sites of initiated hair follicle downgrowth and ultimately blocks hair follicle morphogenesis (Jamora et al., 2003).

Classical cadherins also play an important role in the orientation of the plane of cell division, a process critical to directional expansion of tissues and stem cell maintenance and activation. In the *Drosophila* testis, cadherin-mediated adhesion between germ stem cells (GSCs) and somatic "hub" cells within the stem cell niche has been implicated in the proper orientation of the division plane of GSCs, through a process that appears to involve anchoring astral microtubules of the mitotic spindle (Yamashita et al., 2003). Cadherin-mediated adhesion has also been implicated as a general mechanism in anchoring stem cells to their niches. Thus, mosaic reductions in DE-cadherin in GSCs of the *Drosophila* ovary disrupts intercellular adhesion between somatic

cap cells within the niche and results in stem cell loss (Song et al., 2002). Similar defects have been observed with removal of DE-cadherin in somatic stem cells, a type of epithelial stem cell within the *Drosophila* ovary (Song and Xie, 2002). Evidence from studies of mammalian hematopoietic stem cells has also implicated N-cadherin in polarized divisions and stem cell maintenance (Zhang et al., 2003), although this has been challenged recently (Kiel et al., 2007).

Finally, a number of classical cadherins have been shown to interact with and modulate the signaling of distinct receptor tyrosine kinases (Williams et al., 1994; Carmeliet et al., 1999; Qian et al., 2004). In vitro, E-cadherin homophilic ligation appears to inhibit epidermal growth factor-mediated signaling and cell growth, while a genetic interaction between DE-cadherin and EGFR supporting these findings has been established during development of the *Drosophila* visual system (Dumstrei et al., 2002; Perrais et al., 2007). VE-cadherin regulates endothelial cell survival by promoting signaling via the vascular endothelial growth factor receptor in the mouse vasculature (Carmeliet et al., 1999). Mammalian neurite outgrowth is mediated by N-cadherin, at least in part, through it's interaction with and regulation of the fibroblast growth factor receptor (FGFR) (Williams et al., 1994). Interestingly, the interaction between N-cadherin and FGFR occurs between their extracellular domains, notably the fourth EC repeat domain of N-cadherin (Williams et al., 2001). Thus, in some cases cadherins, independent of their associated catenins, appear to directly influence signaling pathways important in developmental processes.

**p120-catenin Function *In Vivo***

Given the important roles p120-catenin plays in the regulation of the adhesive strength and stability of cadherins at the membrane, it is quite surprising that loss of p120-catenin in both flies and worms does not significantly alter animal physiology or adherens junction structure or function (Myster et al., 2003; Pettitt et al., 2003). This is especially striking as these model organisms possess only one p120 family member. That said, both studies have revealed supportive roles for p120-catenin in cadherin-based adhesion, as p120-catenin mutations enhance cell adhesion defects observed in animals harboring mutations in other adherens junction components. Thus, it appears in lower organisms either unrecognized functional redundancy exists, or p120 may not function as an essential "core" component of adherens junction-dependent intercellular adhesion.

Loss of function studies in vertebrates, however, have revealed a critical requirement for p120-catenin function during embryonic development. Yet interestingly, work in both the frog and mouse suggests this dependency stems from p120-catenin's ability to regulate Rho GTPases as well as Wnt signaling, rather than intercellular adhesion per se (Fang et al., 2004; Kim et al., 2004; Elia et al., 2006; Perez-Moreno et al., 2006). Morpholino-mediated depletion of p120 disrupts gastrulation and axial elongation in *Xenopus* embryos, while intercellular adhesion does not appear to be affected (Fang et al., 2004). These defects can be rescued through expression of dominant negative RhoA or dominant-active

Rac. Additionally, p120 interacts with and modulates the function of the transcriptional repressor Kaiso, which functions to regulate both canonical and non-canonical Wnt signaling, an evolutionarily conserved developmental pathway (Kim et al., 2004; Park et al., 2005). Both pathways of Wnt signaling and the activity of Rho GTPases play critical roles in the process of gastrulation, and have thus been postulated to account for the defects observed in p120-catenin depleted embryos.

Targeted deletion of p120-catenin in several mouse tissues has demonstrated additional insight into p120-catenin function *in vivo*. Loss of p120-catenin in the developing epidermis results in the activation of NF-κB signaling which appears to promote hyperplasia and chronic inflammation (Perez-Moreno et al., 2006). As observed in other model systems, loss of p120-catenin does not overtly affect cell adhesion and epidermal barrier function is unperturbed. While the underlying mechanism is unclear, p120 appears to regulate NF-κB activation and immune homeostasis, at least in part, through regulation of Rho GTPases. Perturbations in the activity of RhoGTPases were also observed following ablation of p120-catenin in hippocampal neurons, and these alterations appear to largely account for the observed defects in dendritic spine and synapse formation (Elia et al., 2006).

While signaling functions of p120-catenin are exciting, it is important to point out that in each vertebrate loss of function study cited, cadherin levels were reduced, further supporting a central role for p120 in the regulation of cadherin

stability as well. Thus, subtle changes in intercellular adhesion strength and any resultant alterations in signaling may also contribute to the observed phenotypes. A central question for the future then is to what extent p120 regulates Rho GTPase and Kaiso separately from its interaction with cadherins.

### β-catenin Function *In Vivo*

Armadillo, the fly homologue of β-catenin, was initially described as a regulator of the *wingless* growth factor signaling pathway (Wieschaus et al., 1984; Peifer et al., 1991), which regulates embryonic patterning, epithelial-mesenchymal interactions, and stem cell maintenance (Clevers, 2006). Subsequent work in a variety of organisms has detailed a conserved pathway in which extracellular Wnt signaling proteins induce the stabilization and nuclear localization of β-catenin where it interacts with the TCF/Lef family of transcription factors to modulate target gene expression (Nelson and Nusse, 2004). Thus β-catenin acts a central player in both a conserved developmental signaling pathway and adherens junction-mediated intercellular adhesion. These seemingly distinct functions, along with the presence of the closely related family member plakoglobin in many species, have complicated genetic dissection of this multifaceted molecule.

Mutagenesis of the *armadillo* locus in *Drosophila* and depletion of β-catenin in *Xenopus* embryos results in embryonic patterning defects, whereas intercellular adhesion is unaffected (Wieschaus et al., 1984; Heasman et al.,

1994b). While these results define a role for β-catenin in developmental patterning, a common finding between these studies is the persistence of functional membrane-associated β-catenin, either due to maternal contribution or incomplete depletion (Riggleman et al., 1990; Kofron et al., 1997).

Generation of germline clones completely lacking β-catenin function and embryos expressing maternal and zygotic intermediate *arm* alleles has addressed this issue in the fly and has demonstrated that armadillo is also required for adherens junction formation and intercellular adhesion (Peifer et al., 1993; Cox et al., 1996). Functional redundancy is not an issue in this case, as flies do not have a plakoglobin homolog.

While complete loss of function of β-catenin in Xenopus has not been achieved, studies of plakoglobin function have demonstrated important differences between these two homologs. Effective depletion of plakoglobin in *Xenopus* embryos does not result in patterning defects (Kofron et al., 1997; Kofron et al., 2002), and the ability to induce axis duplication, a common phenotype resulting from elevated Wnt-β-catenin signaling, via overexpression of plakoglobin appears to be due to a subsequent rise in signaling-competent β-catenin levels rather than plakoglobin function itself (Miller and Moon, 1997). These results suggest that β-catenin's signaling function within the context of Wnt signaling is unique and particularly sensitive to reductions in β-catenin levels, while maternal contributions appear to be sufficient for adherens junction function.

Maternal contribution and functional redundancy has also partially obscured the role of β-catenin protein in early mouse embryonic development. Genetic ablation of β-catenin results in embryonic lethality as gastrulation commences resulting from a block in anterior-posterior axis formation, again indicative of a signaling function in the Wnt pathway (Huelsken et al., 2000). Interestingly, intercellular adhesion is maintained, presumably due at least in part to the upregulation of plakoglobin and its redistribution to adherens junctions (Huelsken et al., 2000).

Recently, the role of maternal β-catenin during embryonic development was examined to overcome the potential additive effects of maternal expression. Partial gene deletion of the maternal β-catenin allele completely disrupts blastomere cell adhesion, however, as observed in embryos lacking maternal E-cadherin, blastomeres are held together by the zona pellucida and subsequent intercellular adhesion is restored at the morula stage by expression of the paternal allele (De Vries et al., 2004). It should be pointed out, however, that this particular mutation results only an amino-terminal truncated form of β-catenin, which could potentially interact with cadherins but not α-catenin. Such a molecule could act in a trans-dominant manner to disrupt both β-catenin and plakoglobin-mediated cadherin-based adhesion (Huelsken et al., 2000).

Patterning defects in a variety of tissues targeted for conditional ablation of β-catenin have demonstrated the extensive and reiterative use of the Wnt/β-catenin signaling pathway in the vertebrate embryo. For example, deletion of β-

catenin in the limb ectoderm disrupts formation of the apical ectodermal ridge (Hill et al., 2006), it's absence in neural crest stem cells results in loss of melanocytes and dorsal root ganglia (Hari et al., 2002), and ablation in the developing epidermis blocks hair follicle morphogenesis (Huelsken et al., 2001). Importantly, in some cases alterations in cell adhesion are also observed (Cattelino et al., 2003; Junghans et al., 2005), yet in the case of epidermal ablation, keratinocyte adhesion appears to be maintained through plakoglobin expression (Huelsken et al., 2001).

Yet, as is the case in Wnt signaling function, the adhesive function of β-catenin and plakoglobin does not appear to be completely interchangeable. Genetic ablation of plakoglobin in both mice and frogs results in perturbations in cell adhesion (Ruiz et al., 1996; Kofron et al., 1997; Bierkamp et al., 1999), and in the developing mouse embryo the effects appear to be due to disruptions in desmosome integrity in both the myocardium and epidermis. While β-catenin re-localizes to and interacts with components of desmosomes in epidermis lacking plakoglobin, these compensatory changes appear incomplete as intercellular adhesion defects persist and embryonic lethality is observed.

### α-catenin Function *In Vivo*

Of all the catenins associated with adherens junctions, α-catenin's function most closely parallels that of classical cadherins. For example, in the frog, depletion of maternal α-catenin leads to blastomere dissagregation (Kofron

et al., 1997), while in the mouse, zygotic loss of function of αE-catenin, the form most prevalent in epithelia, results in dissociation of embryonic blastomeres and failure in trophectoderm formation (Torres et al., 1997). Additionally, while loss of function mutations of α-catenin have yet to be developed in *Drosophila* (which has only one α-catenin gene), α-catenin-specific RNAi treated embryos do not undergo cell shape changes associated with gastrulation and fail to form recognizable structures (Magie et al., 2002). Mammalian αN-catenin, which is restricted to neural tissue, is required for proper synapse formation and neuron and axon migration in restricted regions within the brain (Park et al., 2002; Abe et al., 2004; Uemura and Takeichi, 2006), while the *in vivo* role of the testis and heart tissue-restricted αT-catenin is unknown (Janssens et al., 2001).

Interestingly, the loss of function approach employed for αE-catenin in the mouse embryo results in the deletion of the carboxy-terminal third of the protein, a region shown to be required for α-catenin's interaction with F-actin (Rimm et al., 1995; Weiss et al., 1998; Pokutta et al., 2002). This truncated mutant protein appears to localize at cell junctions, and the levels, localization and interaction between E-cadherin and β-catenin are largely unaffected in mutant embryos (Torres et al., 1997). Similarly, site-specific mutations in the α-catenin binding site within the fly β-catenin protein results in disruptions in both epithelial cell adhesion and actin organization (Orsulic and Peifer, 1996). These results strongly suggest that the recruitment of α-catenin to cadherin-based sites of contact and it's interaction with the actin cytoskeleton are required for proper

intercellular adhesion, although work from the Nelson and Weis groups suggest that these properties of α-catenin are mutually exclusive.

Not to be outdone by the other cadherin-associated catenins, however, the function of α-catenin has expanded to also include important signaling and regulatory roles. The newly discovered role of α-catenin in the dynamic regulation of the cortical actin cytoskeleton *in vitro* has previously been discussed (see above). Examination of α-catenin function through targeted deletion in the developing epidermis and neural progenitors has revealed that α-catenin, depending on the cellular context, also acts as a negative regulator of the hedgehog (Lien et al., 2006), insulin growth factor (IGF) (Vasioukhin et al., 2001a), and NF-κB pathways (Kobielak and Fuchs, 2006).

While adherens junction formation and intercellular adhesion are disrupted, perhaps the most surprising outcome from these studies is the dramatic increase in the proliferation observed in α-catenin-null cells. Interestingly, hyperproliferation appears to arise from the direct activation of these pathways and occurs independently of the effects on intercellular adhesion and of β-catenin-TCF/Lef-mediated transcription. Thus, while it has traditionally been viewed as simply a structural link between membrane-associated cadherin and the actin cytoskeleton, α-catenin also appears to influence proliferation via the regulation of a diverse number of signaling pathways. As is the case with p120-catenin, one of the challenges now is to define the extent to which this function occurs independently of its association with cadherin.

In summary, adherens junction proteins play fundamental roles in both intercellular adhesion and multiple developmental signaling pathways, and as such are central to virtually all aspects of tissue morphogenesis. The position and composition of the adherens junction ensures that cells receive and transmit information from both the extracellular and intracellular environment. Classical cadherins promote the formation and maintenance of tissue boundaries through differential adhesion based on cadherin subtype and level. In turn, cellular signals that impinge on catenins are transduced to intracellular regulators of the cytoskeleton, protein kinases and phosphotases, and transcriptional cofactors. The outcome of many of these intracellular signaling events serves then to modulate cadherin activity through changes at the level of gene transcription, protein trafficking, and organization at the plasma membrane to facilitate coordinated cell movements within a tissue. A major hurdle, and hence a strong focus of research, in cadherin-mediate intercellular adhesion is the extent to which adhesion or signaling contributes to observed phenotypes in cadherin-catenin mutant animals.

**Mammalian Epidermis As A Model System**

The mammalian epidermis provides an excellent model system for the study of intercellular adhesion in general and classical cadherin function in particular. The epidermis is a continually renewing, stratified squamous epithelium that forms the protective layer of the skin and is essential for providing

a bidirectional barrier to prevent environmental insult as well as fluid loss (Fuchs, 1990). A tightly regulated balance between growth and differentiation is required to ensure effective barrier formation and requisite organ renewal (Fuchs and Raghavan, 2002). The highly dynamic movements between keratinocytes that take place as the epidermis turns over also require an exquisite balance between the formation, modification and disassembly of intercellular junctions.

The epidermis is derived from the neuroectoderm, a single layer of cells that covers the embryo. Epidermal fate is specified by the expression of bone morphogenic proteins (BMPs) in early ectodermal progenitors, which is facilitated by Wnt/β-catenin-dependent inhibition of FGF signaling (Stern, 2005). Reciprocal interactions between the overlying multipotent ectoderm and the mesenchymal derived dermis initiate further specification of the ectoderm into two distinct lineages: epidermis and hair.

The program of epidermal terminal differentiation is thought to commence when mitotically active cells in the basal layer withdraw from the cell cycle, detach from the basement membrane and initiate a series of biochemical and morphological changes as they move towards the skin surface (Alonso and Fuchs, 2003) (Fig. 1.6). Basal layer cells are typified by the expression of two keratin proteins, K5 and K14, which form obligate heterodimers and assemble into keratin intermediate filaments. These keratin filaments connect to α6β4 integrin-containing hemidesmosomes which help anchor basal cells to the basement membrane. Basal cells utilize a dynamic cytoskeletal network of actin

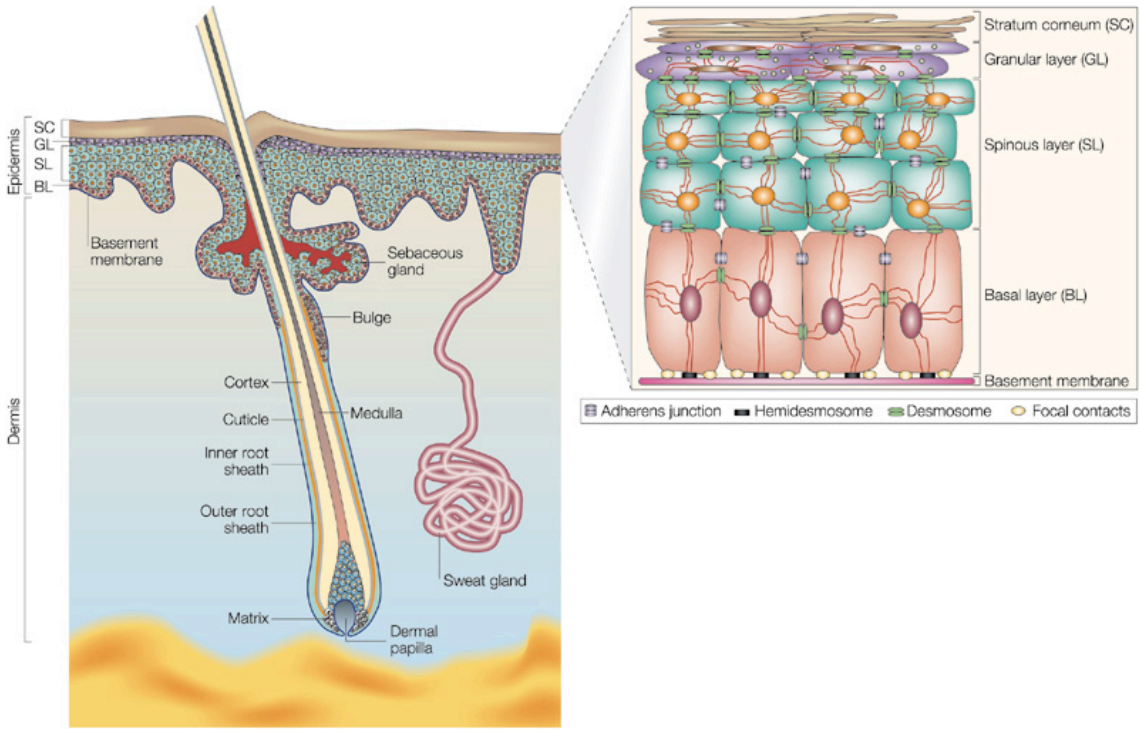



**Figure 1.6** *Schematic representation of mammalian skin.* The mammalian skin is made up of a stratified epithelia, termed the epidermis, and a mesenchyme-derived dermis. The mitotically active basal layer (BL) of the epidermis is anchored to a basement membrane through hemidesmosomes and focal adhesions. Intercellular adhesion is mediated by adherens junctions and desmosomes, the later of which are particularly abundant in the upper layers of the epidermis. During the process of stratification, the basal layer differentiates to form the spinous layer (SL), granular layer (GL), and the stratum corneum (SC), or cornified layer. The epidermis contains three appendages: the hair follicle, the sebaceous gland, and the sweat gland. The hair follicle consists of concentric layers of cells that are thought to be derived from multipotent stem cells that reside in a region within in the upper part of the hair follicle called the bulge. Reciprocal interactions between the matrix cells at the base of the follicle and a specialized region of the dermis called the dermal papilla, promotes hair follicle differentiation. (adapted from Fuchs and Raghavan, 2002).

filaments that interface through α- and β-catenins to E- and P-cadherin-mediated adherens junctions. Expression of the nonclassical T-cadherin, which lacks the highly conserved cytoplasmic domain, is also observed in the basal layer, however it's absence does not effect mouse development and no defects in skin have been reported (Hebbard et al., 2008).

Cells that come to occupy the most proximal suprabasal layer, the spinous layer, are post-mitotic but remain transcriptionally active. One of the first changes in the transition from basal to suprabasal cells is the production of two new keratins, K1 and K10, which form intermediate filaments that associate with and stabilize desmosomes. As a result, desmosomes are especially abundant in suprabasal cells, where they provide robust intercellular adhesion designed to withstand a high degree of mechanical stress (Alonso and Fuchs, 2003). As P-cadherin is restricted to the basal layer, suprabasal adherens junctions are mediated exclusively by E-cadherin. A striking morphological change is also observed, as keratinocytes transition from the cuboidal shape of the basal layer and adopt a flattened squamous shape. Additionally, a number of structural proteins, including involucrin, are deposited beneath the plasma membrane to form the initial scaffold of the cornified envelope (Segre, 2003). Spinous cells also make lamellar bodies, which contain lipids that will eventually coat the cornified envelope.

As cells continue to move toward the skin surface and come to occupy the granular layer, the production of keratohylin granules becomes apparent. Within

these structures keratin filaments are bundled into macrofibrilar cables, a process aided by the production of the protein filaggrin (Dale et al., 1978). Loricrin, a major component of the cornified envelope, is also produced in the granular layer (Fuchs, 1990). Finally, an influx of calcium activates the enzyme transglutaminase 1, resulting in biochemical crosslinking of cornified envelope proteins and the extrusion of lipids from lamellar bodies onto this scaffold (Fuchs and Raghavan, 2002).

The resulting insoluble cornified envelope and lipid matrix within the outermost, or cornified, layer help form the epidermal barrier. Until relatively recently, the epidermal barrier was thought to consist exclusively of these macromolecular complexes. However, loss of function studies and detailed ultrastructural examination have also implicated tight junctions, which become fully functional in the granular layer, as critical components of the “inside-out” epidermal barrier function (Furuse et al., 2002; Schluter et al., 2004) Epidermal terminal differentiation culminates with the sloughing (desquamation) of the dead, enucleated keratinocytes.

Undifferentiated cells of the basal layer adopt the hair lineage upon receiving a mesenchyme-derived inductive signal (Hardy, 1992). Genetic studies have demonstrated that epidermal Wnt-β-catenin signaling is required for both the specification and maintenance of the hair cell lineage (van Genderen et al., 1994; Zhou et al., 1995; Huelsken et al., 2001; Andl et al., 2002; Lowry et al., 2005). Initiation of hair follicle morphogenesis results in placode formation,

defined as spatially isolated thickenings of groups of basal keratinocytes. Hair placodes eventually invaginate as a bud-like structure into the underlying dermis. During this process reciprocal changes are seen in epidermal cadherin expression, as E-cadherin is downregulated at the advancing edge of the hair follicle while P-cadherin becomes strongly upregulated. The initial stages of hair follicle morphogenesis appear to require a finely tuned balance of intercellular adhesion as both loss of epidermal $\alpha$-catenin (ie, too few adherens junctions) and overexpression of E-cadherin (ie, too many adherens junctions) blocks hair follicle downgrowth (Vasioukhin et al., 2001a; Jamora et al., 2003).

Maturation of the hair follicle is driven by signals between a group of highly proliferative follicular cells, known as matrix cells, located at the base of the follicle, and a closely associated cluster of dermal cells, known as the dermal papilla (reviewed in Fuchs and Horsley, 2008). An analogous program of differentiation to that of the epidermis takes place as matrix cells move upward and give rise to the concentric rings of cells that constitute two distinct regions within the hair follicle: the inner root sheath and hair shaft. The inner root sheath acts as a channel that guides the developing hair shaft, which ultimately penetrates the skin and is the visible part of the hair follicle. The most external layer of the hair follicle, or outer root sheath, is continuous with the basal layer of the epidermis and contains a specialized region of the hair follicle in which multipotent stem cells reside (Blanpain and Fuchs, 2006; Cotsarelis, 2006).

## Specific Aims

The development and maintenance of the mammalian epidermis requires a complex and highly dynamic interplay between cell-cell and cell-matrix adhesion systems. Major morphogenetic events including the transition from cuboidal to squamous morphology of interfollicular keratinocytes and the initiation and subsequent development of the hair follicle rely on these systems to integrate cell movement across the entire epidermal organ. Additionally, the ability of the skin to function as the organism's first line of defense depends on the integrity of these adhesion systems and the link to the cytoskeleton that they provide.

The mouse epidermis represents a unique epithelial system to probe the physiological importance of classical cadherins, a core component of epidermal intercellular adhesion, as *$\alpha$-catenin, p120-catenin* and *$\beta$-catenin* have all been targeted for ablation (Huelsken et al., 2001; Vasioukhin et al., 2001a; Perez-Moreno et al., 2006). However, a limiting factor in the analysis of each of these mutant mouse models is that cadherin-catenin complexes still form and localize to keratinocyte membranes. As a result, the functional relevance of the loss of classical cadherins in epidermal intercellular adhesion and morphogenesis remains unknown. Genetic dissection of cadherin function is further complicated by the fact that E- and P-cadherin genes are tightly linked, precluding conventional conditional knockout strategies.

I have combined conditional gene ablation, in vitro analysis of de novo epithelial sheet formation, and transgenic RNA interference to more fully understand the contribution of classical cadherins to epidermal physiology. To address E-cadherin function, a skin-specific conditional knockout was generated and characterized. Loss of *E-cadherin* in embryonic skin results in loss of structural integrity within the inner layers of hair follicles, but epidermal architecture is surprisingly unaffected, a feature I have traced to the upregulation of P-cadherin within the basal layer. E-cadherin function in intercellular junction and epidermal sheet formation was subsequently analyzed in primary keratinocytes derived from E-cadherin conditional null mice. I show that *in vitro,* loss of E-cadherin results in elevated P-cadherin, yet keratinocytes display delayed epidermal sheet formation.

To examine the potential compensation by P-cadherin, RNA interference was employed to specifically and effectively reduce P-cadherin protein. In contrast to E-cadherin loss alone, suppressing both cadherins *in vitro* inhibits not only adherens junctions, but also desmosomes, tight junctions and cortical actin dynamics associated with sheet formation. Using different rescue strategies, I show that cadherin level rather than subtype is critical. By devising novel skin-specific RNAi technology, I demonstrate that cadherin inhibition in vivo impairs junction formation and intercellular adhesion resulting in increased apoptosis and defects in epidermal barrier and tissue integrity. Finally, by contrasting conditional loss-of-function studies of epidermal catenins and cadherins, I have

been able to define cadherin-dependent and independent roles of AJ components in epidermal physiology.

# Chapter 2: Conditional Targeting of E-cadherin in Skin: Insights into Hyperproliferative and Degenerative Responses

### Contributors To This Work

Dr. Terry Lechler and I worked together in the experimental design and execution. Transmission electron microscopy and ultrastructural analysis was provided by Dr. Amalia Pasolli. Mouse breedings were done by Lisa Polak and Roger Huang.


### Abstract

Loss of E-cadherin has been associated with human cancers, and yet in the early mouse embryo and the lactating mammary gland, the E-cadherin null state results in tissue dysfunction and cell death. Here we targeted loss of E-cadherin in skin epithelium. The epidermal basal layer responded by elevating P-cadherin, enabling these cells to maintain adherens junctions. Suprabasal layers upregulated desmosomal cadherins, but without classical cadherins, terminal differentiation was impaired. Progressive hyperplasia developed with age, a possible consequence of proliferative maintenance in basal cells coupled with defects in terminal differentiation. In contrast, hair follicles lost integrity of the inner root sheath and hair cuticle without apparent elevation of cadherins. These findings suggest that if no compensatory mechanisms exist, E-cadherin loss may be incompatible with epithelial tissue survival, whereas partial compensation can result in alterations in differentiation and proliferation.

## Introduction

Classical cadherins function in intercellular adhesion, polarization, and differentiation. The prototype is E-cadherin, a transmembrane protein that makes calcium-dependent homotypic adhesive interactions, known as adherens junctions (AJs) (Perez-Moreno et al., 2003; Yap and Kovacs, 2003). To establish efficient cell–cell junctions, E-cadherin uses its cytoplasmic domain to couple to catenins and the actin cytoskeleton. This association sets the classical cadherins apart from desmosomal cadherins, which form a more robust adhesive interaction (desmosome) through linking to plakoglobin, desmoplakin, and the intermediate filament cytoskeleton (Kowalczyk et al., 1999; Huber, 2003).

Many cells display AJs and desmosomes, which function coordinately in epithelial sheet formation (Vasioukhin et al., 2001b). Whereas desmosomes are particularly important in tissues such as muscle and epidermis that undergo substantial mechanical stress, AJs have a prominent role in remodeling epithelial cell–cell interactions. This is especially critical in early development, as reflected by the blastula defects that occur when E-cadherin is targeted for ablation in mouse (Ohsugi et al., 1997).

AJs also participate in regulating the balance between proliferation and differentiation. Down-regulation and/or mutations in E-cadherin and β-catenin occur in a number of different tumors (Cavallaro and Christofori, 2001; Conacci-Sorrell et al., 2002), and multiple mechanisms have been uncovered in controlling this link (Gottardi et al., 2001; Vasioukhin et al., 2001a; Wong and Gumbiner,

2003). Thus it is surprising that loss-of-function mutations in the *E-cadherin* gene result in cell death in mouse blastocysts (Ohsugi et al., 1997) and in lactating mammary gland (Boussadia et al., 2002). Adding to these complexities, many cell types express multiple cadherins, which in at least some cases do not seem to be functionally equivalent (Hirai et al., 1989; Radice et al., 1997b; Cavallaro and Christofori, 2001; Boussadia et al., 2002; Wong and Gumbiner, 2003).

To gain further insights into E-cadherin function, we have engineered and analyzed a conditional knockout (KO) of *E-cadherin* in epidermis and its appendages. Here, we report on the striking differences by which epidermis and hair follicles handle the loss of this critical protein. Through analyses of these different stem cell lineages in skin, we have obtained insights into our understanding of how some tissues respond to E-cadherin loss by progression to hyperproliferation, whereas others lose integrity and degenerate.

## Results

Mice lacking E-cadherin in the skin epithelium were made by mating keratin 14-Cre recombinase mice (Vasioukhin et al., 1999) with *E-cadherin* mice harboring loxP sites flanking exons 6–10 (Boussadia et al., 2002). Newborn pups were smaller and displayed reduced activity as well as alterations in whisker size and number (Fig. 2.1A; see below). Pups with these phenotypic aberrations were genotypically null for the normal *E-cadherin* allele, which resulted in near quantitative loss of protein (Fig. 2.1B). Immunofluorescence revealed the

absence of anti-E-cadherin labeling in skin epithelium (Fig. 2.1C). These data corroborated the efficacy of the targeting event.

Toluidine blue-stained semithin sections of newborn KO skin revealed only minor changes in epidermal architecture (Fig. 2.1, D and D'). All four morphologically distinct differentiation stages were visible: a single basal layer of proliferative cells, differentiating desmosome-rich spinous layers, a keratohyalin granule-rich granular layer, and the dead enucleated stratum corneum layers at the skin surface. In some regions of newborn KO skin (e.g., those shown here), the epidermis appeared similar to WT, but in others, it was thickened. In addition, intercellular membrane gaps were sometimes seen between basal and spinous layers and often near downgrowths of developing KO follicles (arrows in Fig. 2.1E'). However, most cells within the basal layer appeared to establish normal contacts even at the ultrastructural level (not shown).

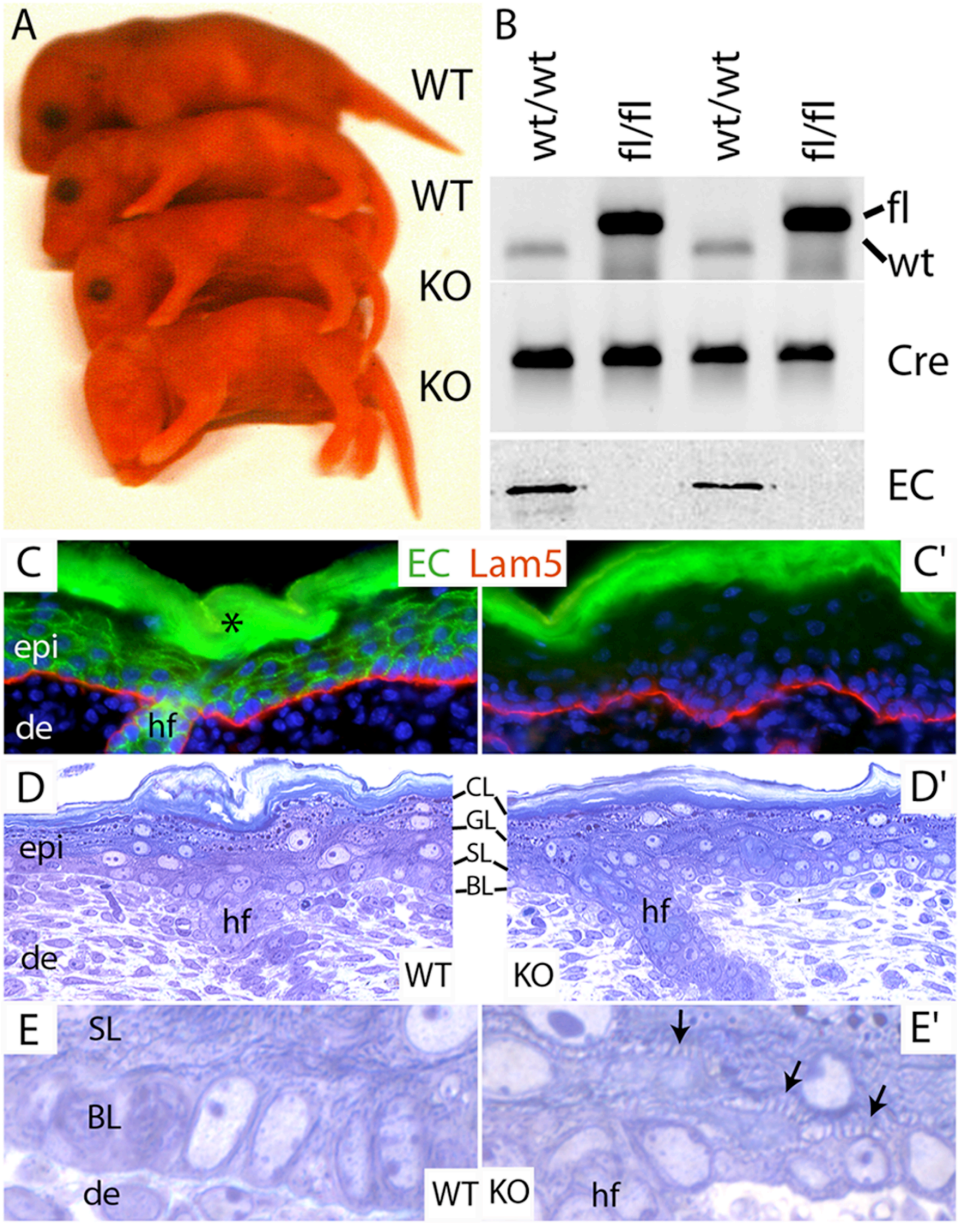



**Figure 2.1** *Generation of mice conditionally null for E-cadherin in skin epithelium.* **(A)** Animals whose epidermis is either heterozygous or homozygous for the WT *E-cadherin* allele are referred to as WT; those whose epidermis is homozygous for the K14-Cre-recombined mutant *E-cadherin* allele are called KO. Shown are newborn pups. **(B)** PCR confirmation of genotype (Top and Middle) and anti-E-cadherin (EC) Western blot of epidermal proteins (Bottom). **(C and C')** Immunofluorescence of skin sections labeled with Abs indicated (color coding is according to secondary antibodies). * denotes nonspecific staining of

cornified layer with the secondary FITC-conjugated Ab. **(D–E')** Toluidine blue-stained semithin sections (1 $\mu$m) of newborn backskin. BL, basal layer; SL, spinous layer; GL, granular layer; CL, cornified layer. (E and E' are 5x the magnification of D and D'.) Arrows denote regions where intercellular spaces are widened. epi, epidermis, de, dermis; hf, hair follicle.

Overall, the morphological defects in *E-cadherin* null epidermis were significantly less severe than those of $\alpha$-catenin null epidermis, conditionally targeted with the same K14-Cre animals (Vasioukhin et al., 2001a). To probe more deeply into the molecular explanation underlying this difference, we first tested for possible expression of other classical cadherin proteins. Anti-N-cadherin immunofluorescence was not detected in either WT or KO newborn skin epithelium (data not shown). Intriguingly, however, whereas anti-P-cadherin immunoreactivity is normally strong only in hair follicle downgrowths (Muller-Rover et al., 1999), it was strong throughout the KO basal epidermal layer (Fig. 2.2A). Although overexposure revealed only very weak anti-P-cadherin in the KO suprabasal cells, this was not seen in WT skin. Western blot analyses verified that this change was not merely a reflection of antigen unmasking as a consequence of the loss of E-cadherin but rather a bona fide increase in P-cadherin protein (Fig. 2.2I).

The paucity of P-cadherin and absence of E-cadherin in the suprabasal layers led us to wonder whether AJ formation might be compromised in spinous cells. To test this possibility, we conducted immunofluorescence with antibodies

against other AJ proteins. As shown in Fig. 2.2 B-D, the basal layer of both WT and KO epidermis displayed similar fluorescence intensities with antibodies against α-, β-, and p120-catenin. In contrast, suprabasal layers of KO, but not WT, epidermis exhibited a marked decrease in AJ antibody fluorescence. As judged by Western blot analysis, α- and β-catenin levels were consistently decreased in KO epidermis (Fig. 2.2I). The reduction was always more striking for α-catenin, consistent with the facts that (i) β- but not α-catenin can associate with desmosomes (Bierkamp et al., 1999), and (ii) the cytoplasmic pools of β-catenin are larger (Gottardi et al., 2001). These factors may also explain why p120-catenin protein levels were largely similar between WT and KO epidermis, despite a reduction in intensity of anti-p120 immunoreactivity in suprabasal KO cells.

Although overall AJ protein levels were down-regulated suprabasally, desmosomal cadherins were up-regulated in KO spinous layers. This was true at the levels of both immunofluorescence (Fig. 2.2E) and Western blot (Fig. 2.2I). Although overall levels of desmoplakin and plakoglobin were largely similar between KO and WT epidermis (Fig. 2.2, F and G), these proteins are not components of the core plaque but rather are involved in linkage of desmosomes to the intermediate filament cytoskeleton (Kowalczyk et al., 1999; Huber, 2003).

In addition to the decrease in AJ proteins and increase in desmosomal cadherins, anti-keratin 6 immunoreactivity was seen in some suprabasal cells of *E-cadherin* null epidermis (Fig. 2.2, H and I). Characteristic of hyperproliferative

skin, this feature correlated with the increased thickness of KO epidermis, which became more pronounced with age (see below).

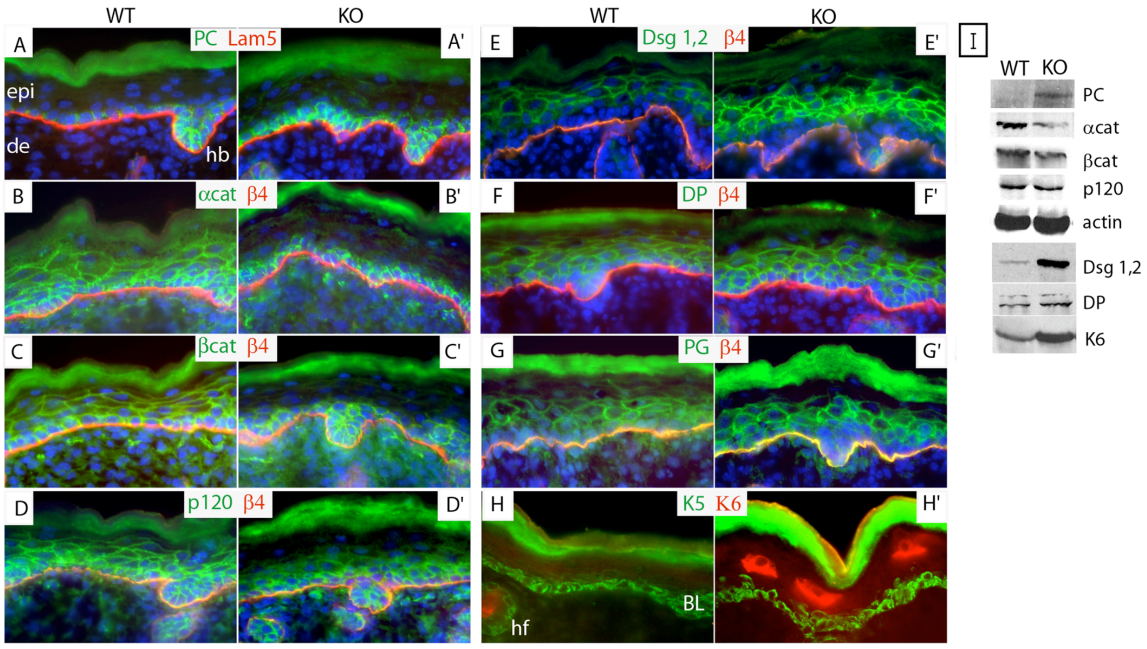



**Figure 2.2** *Up-regulation of P-cadherin in basal but not suprabasal cells results in a selective loss of AJ components in the differentiating cells of E-cadherin null epidermis.* **(A–H')** Sections of newborn backskin were processed for indirect immunofluorescence microscopy using the Abs indicated. In some cases, 4',6-diamidino-2-phenylindole was used to identify the nuclei. Markers are specific for: P-cadherin (PC), AJs; Laminin 5 (Lam5), basement membrane; β4 integrin (β4) and keratin 5 (K5), basal layer (BL); α-catenin (cat), β-catenin (cat), and p120-catenin (p120), AJs; desmogleins 1 and 2 (Dsg1,2), desmoplakin (DP), and plakoglobin (PG), desmosomes; keratin 6 (K6), suprabasal cells of hyperproliferative epidermis and the companion layer of hair follicles (hf). **(I)** Western blot analyses of epidermal proteins. Monospecific Abs used to probe the blots are indicated (Right).

To assess whether the changes in adhesive components of KO skin epidermis might influence other aspects of differentiation, we examined normal differentiation-specific markers of the tissue. Because basal cells withdraw from the cell cycle and commit to differentiate, they switch off expression of keratins 5 and 14 and switch on K1 and K10. KO epidermis still executed this switch at the proper stage in differentiation (Fig. 2.3 A and A'). Similarly, the spinous cell marker involucrin was also expressed in a pattern similar to WT epidermis (Fig. 2.3, B and B').

As cells move toward the skin surface, they remain transcriptionally active, and in the granular layer, they express profilaggrin, which is processed to filaggrin as keratinohyalin granules form. Granular cells also express loricrin, a major constituent of the cornified envelope that serves as a scaffold to organize the lipids of the epidermal barrier. Despite the morphological presence of a granular layer, anti-filaggrin and anti-loricrin immunoreactivity were down-regulated in KO relative to WT epidermis (Fig. 2.3, C and D). Western blot analyses revealed very little processed filaggrin (27 kDa) as well as reduced loricrin levels (Fig. 2.3E). These granular layer perturbations were suggestive of a defect in the epidermal barrier. The defect was likely to be mild, because animals with overt barrier function defects dehydrate and die shortly after birth (Segre et al., 1999), whereas *E-cadherin* conditionally null mice sometimes survived to adulthood. We return to this point below.

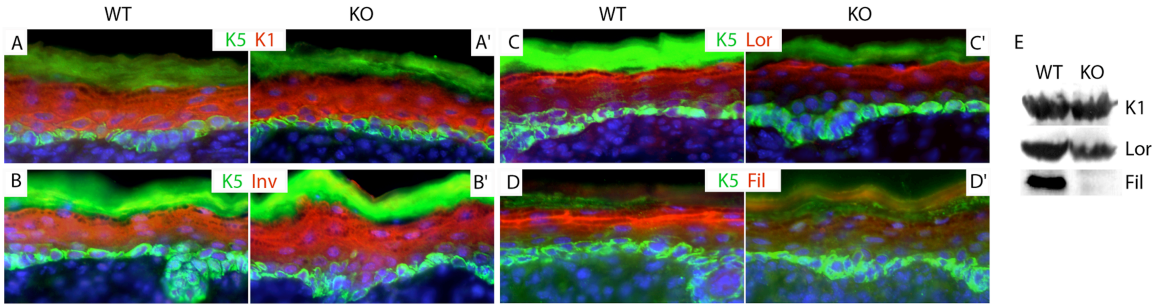



**Figure 2.3** *Reduced terminal differentiation in E-cadherin null epidermis.* **(A–D')** Immunofluorescence microscopy of sections of newborn backskins labeled with the Abs indicated: K5, specific for the basal epidermal layer and follicle ORS; K1, specific for suprabasal layers; Inv, involucrin, expressed in the spinous layer but an early marker of the cornified envelope; Lor, loricrin, a granular marker of the cornified envelope; Fil, filaggrin, expressed as profilaggrin in the granular layer and then processed near or at the end of terminal differentiation. **(E)** Western blot analyses of epidermal proteins. Abs used to probe the blots are indicated on the right.

In the adult, the most dramatic defect of *E-cadherin* conditionally null animals was not at the skin surface, but rather in their hair coat (Fig. 2.4). Animals also tended to be smaller and displayed abnormally short and misshapen whiskers and sparse pelage hairs. Such abnormalities made mosaic animals distinctive.

The defects in hair coat were not due to an inability to initiate placodes or progress through the early stages of follicle morphogenesis. By postnatal day 10, mature follicles had formed in normal numbers and spacing and at first glance, WT and KO skin appeared similar (Fig. 2.4, E and F). Closer inspection revealed that hairs lacking E-cadherin were abnormally short and fragile, and the orifices

were not always properly formed (arrows in Fig. 2.4, E and F). Additionally, whereas the long WT follicles of the growth (anagen) phase were readily captured within the plane of sectioning, this was not the case for KO follicles, reflecting their bent irregular shape (arrowheads in Fig. 2.4, F and G).

The second hair cycle normally initiates at ~3 wk of age. Thereafter, follicles cycle regularly although less synchronously, leading to a mixture of cycling and resting follicles in adult backskin. At these ages, it was readily apparent that with each postnatal hair cycle, the *E-cadherin* conditionally null follicles became progressively more aberrant. By 1.5 mo, the divergence in angling was striking (Fig. 2.4G, arrowheads). In addition, whereas 1.5-mo WT skin displayed regularly spaced hair follicles (not shown), KO skin exhibited expanded interfollicular gaps, and by 4 mo, only remnants of follicles were detected (Fig. 2.4, G and H). In addition, increased cellularity was detected in the dermis along with a reduction in subcutaneous fat.

Normal adult skin is typically thinner than newborn skin, a difference manifested primarily in the suprabasal layers (data not shown; see ref. Fuchs and Raghavan, 2002). As *E-cadherin* null epidermis aged, however, many regions remained thickened (Fig. 2.4H). Adult KO epidermis displayed suprabasal anti-K6 immunoreactivity (not shown; see Fig. 2.2H), as well as a marked increase in the number of basal cell nuclei positive for the proliferating nuclear antigen Ki67 (Fig. 2.4, I and J). Overall, these differences were indicative of a hyperproliferative state.

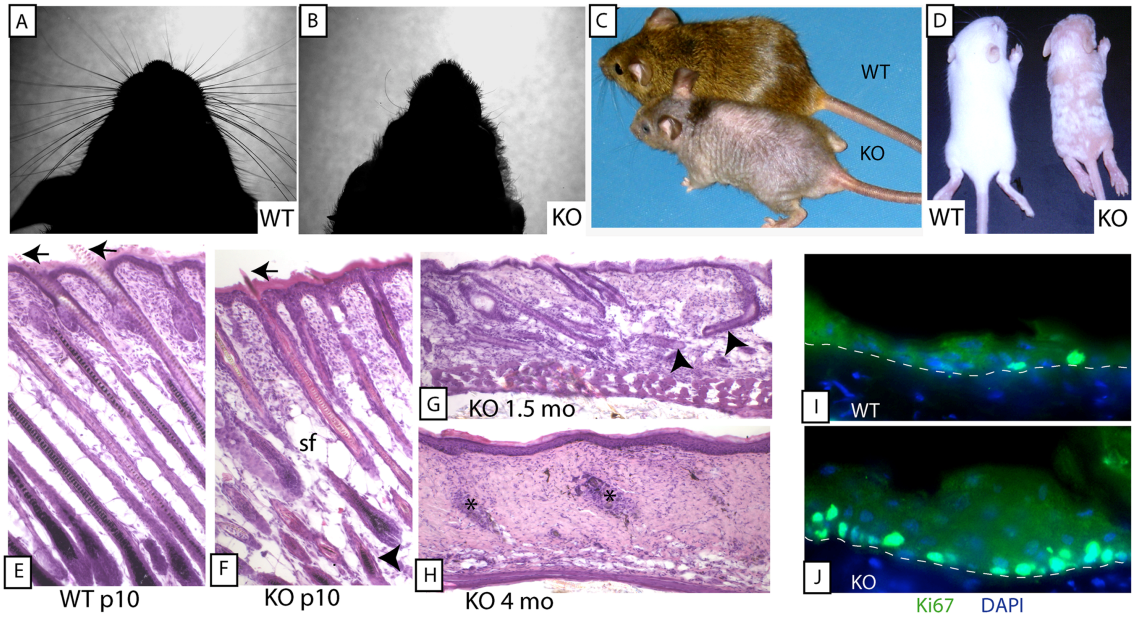



**Figure 2.4** *Hair and epidermal abnormalities in adult E-cadherin conditionally null mice.* **(A and B)** Backlit images of adult snouts. **(C and D)** Example of a full adult KO (*C*) and mosaic (*D*) mice from a postnatal day 10 litter. **(E–H)** Hematoxylin/eosin-stained sections of frozen backskins of the ages indicated. Arrows denote hairs that broke through the skin surface (rare in KO skin). Arrowheads denote misangled KO follicles; asterisks, follicle remnants. epi, epidermis; de, dermis, sf, subcutaneous fat. **(I and J)** Immunofluorescence of sections of adult backskins labeled with Abs against the proliferation marker, Ki67. Nuclei were counterlabeled with 4',6-diamidino-2-phenylindole (DAPI). Dotted line indicates basement membrane.

To begin to understand how a loss of E-cadherin could lead to progressive hyperproliferation in epidermis but progressive loss of hair follicles, we analyzed the expression patterns of cadherins and differentiation-specific markers in the hair follicle. Fig. 2.5A provides a schematic of the concentric rings of differentiating hair follicle cells that arise from the proliferative compartment

(matrix), which is in contact with the specialized mesenchyme (dermal papilla) at the base of the follicle. Because the program of differential cadherin expression is comparable in whisker and backskin follicles, we show data for whiskers, where layers are more readily distinguished.

In normal skin, matrix, outer root sheath (ORS), cortex, and medulla express both P- and E-cadherin (Fig. 2.5, B and C) (Jamora et al., 2003). In contrast, other follicle regions express E- and not P-cadherin (Muller-Rover et al., 1999). In *E-cadherin* null follicles, the pattern and levels of P-cadherin appeared largely unchanged (Fig. 2.5D), as did that of Dsg1,2 (data not shown). In this regard, KO follicles behaved differently than KO epidermis.

Colabeling with anti-P-cadherin and antibodies against the differentiation-specific markers enabled us to assess differentiation and identify precisely which KO follicle layers lacked both E- and P-cadherin. In contrast to the epidermis, the programs of differentiation were largely indistinguishable for KO and WT follicles, at least at early stages, which permitted assessment of primary rather than secondary effects (Fig. 2.5 E-G; data shown for KO follicles).

The single layer of companion cells is readily identified by anti-K6 antibodies (Fig. 2.5E). This layer marked the boundary between P-cadherin-positive ORS and -negative inner root sheath (IRS). The transcription factor GATA-3, which identifies the Huxley layer and IRS cuticle (Kaufman et al., 2003), revealed a two-layer gap between GATA-3-positive cells and P-cadherin-positive ORS (Fig. 2.5F). These two P-cadherin-negative layers are the Henley and

companion layers (Fig. 2.5A). Finally, colabeling with the hair keratin marker AE13 showed that all but the outermost (cuticle) layer of the shaft was also positive for P-cadherin (Fig. 2.5G). Taken together, these data established a zone of P- and E-cadherin-negative cells that encompassed the companion layer, IRS, and hair shaft cuticle of the KO follicle.

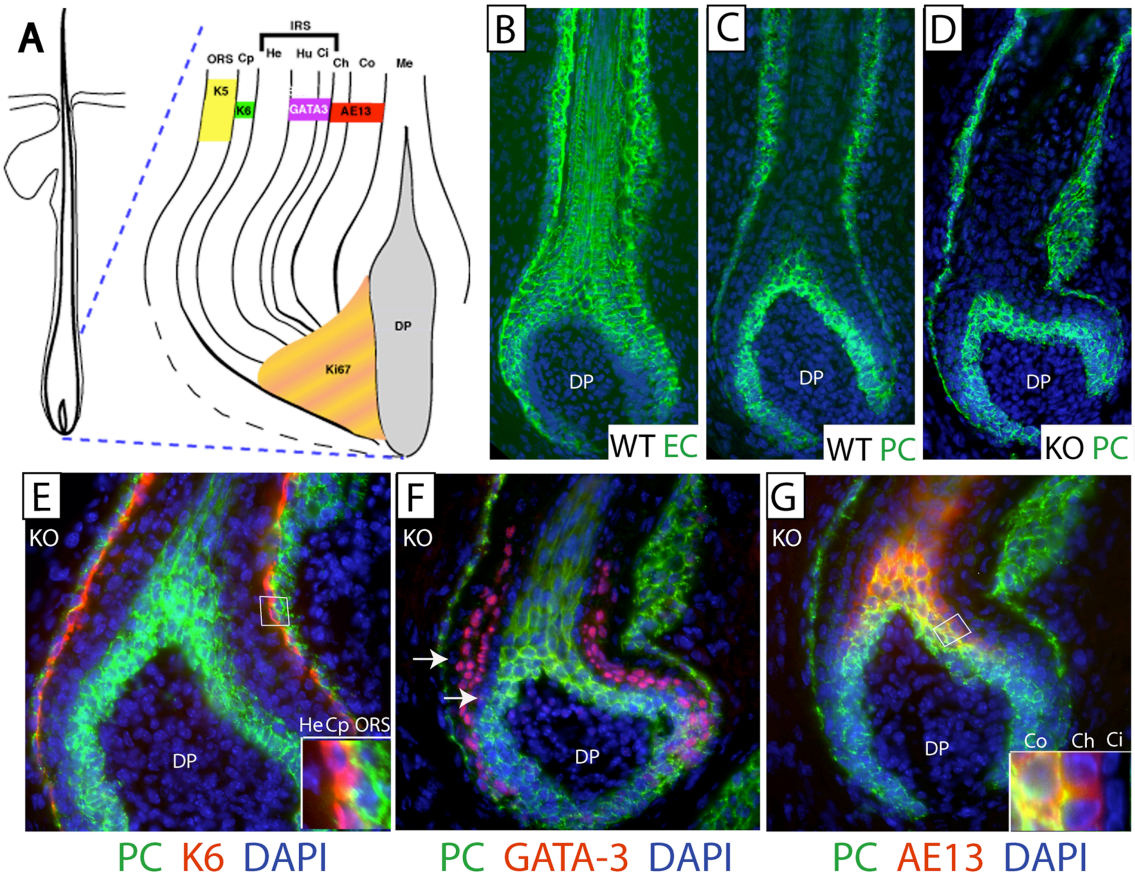



**Figure 2.5** *Expression of cadherins and differentiation markers in hair follicles.* **(A)** Diagram of a hair follicle highlighting the different cell layers and their associated differentiation markers (Kaufman et al., 2003; Kobielak et al., 2003). ORS, outer root sheath; Cp, companion layer; IRS, inner root sheath; He, Henle layer; Hu, Huxley layer; Ci, cuticle of the IRS; Cu, cuticle of the hair shaft; Co, cortex; Me, medulla; DP, dermal papilla. **(B–G)** Immunofluorescence of sections

of newborn whisker follicles labeled with the indicated Abs: EC, E-cadherin; PC, P-cadherin; AE13, specific for the hair shaft keratins; GATA-3 and K6, 4',6-diamidino-2-phenylindole (DAPI) in blue. (Insets) Magnified views of boxed regions. Arrows denote P-cadherin-negative cells layers flanking the GATA-3 positive Huxley and IRS cuticle layers. (A) is reproduced with permission from ref. (Kobielak et al., 2003) (Copyright 2003, The Rockefeller University Press).

Ultrastructural analyses revealed the structural consequences associated with the loss of both P- and E-cadherin. As expected, the bulb of the *E-cadherin* null follicle displayed intact intercellular adhesion with normal AJs (Fig. 2.6A; for extensive data on WT follicles, see refs. (Kaufman et al., 2003) and (Kobielak et al., 2003). Just above the pocket of dermal papilla cells, however, adhesive defects were obvious. Although the largest intercellular gaps (Fig. 2.6A, asterisks) were in the Huxley layer of the IRS, AJs were missing between the companion layer and the electron-dense Henle layer and between the Henle layer and the trichohyalin-rich Huxley layer (Fig. 2.6B). The finger-like connections between these layers were desmosomes, which were intact and in comparable numbers to WT follicles. Desmosomes were abundant in KO Henle–Henle cell junctions as they are in WT, and correspondingly, adhesion between Henle cells was largely intact (Fig. 2.6, B and C). A paucity of AJs was also seen in intercellular contacts between the trichohyalin-rich IRS cuticle and the hair shaft cuticle (Fig. 2.6D; compare with Fig. 6E). Thus, layers that lacked

both P- and E-cadherin exhibited significant defects in AJs, leading to a loss of integrity and a corresponding distortion of follicle structure.

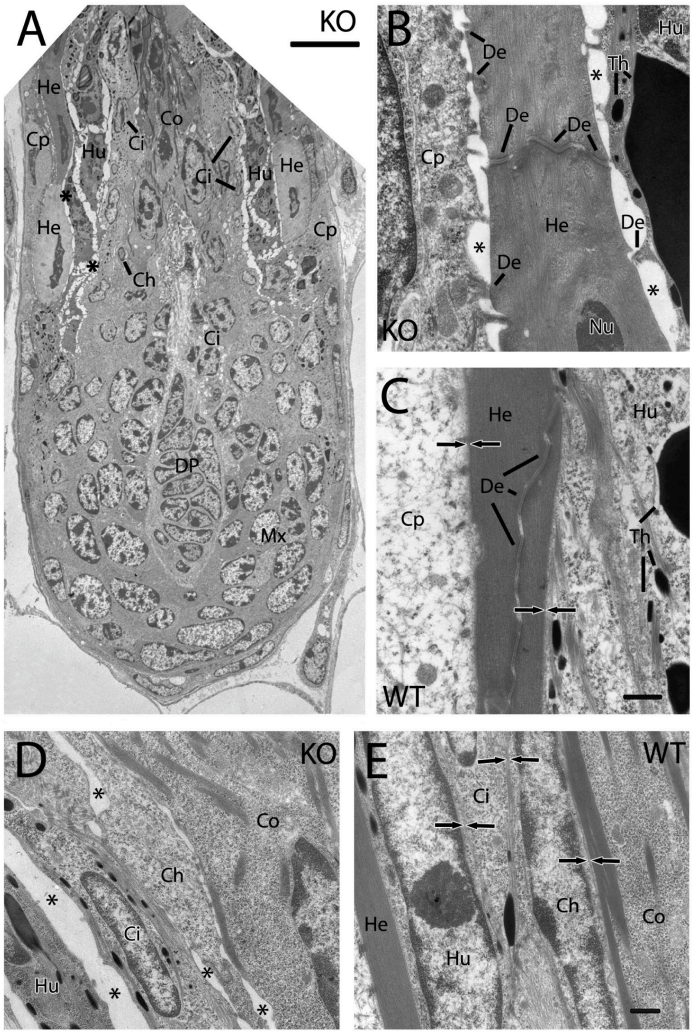



**Figure 2.6** *Ultrastructural abnormalities in E-cadherin null follicles.* Backskins of postnatal day 11 animals were processed for transmission electron microscopy. Sagittal sections of follicles are oriented with the skin surface toward the top of each frame. Asterisks denote intercellular gaps, reflective of a loss of membrane sealing (opposing arrows). Mx, matrix; IRS, inner root sheath; Hu, Huxley; He, Henle; Ci, IRS cuticle; Cp, companion layer; Ch, hair shaft cuticle; Co, cortex; De, desmosomes; Th, trichohyalin. [Bars = 10 $\mu$m(*A*); 500 nm (*B* and *C*); 500 nm (*D* and *E*).]

## Discussion

Defects in skin epithelium have been described for loss-of-function mutations in α- and β-catenin (Vasioukhin et al., 2001a; Huelsken and Behrens, 2002). However, in those cases, E-cadherin remained at cell–cell borders, and either the Ras-MAPK pathway (α-catenin) or Lef1/-catenin-mediated signaling (β-catenin) was affected. This has made it difficult to assess the extent to which catenin-related defects arise from alterations in cell–cell junctions per se. Moreover, null mutations in P-cadherin resulted in no overt abnormalities in skin, and even when desmoglein 3 was also missing, no new skin defects surfaced (Lenox et al., 2000). Thus, although genetic studies have documented the importance of desmosomes in skin (Kowalczyk et al., 1999; Huber, 2003; Kljuic et al., 2003), the role of AJs in this tissue has remained elusive.

Our studies now reveal the deleterious consequences of reducing AJs in the IRS, hair cuticle, and suprabasal epidermal layers. Interestingly, although normal cellular function was compromised in all these compartments, the outcomes were markedly different. In the lower IRS and hair cuticle, desmosomes were too few to maintain membrane sealing and prevent structural aberrations. Although additional explanations are possible, the selective loss of IRS and hair cuticle integrity is sufficient to cause a secondary loss of hair follicles, because ORS, cortex, and medulla alone cannot sustain follicle structure (Byrne et al., 2003).

The hyperproliferative defects in epidermis were distinct not only from the IRS but also from the lactating mammary gland (Boussadia et al., 2002) and blastocyst (Ohsugi et al., 1997), all of which exhibited cell degeneration and/or loss of tissue integrity. They also differed from P-cadherin null mice, which displayed precocious differentiation in mammary gland and no skin defects (Radice et al., 1997a; Lenox et al., 2000). The molecular explanation underlying this paradoxical difference is complex. In the suprabasal layers, the loss of E-cadherin was accompanied by a reduction in $\alpha$- and $\beta$-catenins, reflective of a corresponding reduction in AJs. However, the concomitant increase in desmosomal cadherins coupled with the natural abundance of desmosomes in spinous layers seemed to explain the preservation of KO spinous layer integrity. This said, these adhesive changes were not fully compensatory, as best exemplified by the failure to properly execute terminal differentiation. Although quantitative loss of epidermal barrier results in dehydration and death, mild defects are often counterbalanced by hyperproliferation and thickening (Byrne et al., 2003; Madison, 2003), similar to what we observed in older *E-cadherin* null skin.

Precisely how hyperproliferation arises in aging *E-cadherin* null epidermis is likely to be important in understanding why decreases in cadherin expression are widely associated with human cancers. It has been speculated that by uncoupling $\beta$-catenin from its partner cadherin, Wnt signaling could be activated, contributing to tumorigenesis (Conacci-Sorrell et al., 2002). In skin epidermis,

constitutively stabilized β-catenin can lead to hyperproliferation and thickening, but it is also accompanied by follicle-like down-growths (Gat et al., 1998) not observed in our KO animals. An alternative possibility is that by up-regulating P-cadherin, KO basal cells maintain AJs, survive, and counterbalance the terminal differentiation defects by hyperproliferating.

Our results are interesting in light of the many cancers involving alterations in the expression of classical cadherins (Cavallaro and Christofori, 2001; Conacci-Sorrell et al., 2002). Although future studies will be necessary to fully appreciate the functional relation between E- and P-cadherins, our studies suggest that if mutations in E-cadherin are accompanied by cadherin compensation in progenitor cells but not their differentiating offspring, the prognosis can be prolonged hyperplasia and/or dysplasia, which could in turn lead to tumor progression.

## Materials and Methods

### Immunofluorescence and Antibodies

Primary antibodies used were E-cadherin (M. Takeichi, Zymed), P-cadherin and p120 (Zymed), α- and β-catenin (Sigma), desmoglein 1,2 (1:100, W. Franke German Cancer Research Center, Heidelberg), desmoplakin (Research Diagnostic, Flanders, NJ), laminin 5 (1:100, R. Burgeson University School of Medicine, Chiba, Japan), β4-integrin (Pharmingen), keratin 1 (1:250, E.F.), keratin 5 (1:100, E.F.), keratin 6 (1:500, P. Coulombe Johns Hopkins School of

Medicine, Baltimore), Ki67 (1:200, NovoCastra, Newcastle, U.K.), plakoglobin (1:50, P. Cowin New York University School of Medicine, New York), AE13 (1:10, T. T. Sun New York University School of Medicine, New York), GATA-3 (1:25, Santa Cruz Biotechnology, HCG3), involucrin (1:100, Babco, Richmond, CA), filaggrin (Covance, Berkeley, CA, PRB-417P), and loricrin (1:100, E.F.). Alexa 488 (Molecular Probes) or Texas red (The Jackson Laboratory) conjugated secondary antibodies were used for detection. Additional reagents were TRITC-Phalloidin and 4',6-diamidino-2-phenylindole (DAPI; Sigma). Unless stated, dilutions were according to the manufacturer's recommendations.

Tissues were frozen, embedded in OCT compound, and sectioned (10 $\mu$m). Fixation was with 4% formaldehyde in PBS for 8 min. Slides were blocked with PBS, 0.2% Triton X-100, 1% BSA, 5% normal goat serum, 5% normal donkey serum, or MOM Basic kit (Vector Laboratories).

**Electron Microscopy**

Tissues were fixed for 1 h in 2% glutaraldehyde, 4% formaldehyde, 2 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer, and then processed for Epon embedding (Kaufman et al., 2003). Samples were visualized with a Tecnai (Hillsboro, OR) G2 transmission electron microscope.

**Protein Analysis**

Newborn backskin was incubated in dispase for 30 min at 37°C. Epidermis was flash-frozen in liquid nitrogen and then crushed. Typically, proteins were then extracted and processed for Western blot analysis (Vasioukhin et al., 2001a; Vasioukhin et al., 2001b). For detection of E-cadherin, crushed tissue was resuspended in 1 ml of radioimmunoprecipitation assay (RIPA) buffer with 1 mM DTT and protease inhibitors, and after 30 min, extracts were centrifuged at 14,000 x *g* for 15 min. Anti-E-cadherin-coated protein G-Sepharose beads were incubated with extracts for 2 h, washed three times with RIPA buffer, and then boiled in Laemmli buffer to release bound proteins, which were analyzed by SDS/PAGE and anti-E-cadherin Western blotting.

# Chapter 3: New Insights into Cadherin Function in Epidermal Sheet Formation and Maintenance of Tissue Integrity

### Contributors To This Work

Transmission electron microscopy and ultrastructural analysis was provided by Dr. Amalia Pasolli. DNA injection for transgenic mice and mouse breedings were done by Nicole Stokes and Lisa Polak.

### Abstract

Overlapping expression patterns and gene linkage have frequently hampered elucidating the physiological relevance of cadherins in mammalian tissues. Here, we combine conditional gene ablation and transgenic RNA interference to uncover new roles for E and P-cadherins in epidermal sheet formation *in vitro* and maintenance of epidermal integrity *in vivo*. We show that *in vitro,* loss of E-cadherin alone results in elevated P-cadherin but delayed epidermal sheet formation. By contrast, suppressing both cadherins inhibits not only adherens junctions, but also desmosomes, tight junctions and cortical actin dynamics associated with sheet formation. Using different rescue strategies, we show that cadherin level rather than isotype is critical. By devising novel skin-specific RNAi technology, we demonstrate that cadherin inhibition in vivo impairs junction formation and intercellular adhesion resulting in increased apoptosis and defects in epidermal barrier and tissue integrity. Finally, by contrasting conditional loss-of-function studies of epidermal catenins and cadherins, we've been able to

define cadherin-dependent and independent roles of AJ components in tissue physiology.

## Introduction

The formation, maintenance and morphogenesis of tissues within multicellular organisms are dependent on intercellular adhesive contacts (Gumbiner, 2005; Perez-Moreno and Fuchs, 2006). In many vertebrate epithelial cells these interactions are mediated by three major multi-protein complexes: adherens junctions (AJs), desmosomes (Dms), and tight junctions (TJs). In epithelial cells, AJs are particularly important for the dynamic regulation of intercellular adhesion. The transmembrane core of epithelial AJs is composed of E-cadherin, whose ectodomain binds $Ca^{2+}$ to mediate specific adhesive binding through trans-cadherin interactions between neighboring cells (Nose et al., 1988; Patel et al., 2006). The intracellular domain of E-cadherin binds directly to p120-catenin and $\beta$-catenin, which in turn binds to $\alpha$-catenin, unrelated in sequence to other catenins. Collectively, catenins regulate cadherin stability and coordinate associated actin dynamics to ensure efficient cell adhesion (Gumbiner, 2005). AJ formation and stability are also thought to depend upon an additional adhesive complex of the transmembrane protein nectin, which associates with afadin, an actin binding protein (Ogita and Takai, 2008).

The hierarchical process of intercellular adhesion and its dependency on AJs has been unveiled largely by *in vitro* studies (Perez-Moreno and Fuchs,

2006). Thus, if α-catenin is missing (Watabe et al., 1994) or classical cadherin function is inhibited either by blocking antibodies, mutant cadherins, or short hairpin (sh) RNAs (Gumbiner et al., 1988; Amagai et al., 1995; Capaldo and Macara, 2007), then formation of Dms and TJs is also impaired. By contrast, AJs still form in the absence of desmoplakin (Vasioukhin et al., 2001b) or Z0 proteins (Umeda et al., 2006).

In pioneering studies exploring the spatiotemporal organization of cadherins and actin during early steps of intercellular adhesion, it was discovered that cadherin-catenin complexes cluster into puncta, representing stable sites of intercellular contact (McNeill et al., 1993; Yonemura et al., 1995; Adams et al., 1996; Adams et al., 1998). Coincident with puncta formation is the appearance of radial actin cables that associate with puncta and orient perpendicular to sites of contacting membranes. As epithelial sheet formation proceeds, puncta appear to expand and coalesce along sites of opposing cell-cell contacts, and junctional actin reorganizes into a circumferential cortical actin belt oriented parallel to membrane contacts.

When stimulated by calcium, primary epidermal keratinocytes *in vitro* undergo prolonged periods of filopodial and lamellipodial extensions and intercellular contacts prior to establishing the initial punctum (Vasioukhin et al., 2000; Vaezi et al., 2002). Following this rate-limiting step, additional puncta assemble rapidly, as the entire epithelial sheet coordinates actin-AJ dynamics in unison to function as a two-dimensional “tissue.” At the conclusion of sheet

formation, associated actin dynamics wanes. α-catenin is essential for regulating these actin dynamics, since primary keratinocytes cultured from *α-catenin* conditionally null (cKO) epidermis fail to form radial actin cables at early stages of junction formation, nor do they organize cortical actin belts or assemble into epidermal sheets (Vasioukhin et al., 2000; Vaezi et al., 2002; Kobielak et al., 2004). While these *in vitro* experiments have identified key steps in AJ formation and revealed an importance in AJ-actin cytoskeletal dynamics in the regulation of intercellular adhesion, less is known about cadherin-dependent functions within the context of a three-dimensional tissue *in vivo.*

An excellent system to probe the physiological importance of AJ components in a mammalian tissue is the skin epidermis, where *E-cadherin, P-cadherin, α-catenin, p120-catenin* and *β-catenin* have all been targeted for ablation (Radice et al., 1997a; Huelsken et al., 2001; Vasioukhin et al., 2001a; Young et al., 2003; Tinkle et al., 2004; Tunggal et al., 2005; Perez-Moreno et al., 2006). While E-cadherin is expressed throughout epidermis, P-cadherin is restricted to the basal layer where it is enriched at sites of hair follicle (HF) downgrowth (Hirai et al., 1989; Hardy and Vielkind, 1996). Mice lacking P-cadherin are viable and fertile and do not show an obvious epidermal phenotype (Radice et al., 1997a), and combined loss of P-cadherin and desmoglein 3, a desmosomal cadherin, does not result in any new defects in the skin epidermis (Lenox et al., 2000). Conditional ablation of *E-cadherin* in both embryonic and adult skin results in loss of structural integrity within the inner layers of hair

follicles (HFs), but epidermal architecture is surprisingly unaffected, a feature traced to the upregulation of P-cadherin in the *E-cadherin* null basal layer (Young et al., 2003; Tinkle et al., 2004; Tunggal et al., 2005).

In comparing conditional loss of function mutations of β-catenin, p120 catenin and α-catenin, a shared finding is the persistence of cortical localization of E and P-cadherins (Huelsken et al., 2001; Vasioukhin et al., 2001a; Perez-Moreno et al., 2006). Analogously, the most striking phenotypes resulting from catenin loss appear to be largely independent of alterations in cell adhesion. Epidermal integrity is unaltered in the absence of either β-catenin or p120-catenin, yet prominent defects occur in HF morphogenesis and inflammation, respectively. While loss of α-catenin does disrupt epidermal adhesion, the entire skin epithelium systematically transforms to a hyperproliferative, invasive tissue replete with inflammation (Kobielak and Fuchs, 2006).

Such complex phenotypes have made it difficult to dissect cadherin-dependent and independent functions of catenins. Furthermore, a limiting factor in the analysis of each of these mutant mouse models is that cadherin-catenin complexes still form and localize to keratinocyte membranes. As a result, the functional relevance of the loss of classical cadherins in epidermal physiology remains unknown. Genetic dissection of cadherin function is further complicated by the fact that E- and P-cadherin genes are tightly linked, precluding conventional conditional knockout strategies. We have now circumvented this problem in epidermal keratinocytes *in vitro* and skin epidermis *in vivo*. Our

analyses have uncovered defects not observed previously in single loss of function mutations for E-cadherin, P-cadherin, α-catenin, p120-catenin or β-catenin. Most importantly, our studies reveal new insights into the functional significance of E and P-cadherin in the formation of cell adhesion complexes in keratinocytes and maintenance of epidermal integrity *in vivo*, and they have enabled us to dissect those functions dependent upon AJs versus those that are unique to individual AJ components.

## Results

### *E-cadherin* null keratinocytes display delayed kinetics of epidermal sheet formation

To explore how the formation of intercellular junctions, actin dynamics and epidermal sheets depends upon cadherins, we first analyzed cultured primary keratinocytes ($1^0$MK) from newborn (P0) *K14-Cre/E-cadherin* (fl/fl) mice. Under the conditions used, *E-cadherin* null (KO) and WT $1^0$MK were morphologically indistinguishable, and showed no difference in growth or survival *in vitro.* When calcium was increased from low (0.05 mM) to high (1.5 mM) to stimulate homotypic engagement of cadherins and promote junction formation, epidermal sheets formed within 24 hrs, irrespective of genotype. Closer inspection, however, began to reveal differences in the behavior of KO $1^0$MK relative to their WT counterparts.

Previously, we noted that P-cadherin was upregulated in the basal epidermal layer when E-cadherin was absent (Tinkle et al., 2004). This was also true for cultured KO $1^0$MK, demonstrating the cell-autonomous nature of this elevation (Fig. 3.1, A and C). Despite enhanced P-cadherin, however, total catenin levels were reduced in KO $1^0$MK (Fig. 3.1C). In monitoring kinetics of adhesion, we discovered that P-cadherin concomitantly increased at puncta during early stages of junction formation (Fig. 3.1A). Despite this increase, cadherin-associated catenins were diminished at puncta of KO $1^0$MK (Figs. 3.1B; 3.2, A and B). At later stages of the process, this difference disappeared, and catenins localized normally to intercellular contacts within epidermal sheets, albeit it at reduced overall levels.

Previous studies have reported cooperative interactions between cadherin-catenins and nectin-afadin junctional complexes, resulting in the suggestion that nectin-afadin complexes may promote AJ assembly and stabilization (Ogita and Takai, 2008). Thus, it was intriguing that in $1^0$MK, loss of E-cadherin diminished nectin-afadin localization at puncta during early stages of epidermal sheet formation. That said, it did not influence their localization at later stages, nor was the total level of afadin protein altered (Figs. 3.1D; 3.2, C and E).

The delay in epidermal sheet formation suggested that actin dynamics might also be affected when E-cadherin is missing. To test this possibility, we generated *K14-Cre/E-cadherin (fl/fl)/K14-GFPactin mice* (Vaezi et al., 2002) and then derived P0 GFPactin $1^0$MKs either null or WT for *E-cadherin*. Despite the

formation of P-cadherin puncta, KO $1^0$MK failed to display the radial arrangement of actin cables typically associated with E-cadherin puncta (Adams et al., 1998; Vasioukhin et al., 2000) (Fig. 3.1E). However by 24 hr, the organization of F-actin was similar within KO and WT sheets, and $1^0$MKs displayed a typical cortical actin belt beneath what appeared to be mature junctions.

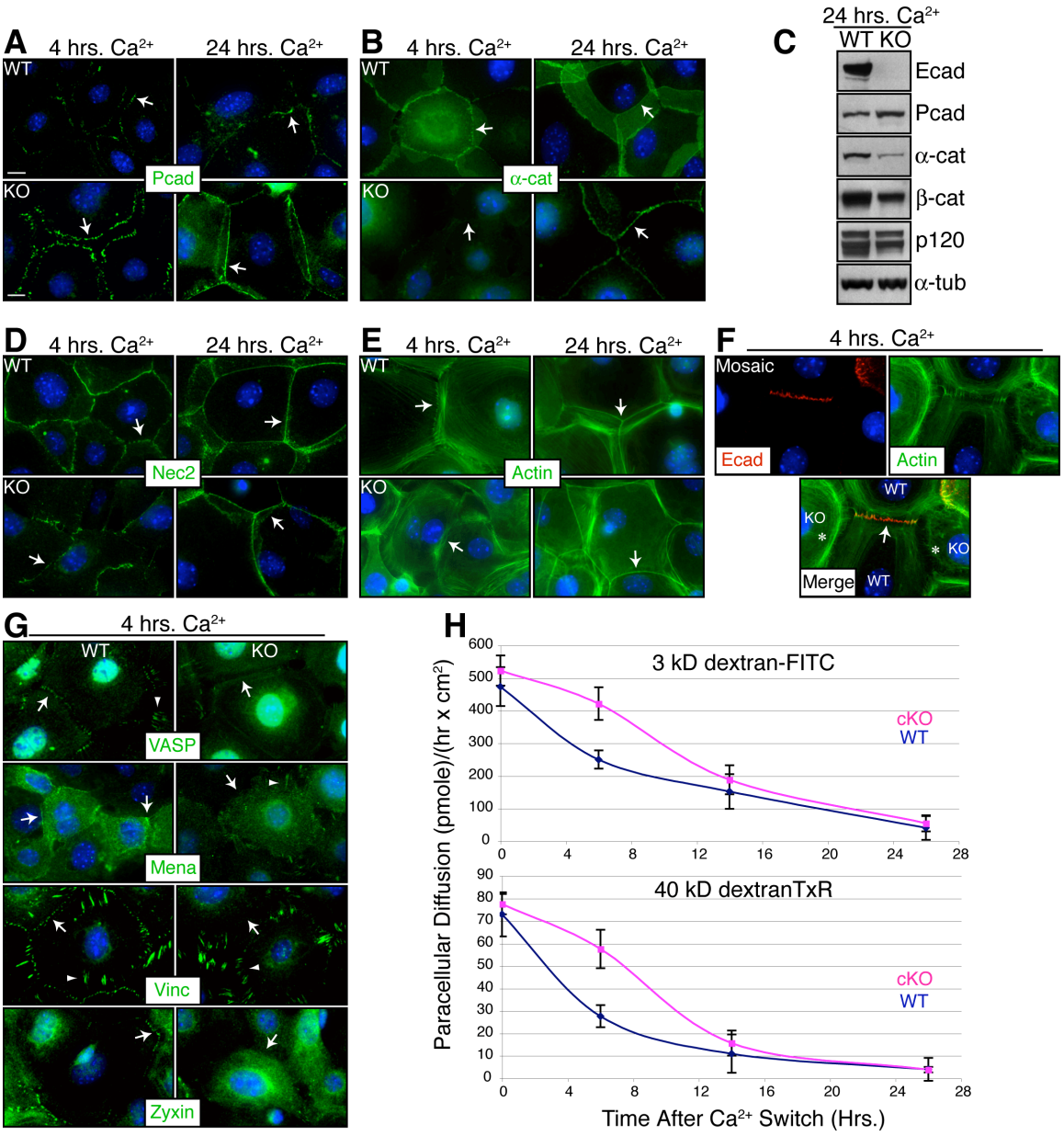


**Figure 3.1** *Loss of E-cadherin results in delayed kinetics of AJ formation, actin organization and barrier acquisition in primary keratinocytes.* **(A-G)** Confluent monolayers of WT and *E-cadherin* null (KO) $1^0$MKs were shifted to high $Ca^{2+}$ media for indicated times prior to processing for either indirect immunofluorescence or immunoblot analyses of total lysates. Abs are indicated and color-coded according to the $2^0$ Abs except for actin (E, F), which represents epifluorescence from K14-GFPactin $1^0$MKs WT, null or mosaic for *E-cadherin*. Abbreviations: Ecad (E-cadherin), Pcad (P-cadherin); $\alpha$-cat ($\alpha$-catenin); $\beta$-cat ($\beta$-catenin); p120 catenin (p120); $\alpha$-tub ($\alpha$-tubulin); Nec2 (nectin-2); Vinc (vinculin). Arrows indicate sites of cell-cell interactions. Asterisks in (F) denote absence of radial actin cables at sites of interaction between one WT and one KO cell. Arrowheads in (G) point to focal adhesions, to which these actin-associated proteins also localize. In all immunofluorescence images, DAPI (blue) marks nuclei. Bars, 10 $\mu$m. **(H)** Paracellular diffusion of 3 kDa and 40 kDa fluorescently-labeled dextrans through confluent $1^0$MK monolayers was measured at times indicated after switch to high $Ca^{+2}$ media. Diffusion was analyzed 2.5 hrs after addition of tracers. Data represent the mean +/- SE of 6 independent cell lines/genotype.

Early alterations in actin organization were further highlighted in $Ca^{2+}$-treated cultures of $1^0$MKs mosaic for loss of E-cadherin. Interestingly, radial actin cables only assembled at sites of intercellular contact between two WT cells (Fig. 3.1F). This contrasted markedly with $\alpha$*-catenin* null $1^0$MKs, where radial actin cables still formed and actin organized normally on the WT side of mosaic adhesions (Vasioukhin et al., 2000). One notable difference was that E-cadherin still assembled at intercellular contact sites between WT and $\alpha$*-catenin*-null

$1^0$MKs. Taken together, these data revealed several important points: first, an importance of intercellular contacts involving transmembrane E-cadherins in enabling α-catenin to organize actin into radial cables in early stages of epidermal sheet formation, and second, the lack of upregulated endogenous P-cadherin to compensate for loss of E-cadherin in the early dynamic stages of intercellular junction formation.

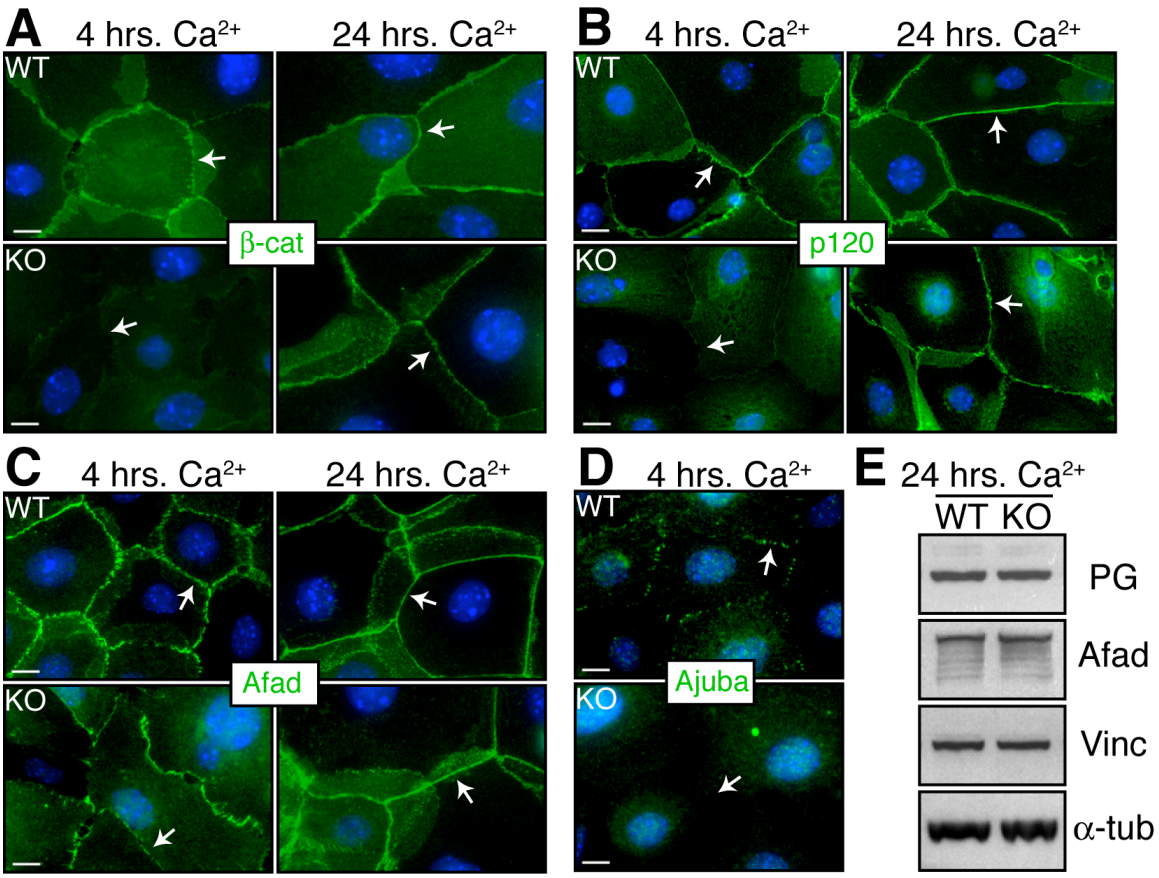



**Figure 3.2** *Loss of E-cadherin results in delayed kinetics of AJ formation.* **(A-E)** Confluent monolayers of WT and KO $1^0$MKs were shifted to high $Ca^{2+}$ media for indicated times prior to processing for indirect immunofluorescence (A-D) and immunoblot blot analyses of total lysates (E) with indicated Abs. Abbreviations: β-cat (β-catenin), p120 (p120 catenin); afad (afadin); PG (plakoglobin); Vinc

(Vinculin); α-tub (α-tubulin). Arrows indicate sites of cell-cell interactions. Bars, 10 μm.

In probing the underlying defects associated with these altered actin dynamics, we discovered that radial actin polymerizing proteins VASP and Mena, as well as the α-catenin associated proteins vinculin, zyxin and Ajuba all failed to associate with developing puncta of KO $1^0$MK (Figs. 3.1G, 3.2D). Since junctional localization of these proteins depends upon α-catenin (Vasioukhin et al., 2000; Marie et al., 2003), these results pointed to inefficient cortical localization of α-catenin as a likely principal source of the altered actin organization observed in *E-cadherin* null cells.

To determine the functional consequences of these alterations in junction-cytoskeletal dynamics, we tested the ability of $Ca^{2+}$-treated $1^0$MK to form an effective barrier to paracellular diffusion of nonionic 3 and 40-kD dextrans (Jou et al., 1998; Mertens et al., 2005). In contrast to their WT counterparts, confluent monolayers of KO $1^0$MKs exhibited an early delay in exclusion of tracers following $Ca^{2+}$ switch (Fig. 3.1H). By 24 hrs however, an intact barrier had formed. Although altered in the normal sequence of events, KO $1^0$MK eventually produced mature intercellular junctions and provided a functional barrier, providing an explanation for why *E-cadherin* null epidermis *in vivo* displayed seemingly normal, sealed intercellular membranes and an intact epidermal barrier (Tinkle et al., 2004).

**Overall cadherin levels are a defining factor in efficient epidermal sheet formation**

A priori, the defects in early intercellular junction formation in KO $1^0$MKs could be due to functional differences between E and P-cadherin or a failure of P-cadherin upregulation to reach requisite levels of total cadherins. Interestingly, in both WT and KO $1^0$MKs, P-cadherin levels rose steadily after $Ca^{2+}$ switch (Fig. 3.3A). Although this differed from that seen with passaged human keratinocytes (Wheelock and Jensen, 1992), it resembled the increase in cadherin 6 protein observed when the epithelial cell line MDCK is exposed to elevated calcium (Stewart et al., 2000). The increase in P-cadherin in *E-cadherin* null $1^0$MKs seemed to have physiological significance as increased puncta localization correlated with the emergence of radial actin cable formation (Fig. 3.3B).

To test whether overall levels of cadherins regulate the rates and efficiencies of AJ assembly, we generated and expressed vectors encoding GFP fusion proteins of E and P-cadherins. As previously shown for E-cadherin-GFP in MDCK cells (Adams et al., 1998), both GFP-cadherins properly localized to intercellular junctions, immunoprecipitated β- and p120-catenin, and were of the expected size and expressed comparably in epidermal MKs (Fig. 3.4, A and B).

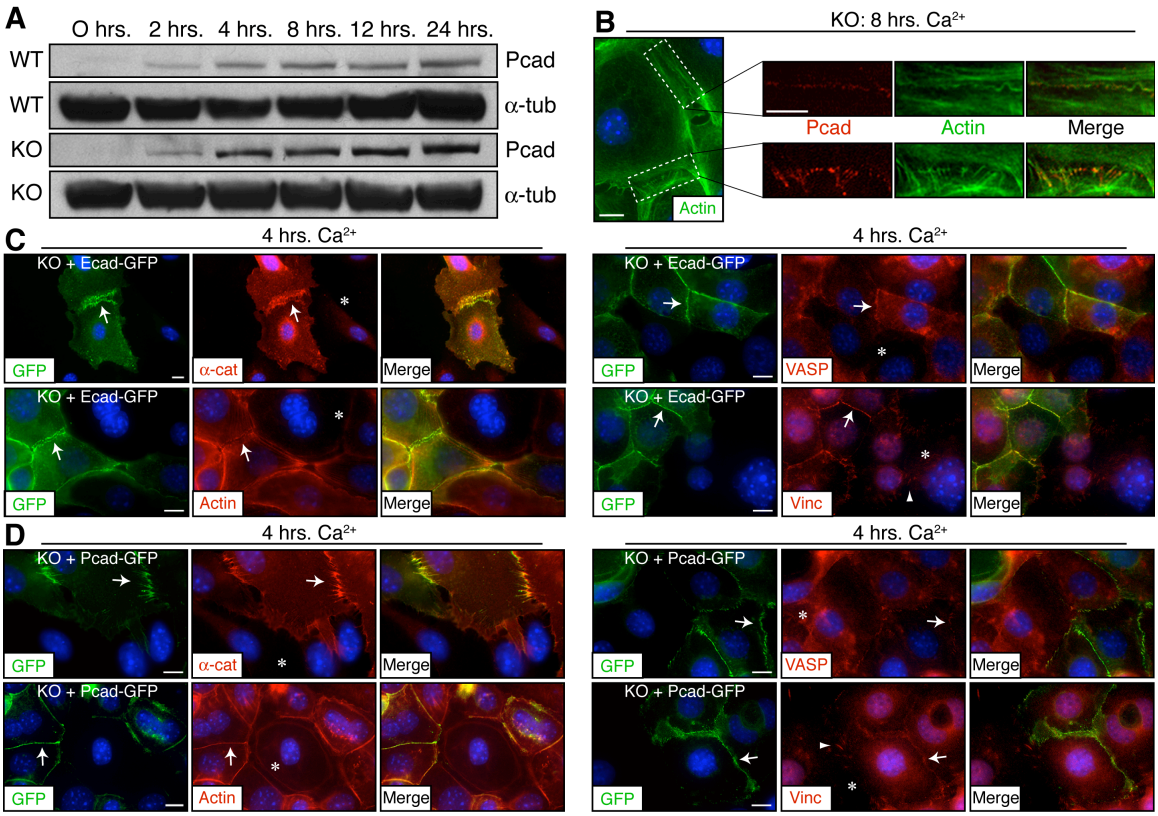



**Figure 3.3** *Overall cadherin levels govern epidermal sheet formation in vitro.* **(A)** Confluent monolayers of WT and KO $1^0$MKs were shifted from low (O hrs.) to high $Ca^{2+}$ media for indicated times prior to processing for immunoblot blot analyses of total lysates with indicated Abs. **(B)** Immuno/epifluorescence revealed initial signs of radial actin cable formation within ~8 hr after $Ca^{2+}$ shift in *E-cadherin* KO/GFPactin $1^0$Mks. These structures were consistently associated with elevated levels of P-cadherin at puncta. **(C and D)** Confluent monolayers of KO $1^0$MKs were monitored for the ability of E-cadherin-GFP or P-cadherin-GFP to rescue the early delay in epidermal sheet formation seen after shift to high $Ca^{2+}$ media. Cells were subjected to GFP epifluorescence, labeling of F-actin with TRITC-phalloidin and indirect immunofluorescence with indicated Abs. Arrows indicate sites of stable cadherin-mediated cell adhesion. Asterisks denote sites of interactions between KO cells that did not express cadherin-GFP. Arrowheads point to focal adhesions, to which these actin-associated proteins also localize. Bars, 10 μm

Underscoring its efficacy, E-cadherin-GFP rescued the delayed kinetics of sheet formation seen in KO $1^0$MKs (Fig. 3.3C; data not shown). E-cadherin-GFP puncta formed within 4 hr of $Ca^{2+}$ exposure with enhanced $\alpha$-, $\beta$- and p120-catenin colocalization. Puncta formation was also accompanied by associated radial actin cables and actin-polymerizing and actin-associated proteins. Interestingly, P-cadherin-GFP also restored all of these features to *E-cadherin* null $1^0$MKs (Fig. 3.3D). The marked influence of overall cadherin levels on the efficiency of AJ assembly and the kinetics of junction formation supported the notion that *E-cadherin* null epidermal sheets form when threshold levels of compensatory P-cadherin are reached.

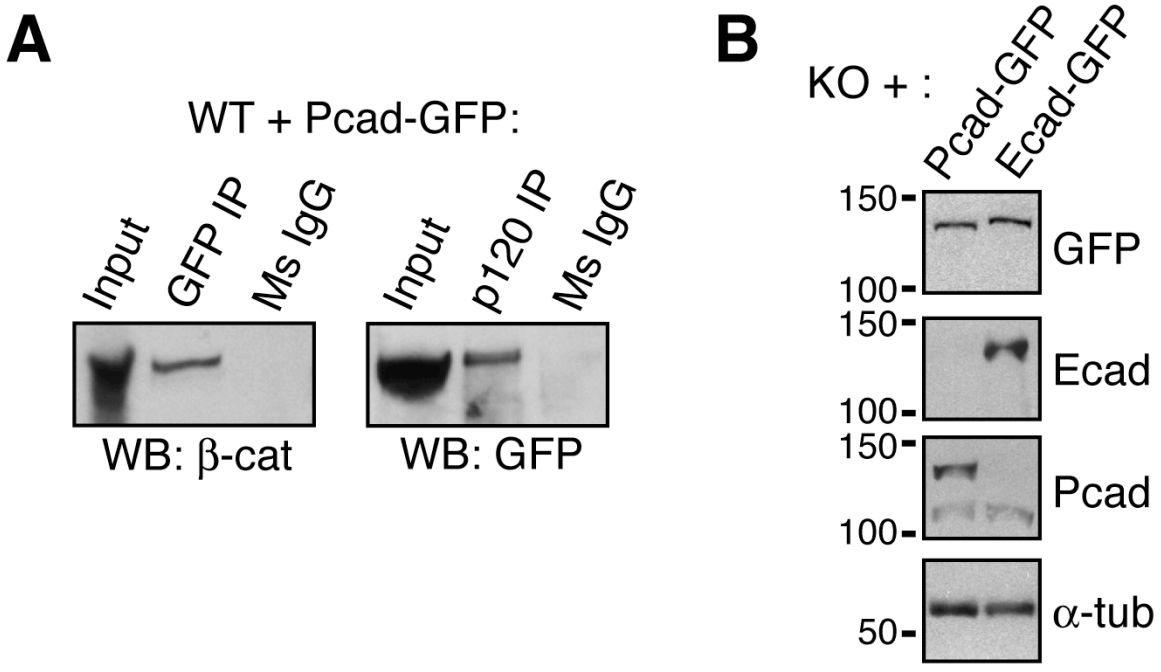



**Figure 3.4** *Cadherin-GFP expression in WT and KO keratinocytes.* **(A)** P-cadherin interacts with endogenous catenins. WT $1^0$MKs stably expressing P-cadherin-GFP were immunoprecipitated with indicated Abs followed by

immunoblot analysis with indicated Abs. **(B)** E-cadherin and P-cadherin-GFP are expressed at similar levels. KO $1^0$MKs stably expressing either E or P-cadherin-GFP were shifted to high $Ca^{2+}$ media for 4 hrs., and then processed for immunoblot analysis of total lysates with indicated Abs.

**Inhibition of classical cadherins blocks epidermal sheet formation *in vitro***

If endogenous P-cadherin compensates for E-cadherin, then its removal should unmask additional functions of classical cadherins in epidermal sheet formation. We tested this *in vitro* by stably expressing shRNAs against *P-cadherin* and control (*luciferase)* mRNAs. shRNAs were delivered by retroviral vectors containing a puromycin-IRES-GFP cassette, which served as a reliable readout for shRNA expression. P-cadherin protein was markedly reduced in KO cells expressing *P-cadherin* RNAi (KO + PcadRNAi), but not control RNAi (KO + LucRNAi) (Figs. 3.5, A and C). Importantly, a pan-cadherin antibody directed to the highly conserved cytoplasmic domain of classical cadherins (Geiger et al., 1990) failed to detect any additional bands (data not shown; see also Fig. 3.10G).

In striking contrast to the loss of E-cadherin alone, inhibition of both cadherins resulted in a failure to recruit catenins to sites of cell contact even after 24 hours after $Ca^{2+}$ switch (Figs. 3.5B; 3.6B). Localization of proteins of nectin-afadin adhesions and proteins that regulate actin dynamics, including VASP and vinculin, were similarly compromised (Figs. 3.5, B and D; 3.6C). Additionally, F-

actin failed to organize into the characteristic cortical bundles associated with sealed membranes (Fig. 3.5E). Protein constituents of Dms and TJs also failed to

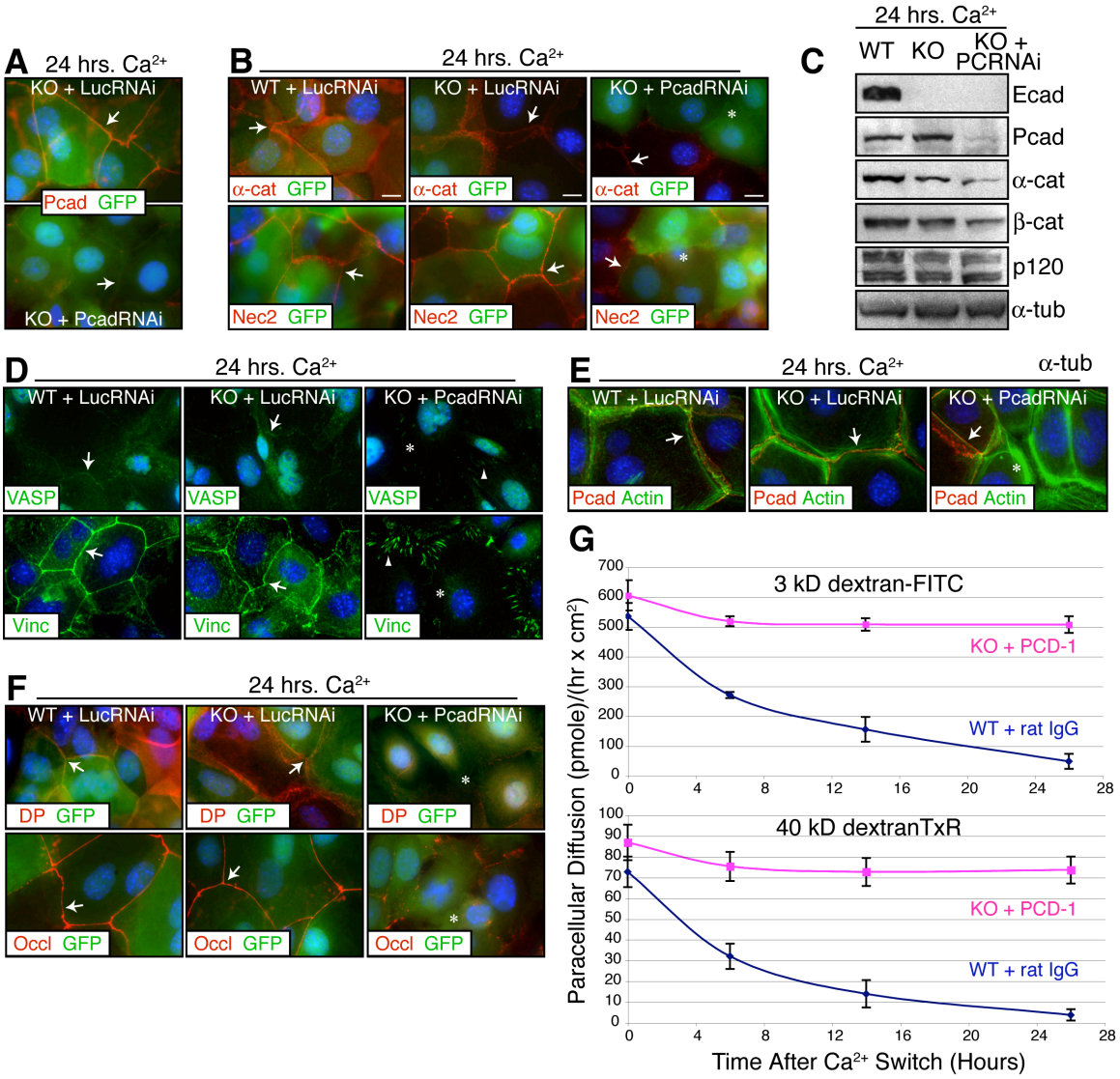



**Figure 3.5** *Inhibition of E and P-cadherins blocks epidermal sheet formation in vitro.* **(A-F)** Confluent monolayers of WT and KO $1^0$MKs stably expressing shRNA against *P-cadherin* (PcadRNAi) or control (*luciferase* ; LucRNAi) with (A, B and F) or without (D and E) GFP reporter cassette were shifted to high $Ca^{2+}$ media for 24 hrs. prior to processing for fluorescence microscopy with Abs or FITC-phalloidin (actin, green) as indicated or immunoblot analyses of total lysates (C). Additional abbreviations: Pan Cad (pan cadherin); DP (desmoplakin);

Occl (occludin). Asterisks specifically indicate sites of interactions between KO + PcadRNAi $1^0$MKs. Arrows indicate sites of cell-cell interactions. Arrowheads in (D) point to focal adhesions, to which these actin-associated proteins also localize. Bars, 10 μm. **(G)** Paracellular diffusion of fluorescently-labeled dextrans through confluent monolayers was measured in WT $1^0$MKs incubated with control rat IgG antibody or in KO $1^0$MKs incubated with PCD-1, a specific blocking Ab to P-cadherin (Nose and Takeichi, 1986). Cells were cultured in high $Ca^{2+}$ media for indicated times and diffusion was measured for 2.5 hours after addition of tracers. Data represent the mean +/- SE of 3 independent cell lines/genotype.

localize in the absence of these classical cadherins (Figs. 3.5F; 3.6, C-E). While total levels of catenins were also markedly reduced, the differences between loss of E-cadherin alone vs both cadherins were modest (Fig. 3.5C). Additionally, total levels of other junctional components were unchanged (Fig. 3.6A).

To ensure that the shRNA-mediated defects we observed were specific to loss of P-cadherin function and not off-target effects, we constructed and tested a *P-cadherin-cherry* expression vector in which silent mutations were introduced within the target *P-cadherin* shRNA sequence. Immunoblot analysis revealed appreciable P-cadherin-cherry protein of the expected size irrespective of whether the cells also expressed shRNA against *P-cadherin* (Fig. 3.7, A and C). Importantly, P-cadherin-cherry restored the ability of KO + PcadRNAi cells to form intercellular junctions, as assessed by the localization of components of AJs (α-catenin; Fig. 3.7D), Dms (desmoplakin; Fig. 3.7F), and TJs (occludin; Fig. 3.7G), and by the cortical actin organization characteristic of mature junctions

(Fig. 3.7E). Based upon these data, we conclude that the sustained junctional defects and failure in sheet formation are a direct consequence of loss of cadherin function in 1° MKs.

The failure to recruit intercellular junction proteins to sites of contact and reorganize F-actin into cortical actin belts in cadherin-deficient 1° MKs had significant functional consequences *in vitro.* These were best visualized by pretreating KO cultures with P-cadherin blocking antibodies (Nose and Takeichi, 1986) so that all MKs within the cultures quantitatively lacked cadherin function. Under these conditions, paracellular diffusion persisted even in confluent cultures exposed to $Ca^{2+}$ for extended periods of time (Figs. 3.5G; 3.6F). Overall, our findings provide compelling evidence that endogenous upregulation of P-cadherin *in vitro* compensates for E-cadherin loss at late stages of junction formation, and when both cadherins are inhibited, $1^0$MK fail to establish functional cell adhesions and acquire an effective barrier.

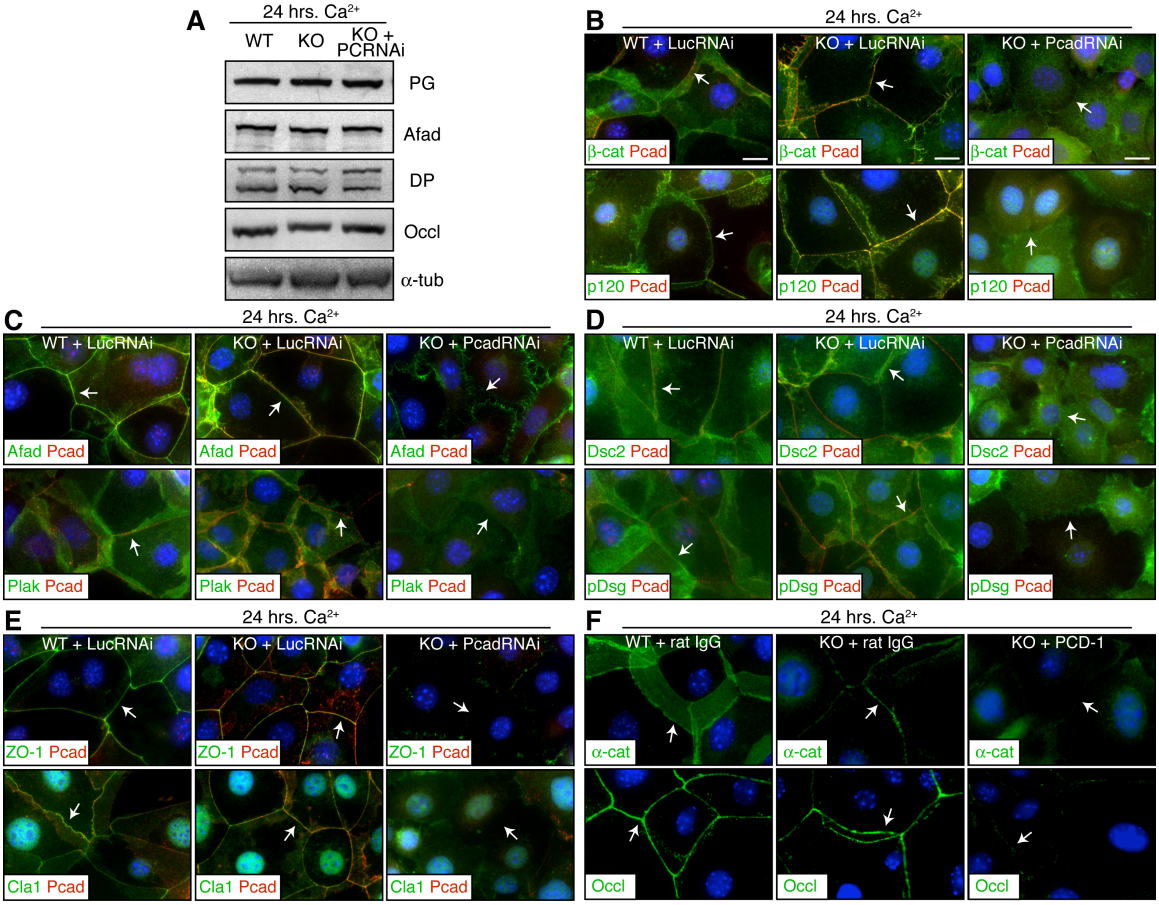



**Figure 3.6** *Inhibition of E and P-cadherins blocks epidermal sheet formation in vitro.* **(A-F)** Confluent monolayers of WT and KO $1^0$MKs stably expressing shRNA against *P-cadherin* (PcadRNAi) or control (*luciferase*; LucRNAi) or treated with PCD-1, a specific blocking Ab to P-cadherin (Nose and Takeichi, 1986) (KO $1^0$MKs), or control Ab (WT $1^0$MKs) were shifted to high $Ca^{2+}$ media for 24 hrs. prior to processing for immunoblot analyses of total lysates (A) and indirect immunofluorescence (B-F) with indicated Abs. Additional abbreviations: Dsc2 (desmocollin 2); pDsg (pan desmoglein); Cla1 (claudin 1). Note similar block in recruitment of junctional components to cell-cell contacts in KO cells with both *P-cadherin* RNAi expression and PCD-1 treatment. Bars, 10 μm.

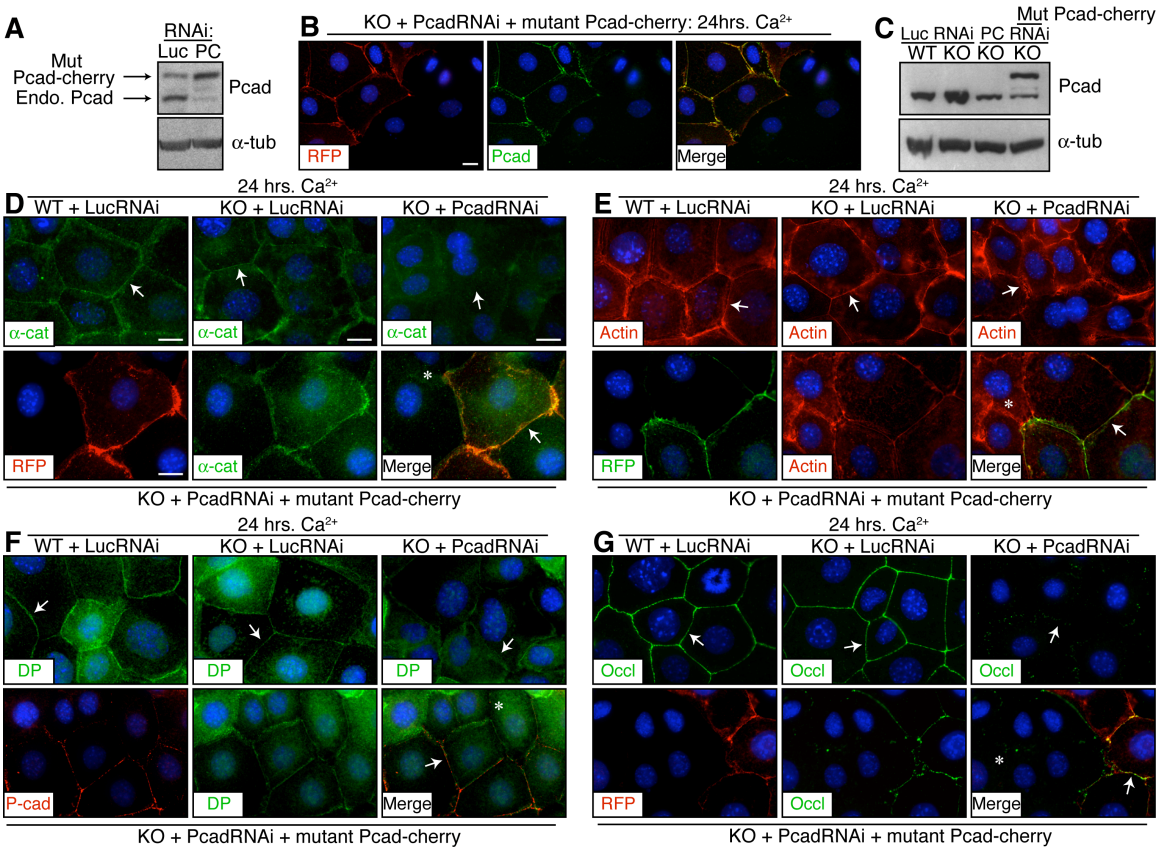



**Figure 3.7** *Expression of RNAi-resistant P-cadherin rescues junction maturation in cells deficient for both E and P-cadherin.* **(A-G)** Confluent monolayers of WT and KO $1^0$MKs stably expressing shRNA against *P-cadherin* (PcadRNAi) or control (*luciferase*; LucRNAi) and mutant P-cadherin-cherry fusion protein were shifted to high $Ca^{2+}$ media for 24 hrs. prior to processing for immunoblot analysis of total lysates (A and C) and fluorescence microscopy with indicated Abs or Alexa 647-phalloidin (actin, red) as indicated. Introduction of silent point mutations within region targeted by shRNA renders mutant *P-cadherin-cherry* resistant to RNAi. Anti-RFP antibody was used to detect mutant P-cadherin protein. Note in (B) precise colocalization of anti-RFP and P-cadherin antibodies, indicating RFP antibody is specific for the mutant protein. Note also in (F) anti-P-cadherin Ab was used to detect mutant protein as anti-desmoplakin and anti-RFP antibodies were raised in the same species. Arrows indicate sites of cell-cell

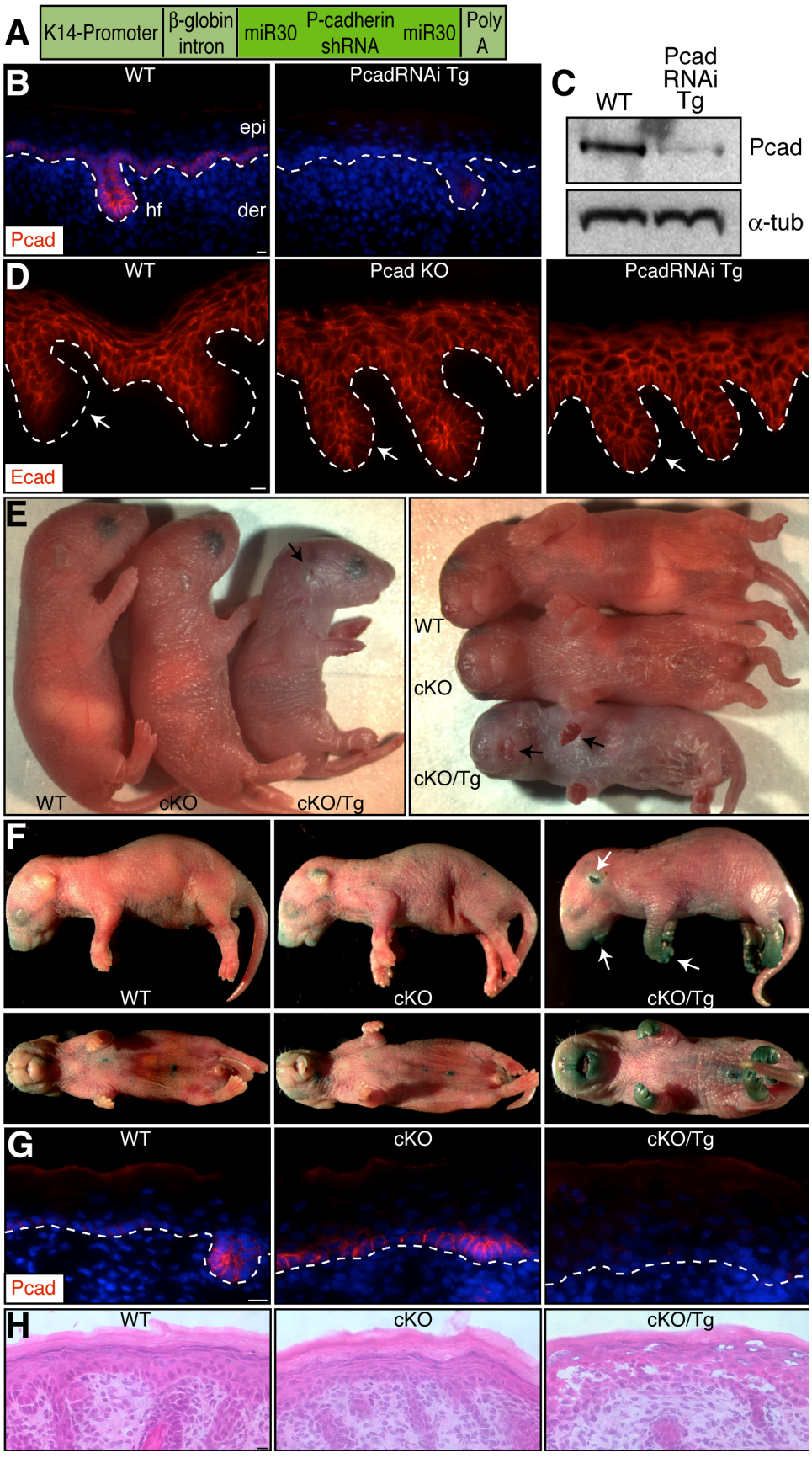
interactions. Asterisks specifically indicate sites of interactions between KO + PcadRNAi cells lacking rescue construct. Bars, 10 μm.

**Inhibition of cadherin function in skin results in cell dissociation, blistering skin lesions and defects in epidermal barrier function**

The widespread alterations in epidermal sheet formation *in vitro* suggested that functional redundancies might also be unveiled *in vivo* when both E and P-cadherin are missing. *E* and *P-cadherin* genes are tightly (32 kb) linked on chromosome 8 (Hatta et al., 1991) and separated by a predicted open reading frame of unknown function. Since this precludes conventional double knockout strategies, we engineered transgenic mice expressing *P-cadherin* shRNA driven by the *K14* epidermal keratin promoter (*PcadRNAi* Tg) (Fig. 3.8A) and then mated them with our *E-cadherin* cKO mice.

Although P0 *PcadRNAi* Tg mice were visibly and histologically indistinguishable from their WT littermates (data not shown), epidermal P-cadherin expression was effectively repressed (Fig. 3.8, B and C). However, a consistent alteration observed in both *PcadRNAi* Tg mice and *P-cadherin* null (Pcad KO) mice (Radice et al., 1997a) was the failure to downregulate E-cadherin at sites of HF downgrowth, a process normally observed during morphogenesis (Hardy and Vielkind, 1996; Jamora et al., 2003) (Fig. 3.8D). This converse compensatory change in E-cadherin level in the absence of P-cadherin

further highlights the importance of modulating cadherin levels in morphogenetically active tissues.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K14-Promoter
β-globin intron
miR30
P-cadherin shRNA
miR30
Poly A
B
WT
PcadRNAi Tg
epi
hf
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C
WT
Pcad RNAi Tg
Pcad
α-tub
D
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Pcad KO
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cKO/Tg

**Figure 3.8** *Inhibition of classical cadherins in mouse epidermis results in cell dissociation, blistering skin lesions, and defective epidermal barrier.* **(A)** The *K14-P-cadherin* RNAi construct used to generate transgenic mice. **(B and D)** P0 tail skins of WT, *K14-P-cadherin* RNAi (PcadRNAi) Tg and *P-cadherin* (Pcad KO) null mice were processed for indirect immunofluorescence with indicated Ab. Arrows denote downregulation of E-cadherin at sites of HF downgrowth in WT, but not Pcad KO or PcadRNAi Tg mice. **(C)** Immunoblot analyses of P0 epidermal lysates with indicated Abs. **(E-H)** *PcadRNAi* Tg and *K14-Cre/E-cadherin* cKO mice were bred to generate P0 mice WT or conditionally null for the *E-cadherin* allele and either negative (cKO) or positive (cKO/Tg) for the *PcadRNAi* transgene. Arrows in (E) denote blistered skin lesions, seen only in cKO/Tg mice. Barrier function analyses in (F) assessed by exclusion of an XGAL-containing solution. Arrows indicate areas, found only in cKO/Tg mice, where skin barrier function is disrupted and endogenous β-galactosidase converts dye to blue. Immunofluorescence in (G) shows absence of P-cadherin in cKO/Tg backskin epidermis (epidermal E-cadherin is completely absent in cKO mice; Tinkle et al., 2004). H&E staining in (H) reveals that such regions lacking both cadherins exhibit gross perturbations in tissue integrity. epi, epidermis; der, dermis; hf, hair follicle. Dotted lines represent junction between epidermis and dermis. Bars, 10 μm.

Numerous phenotypic defects distinguished the *K14-Cre/E-cadherin*(fl/fl)/*K14-P-cadherin-RNAi* (cKO/Tg) mice from their counterparts harboring single loss-of-function mutations in cadherins. cKO/Tg mice died within 1-2 hours after birth and were smaller than WT and *E-cadherin* cKO animals. Their skin was shiny like that of cKO mice, but it was also taut and inflexible and showed considerable flaking on the ventral surface. Additionally, their skin blistered on the paws and

around the mouth, umbilicus and tail (Fig. 3.8E). Moreover, P0 cKO/Tg pups aberrantly adsorbed dye throughout the paws, facial skin, ear buds and lower belly (Fig. 3.8F). Such defects in epidermal barrier function typically result in dehydration, followed by death shortly after birth (Segre et al., 1999).

P-cadherin was not detected over large regions of cKO/Tg skin (Fig. 3.8G). Despite germline transmission, F1 pups still displayed some patches of P-cadherin positive skin, providing an internal control for our analyses. Areas devoid of cadherins selectively displayed severe histological abnormalities not evident in regions lacking only E-cadherin or in cKO epidermis (Fig. 3.8H). HFs were markedly diminished in numbers and those that formed were stunted. Hair placodes that appeared normal were inevitably positive for P-cadherin (see below). Most notable was the loss of epidermal integrity within skin areas lacking both cadherins (Fig. 3.8H).

**Perturbations in global intercellular junctions in epidermis lacking E and P-cadherins**

We next focused on ascertaining the extent to which cadherin loss impacted epidermal morphology and cell junction-cytoskeletal dynamics. Inhibition of cadherin function was associated with gross epidermal hyperthickening, which appeared to stem from distortions in cellular morphology (Fig. 3.9A). Notably, the typical columnar orientation of nuclei and cells within the basal layer was largely lost in cadherin-deficient epidermis as was the flattened

squamous morphology typical of WT suprabasal cells. Adhesive defects were frequently observed but were discontinuous throughout cadherin-deficient epidermis (Fig. 3.9, A and B). Thus while many intercellular membranes appeared to be sealed, focal gaps compromised the continuity of the epithelial sheets.

Interestingly, Dms still formed and were not altered in number (Fig. 3.9B'; data not shown). Moreover, closer inspection revealed that intercellular gaps did not arise from splits within the Dms or the membranes, but rather from degeneration of one of the two neighboring cells (asterisks in Fig. 3.9B'). Such perturbations were often associated with clusters of Dms in some areas and a paucity in others, suggestive of a global collapse in cellular integrity. Dissociated suprabasal cells occasionally exhibited signs of nuclear condensation, a sing of apoptosis (data not shown). Finally, keratin filament organization was often severely perturbed, particularly within the upper epidermal layers (Fig. 3.9C). Overall, these signs were suggestive of an increased mechanical fragility within the cKO/Tg epidermis.

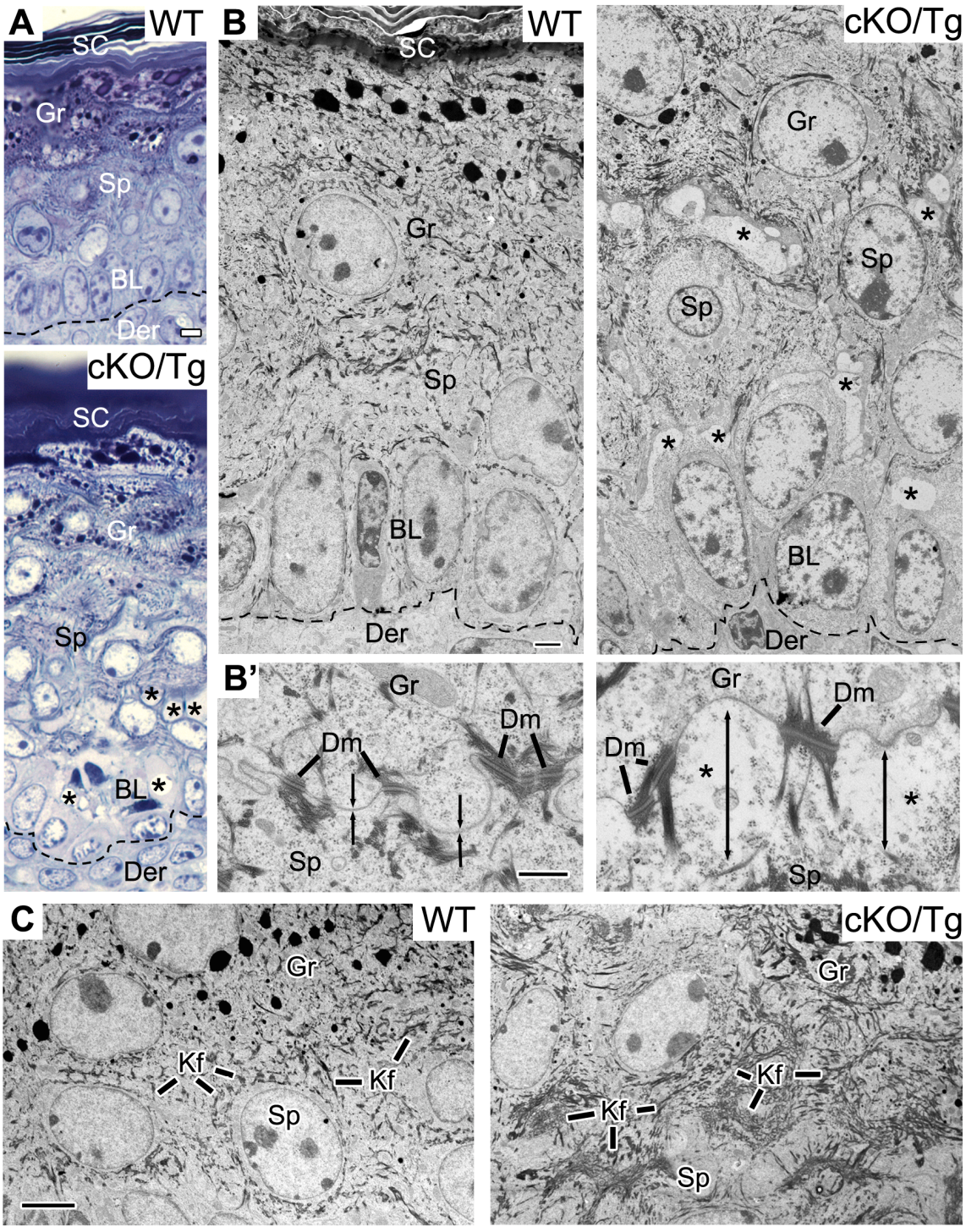



**Figure 3.9** *Ultrastructural abnormalities in cadherin inhibited epidermis.* **(A)** 0.8 μm semi-thin sections of P0 backskins were stained with toluidine blue. Asterisks denote intercellular gaps between keratinocytes of cKO/Tg epidermis. Also note epidermal hyperthickening and marked disorganization and altered cuboidal

morphology of basal cells in cadherin-deficient skin. **(B and C)** Transmission electron microscopy. Asterisks in (B and B') and double arrows in (B') denote intercellular gaps. Despite gaps and signs of cellular degeneration, double membranes appear to persist between cKO/Tg keratinocytes. Note also that desmosomes (Dm), which were not reduced in number, appear to be intact even in areas where intercellular gaps occurred (B'). Region in (C) contrasts the normal desmosome-keratin filament network of suprabasal cells of WT epidermis versus the irregular aggregates of keratin filaments that were frequently observed in cKO/Tg epidermis. Such alterations in keratin organization frequently reflect defects in mechanical integrity. Additional abbreviations: BL (basal layer); Sp (spinous layer); Gr (granular layer); SC (stratum corneum). Dotted lines in (A and B) represent junction between epidermis and dermis. Bars, 5 μm (A); 2 μm (B); 500 nm (B'); 5 μm (C)

Additional insights into these morphological perturbations were obtained by immunofluorescence microscopy and biochemical analyses. Most striking was the complete lack of cortical cadherin-catenin components throughout the entire cadherin deficient epidermis (Figs. 3.10A; 3.11, A and B). This differed from single loss-of-function mutations in E-cadherin, in which basal AJs were maintained through P-cadherin. As was the case in vitro, overall levels of catenins were noticeably reduced compared to WT, yet only a subtle reductions were observed between cKO and cKO/Tg epidermal lysates (Fig. 3.10G).

Another major difference between single and double cadherin mutants was in the actin organization, which was particularly aberrant at sites of suprabasal cell-cell contacts in cKO/Tg epidermis (Fig. 3.10B). The disorganized

and often discontinuous cortical actin belts could explain why suprabasal cells failed to adopt the flattened squamous shape of their WT counterparts. Signs of actin disorganization were also evident in the basal layer, as reflected by altered cellular organization and discontinuous basal localization of the hemidesmosomal integrin β4 (Fig. 3.10B). Immunofluorescence also revealed atypical suprabasal localization of this integrin within cadherin-deficient epidermis.

Antibodies against the desmosomal cadherins desmocollin-2 and desmoglein and the intermediate filament binding protein desmoplakin still showed appreciable cell border localization, and the total levels of these desmosomal proteins were unchanged (Fig. 3.10G). Consistent with our ultrastructural findings, however, staining was discontinuous (Figs. 3.10C; 3.11, C and D). Similarly, antibodies against K1, a major cytoskeletal associate of Dms, often showed intense perinuclear staining (Fig. 3.11D), a reflection of the abnormal intracellular aggregates of keratin filaments observed at the ultrastructural level.

The loss of E and P-cadherins also dramatically altered intercellular border localization of tight junction proteins, including the transmembrane occludin and claudin 1 proteins and the intracellular ZO-1 scaffold protein (Figs. 3.10E; 3.11, E and F). Although some TJ proteins are expressed throughout the epidermis, TJs do not assemble until the granular layer (Furuse et al., 2002; Schluter et al., 2004). The perturbed organization of early components, e.g.

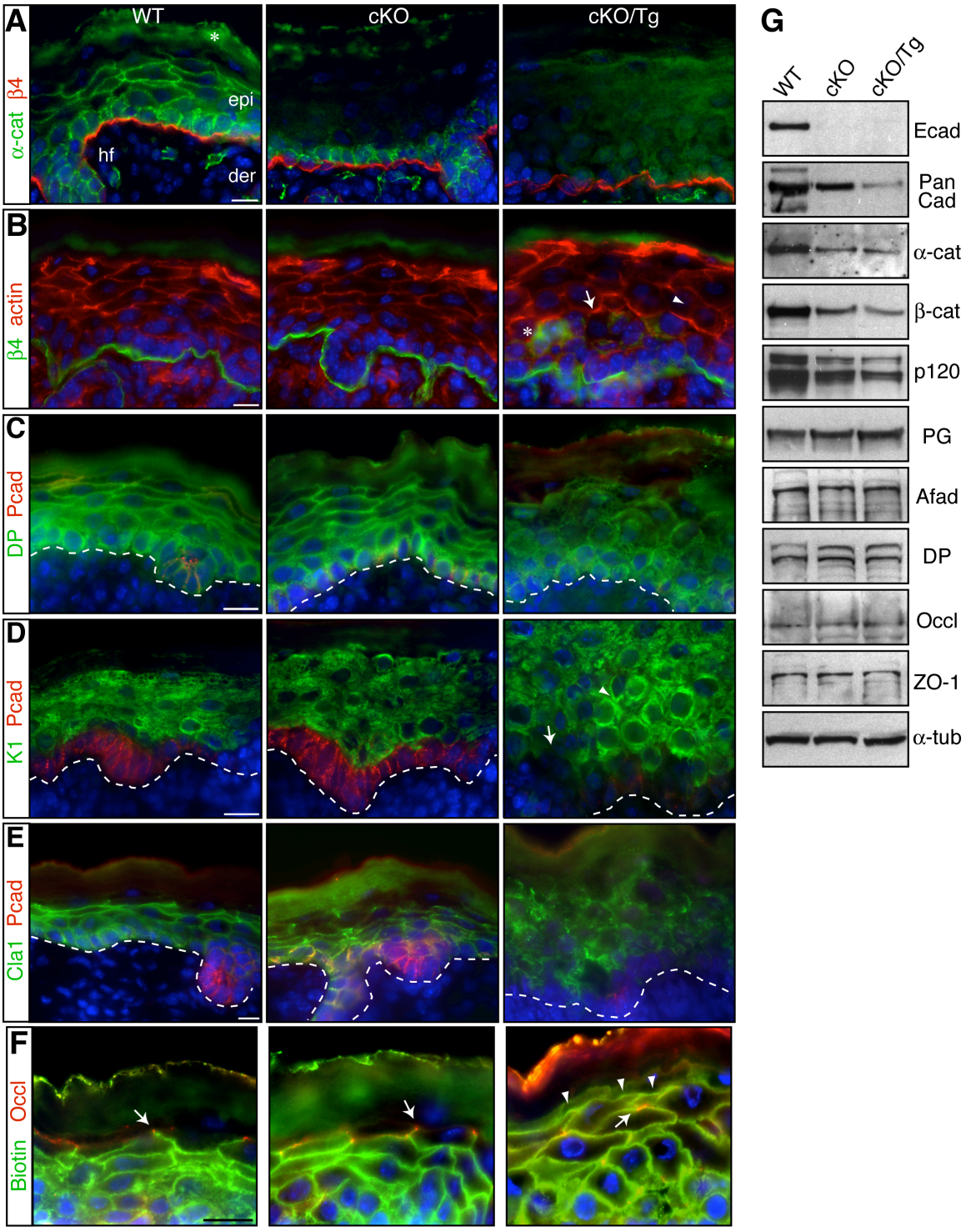
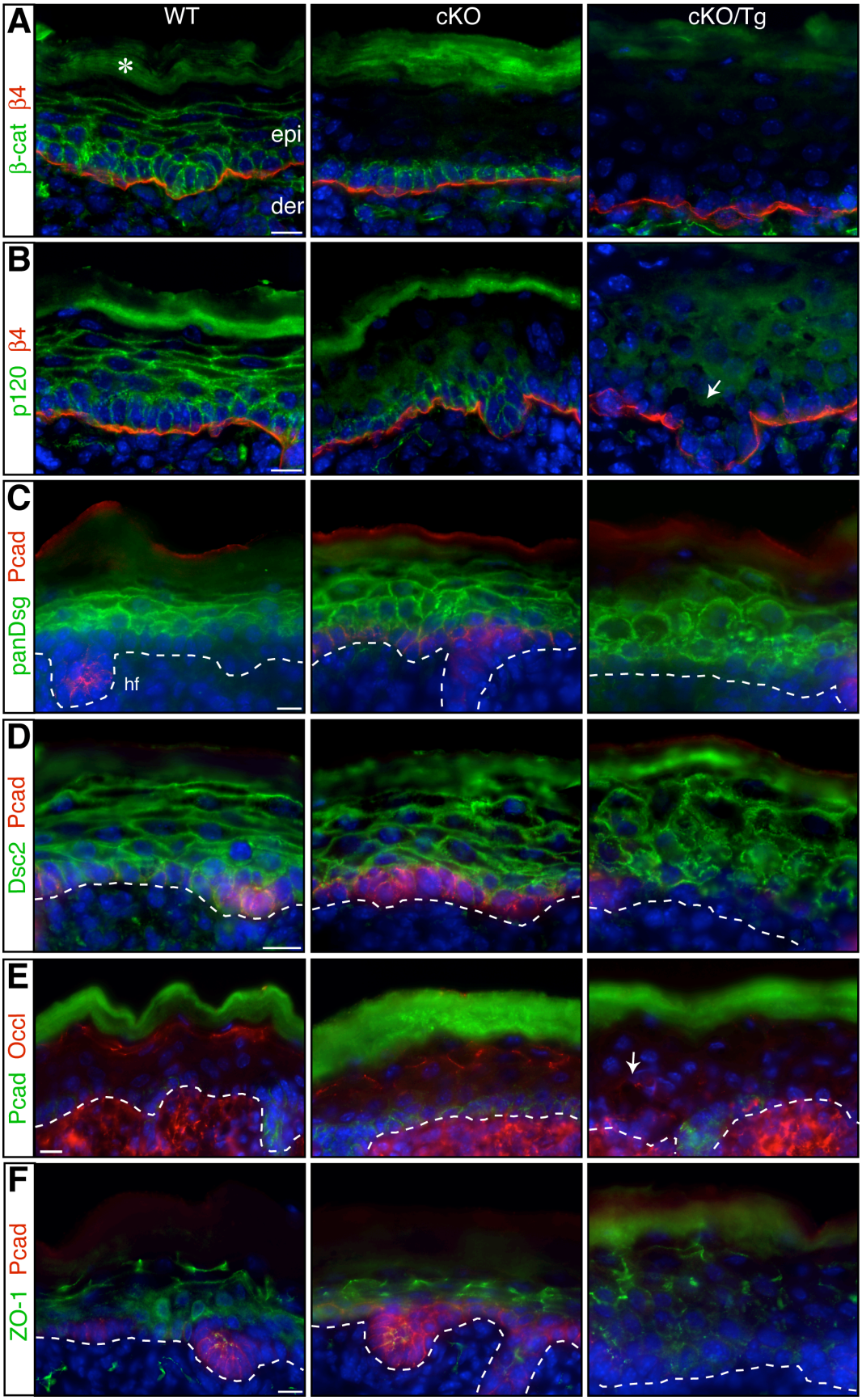



**Figure 3.10** *Perturbations in intercellular junctions, cytoskeleton organization, and tight junction function in epidermis lacking E and P-cadherins.* **(A-F)** P0 skins from tail (D) and back (all others) were processed for fluorescence microscopy with indicated Abs or TRITC-Phalloidin (actin, red). Additional abbreviations: β4

(β4 integrin), hemidesmosomal component restricted to the base of the basal layer; K1 (keratin 1), component of suprabasal intermediate filament network. (A) is a maximum intensity projection. Asterisk in (A) denotes nonspecific $2^0$Ab staining of cornified layer. Abnormalities unique to cKO/Tg cadherin-deficient skin are denoted by: Arrows in (B and D), gaps between basal and suprabasal cells; arrowhead in (B), absence of cortical actin network between two suprabasal keratinocytes; asterisk in (B), expansion of β4 integrin localization into suprabasal layers; arrowhead in (D), abnormal keratin organization in some suprabasal keratinocytes. (F) Inside-out permeability assessed by monitoring impedance to biotin flow at tight junctions in granular layer. Arrows indicate occludin-based tight junctions. Asterisks denote biotin flow past tight junctions in cadherin-deficient epidermis only. **(G)** Immunoblot analysis of total P0 epidermal lysates with indicated Abs. Bars, 10 μm.

claudin 1, as well as late components e.g. occludin, was manifested in the inability of cadherin mutant skin to prevent biotin flow past the granular layer, a reflection that epidermal TJs were functionally disrupted (Furuse et al., 2002) (Fig. 3.10F). These TJ defects were not seen in our mice conditionally targeted for ablation of *E-cadherin* alone and in this regard seemed to differ from similarly generated *E-cadherin* cKO mice engineered by Tunggal et al. (2005). Strain-specific differences aside, the striking differences in single versus double cadherin inhibition underscores the physiological importance of cadherins in the overall formation and/or stability of TJs.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WT
cKO
cKO/Tg
β-cat β4
epi
der
B
p120 β4
C
panDsg Pcad
hf
D
Dsc2 Pcad
E
Pcad Occl
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ZO-1 Pcad

**Figure 3.11** *Perturbations in intercellular junctions, cytoskeleton organization, and tight junction function in epidermis lacking E and P-cadherins.* **(A-F)** P0 backskin was processed for indirect immunofluorescence with indicated Abs. Note markedly reduced cortical localization of catenins and perturbations in cortical localization of components of Dms and TJs in cadherin deficient epidermis. Arrows in (B and E) denote gaps between basal and suprabasal cells. (A and B) are maximum intensity projections. Bars, 10 μm.

Cadherin-based cell adhesion has been shown to play an important role in the establishment of epithelial cell polarity (Nelson, 2003), which is governed by conserved protein complexes, including the Par and Scribble complexes (Macara, 2004). The Par3 complex has also been implicated in tight junction formation in simple epithelia (Suzuki et al., 2001). Additionally, studies in cultured mammalian cells have demonstrated an interdependence between scribble and E-cadherin in the regulation of their cellular localization and function (Navarro et al., 2005; Qin et al., 2005).

The *in vitro* connections identified between polarity proteins and AJs coupled with the striking defects observed in cellular morphology and epidermal barrier led us to investigate whether polarity proteins might be affected in cadherin-deficient epidermis. Interestingly, epidermis lacking both E and P-cadherins showed a marked defect in the localization of Par3, aPKC and Scribble (Fig. 3.12, A-C). By contrast, loss of *E-cadherin* alone did not alter the

distribution of either Par3 (Tunggal et al., 2005) or Scribble (Fig. 3.12, A and C). While our current understanding of these polarity-regulating complexes in stratified epithelia is limited, these findings suggest that cadherin function is required for their proper localization *in vivo*. Overall, our results underscore the global importance of cadherins in the localization and function not only of the three major adhesive complexes, but also proteins implicated in the regulation of cell polarity.

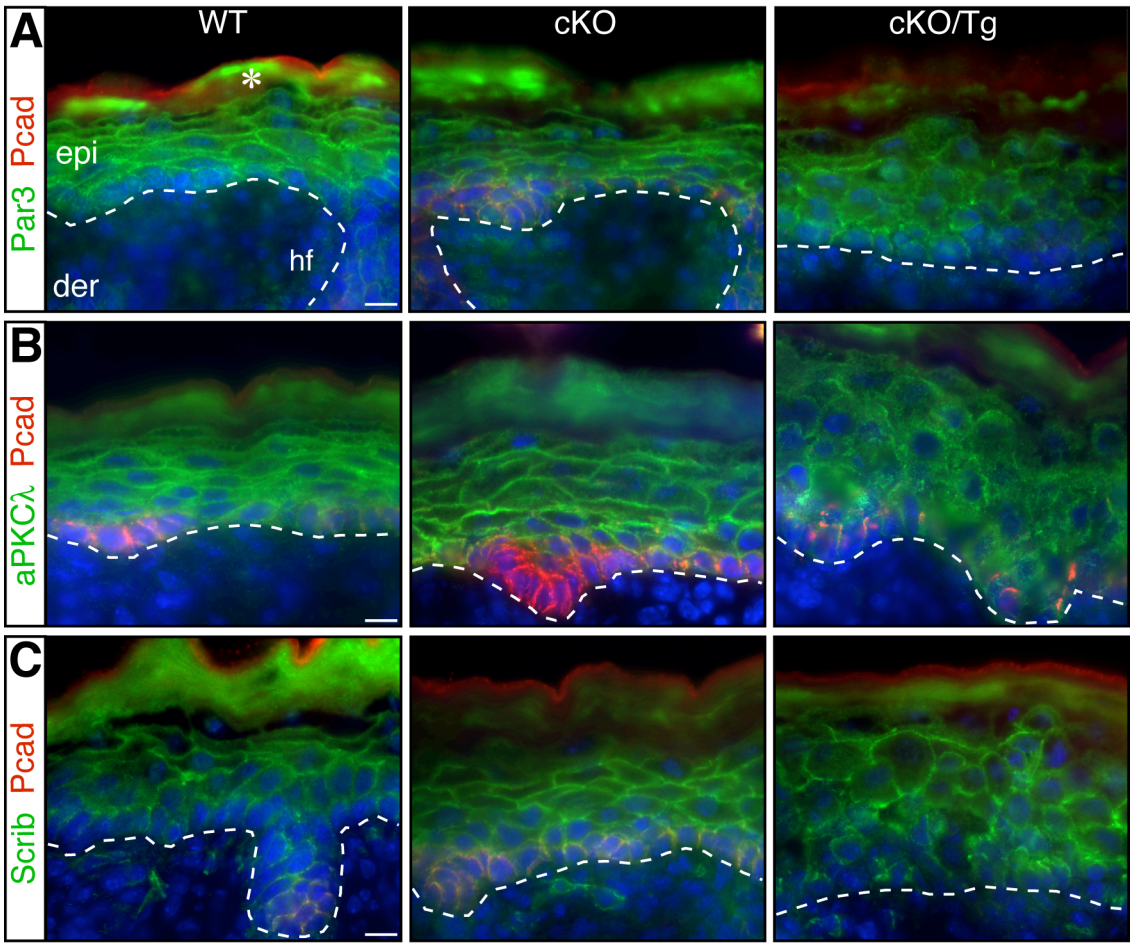


**Figure 3.12** *Classical cadherin inhibition in epidermis alters organization of polarity complexes.* (A-C) P0 backskin was processed for indirect

immunofluorescence with indicated Abs. Additional abbreviations: aPKCλ (atypical protein kinase, lambda); Scrib (Scribble). Note perturbations in cortical localization of components of core polarity complexes in cadherin deficient epidermis. Bars, 10 μm.

**Distinctions between loss of α-catenin and cadherins in the skin: dissecting AJ-dependent vs AJ-independent defects**

The cell adhesion and junctional defects observed in our mice and in cultured $1^0$MK deficient for cadherins were remarkably similar to those we described for skin and $1^0$MK conditionally targeted for the loss of *α-catenin* (Vasioukhin et al., 2000). We therefore addressed whether these similarities extended to other perturbations observed in *α-catenin* cKO epidermis (Vasioukhin et al., 2001a; Kobielak and Fuchs, 2006). Although we have noted mild alterations in the terminal differentiation program within epidermis deficient for E-cadherin (Tinkle et al., 2004), such defects appear to be strain specific, and were not detected in our cadherin deficient animals (Fig. 3.13, A-C). Additionally, the biochemical program of terminal differentiation was largely unaffected upon loss of α-catenin in skin (Vasioukhin et al., 2001a).

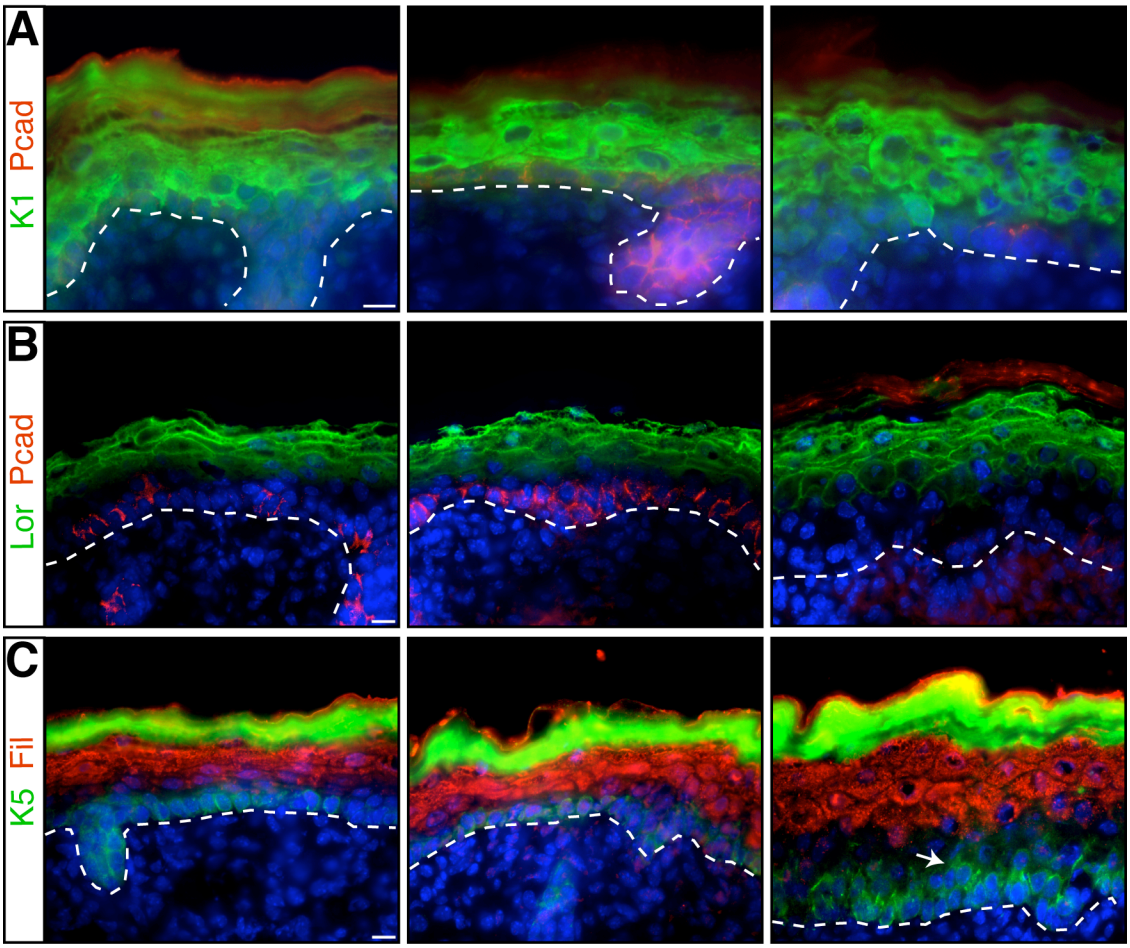


**Figure 3.13** *Classical cadherin inhibition in epidermis does not alter terminal differentiation.* **(A-C)** P0 backskin was processed for indirect immunofluorescence with indicated Abs. Additional abbreviations: Lor (Loricrin) and Fil (Filaggrin), granular layer markers of the cornified envelope; K5 (keratin 5), component of basal layer intermediate filament network. Arrow in (C) indicates suprabasal expansion of K5 detected in some areas of cadherin deficient epidermis. Bars, 10 μm.

By contrast, in both cadherin and $\alpha$-catenin deficient epidermis a significant increase in keratinocyte apoptosis was observed (Fig. 3.14), a finding also documented in E-cadherin deficient mammary gland (Boussadia et al., 2002). TUNEL-positive cells were most frequently suprabasal and were often dissociated from their neighbors (Fig 3.14, A and C). Immunofluorescence and quantification revealed a similar increase in the number and location of cells positive for the activated form of caspase 3, a marker more specifically associated with apoptosis (Fig. 3.14, B and D-F). Since the alterations in apoptosis were not observed in the epidermis of *desmoplakin* cKO mice (Vasioukhin et al., 2001b), but were seen in both *$\alpha$-catenin* cKO and cadherin-deficient epidermis, we conclude that the effects on apoptosis are likely to be rooted in defects specific to AJ formation rather than intercellular adhesion per se.

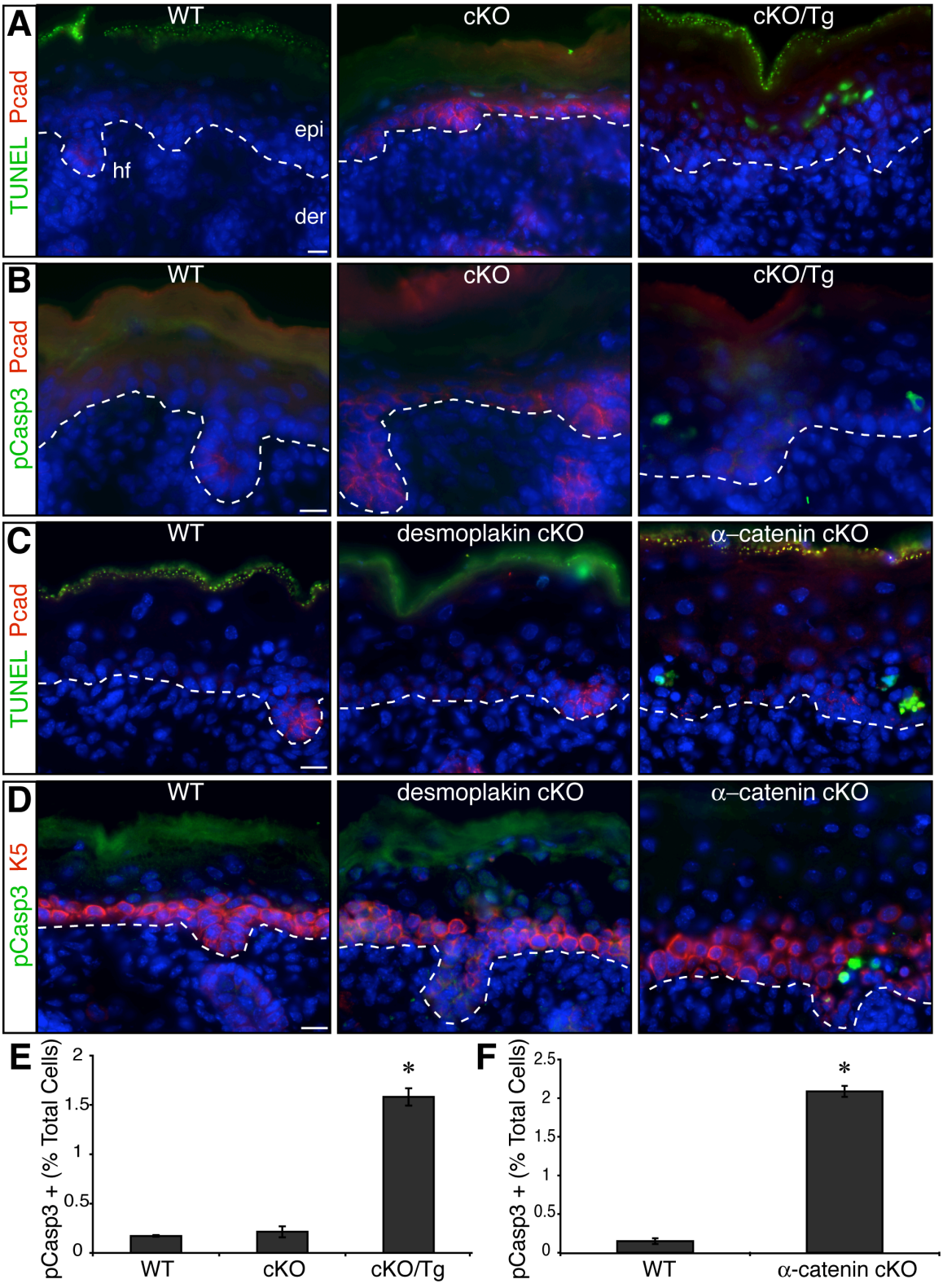


**Figure 3.14** *In vivo cadherin and catenin inhibition results in an increase in epidermal apoptosis.* **(A-D)** P0 backskins and E18.5 embryos were processed for

indirect immunofluorescence with indicated Abs and labeling of fragmented DNA via TUNEL. Additional abbreviations: pCasp3 (active caspase 3). **(E and F)** Quantifications of active-caspase 3 immunofluorescence of WT, cKO and cKO/Tg (E) and $\alpha$*-catenin* cKO and WT (F) littermate samples. Data were collected from two independently processed sets of animals. Results are shown as percent anti-active caspase 3 immunoreactive cells of the total epidermal cells counted. In (E): WT, n=2327; cKO, n=2425; cKO/Tg, n=3290. In (F): WT, n=3336; $\alpha$-catenin cKO, n=5030. Asterisks denote significant difference from WT cells determined by *t* test. In (E): $P < 1 \times 10^{-5}$; In (F): $P < 1 \times 10^{-10}$. Error bars represent SD. Bars, 10 $\mu$m.

The hyperthickened epidermis associated with cadherin loss suggested that the skin might be hyperproliferative as well. Surprisingly, however, incorporation of the thymidine analog BrdU revealed no significant differences in the number or basal location of epidermal cells actively in S-phase (Fig. 3.15A). Similarly, immunofluorescence microscopy with the mitosis marker phospho-histone H3 revealed no differences in the basal location or numbers of positive cells, and no changes were detected in the level of activated MAPK (Fig. 3.15, B and G). Although keratin 6 was induced (Fig. 3.15C), it is not reflective of a hyperproliferative state per se, but rather is a broad indicator of perturbed epidermal biology (Fuchs, 2007).

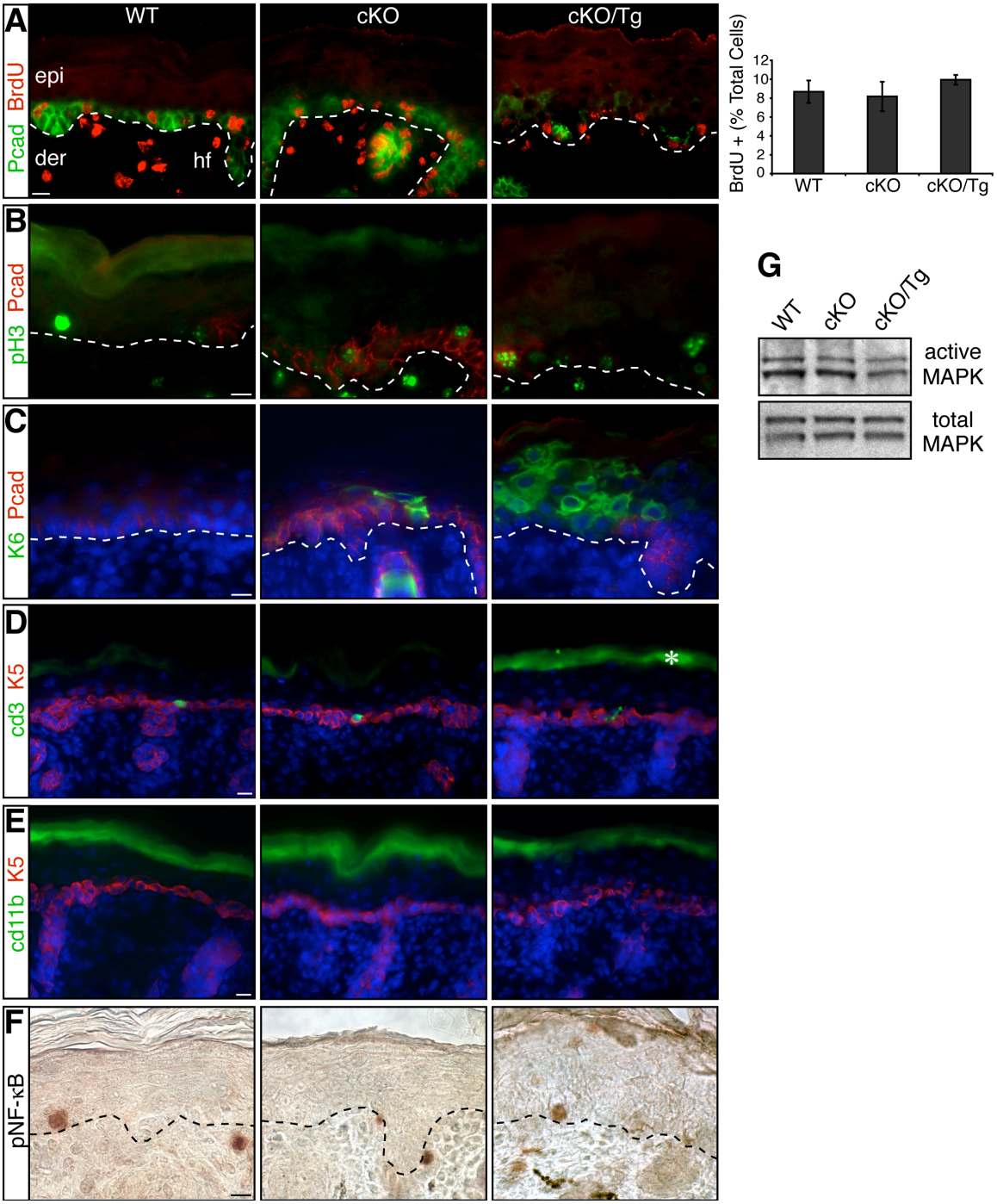


**Figure 3.15** *Depletion of E and P-cadherins does not alter epidermal proliferation or stimulate and inflammatory response.* **(A-F)** P0 backskins were processed for indirect immunofluorescence and immunohistochemistry with indicated Abs. Additional abbreviations: K6 (keratin 6), keratin restricted to companion layer of the hair follicle (hf) and frequently seen in suprabasal cells of

hyperproliferative epidermis; phospho-histone-H3 (pH3), mitosis marker; CD3, T lymphocytes; CD11b, macrophages. In (A) quantification of BrdU-labeling experiments is also shown. P0 mice were injected subcutaneously with 50 μg/g wt BrdU and killed 2 hrs. later. Data were collected from 2 independently labeled sets of animals. Results are shown as percent BrdU-labeled cells of the total epidermal cells counted (WT, n=2250; cKO, n= 2560; cKO/Tg, n=3191). Error bars represent SD. **(G)** P0 epidermal lysates were processed for immunoblot analysis with indicated Abs. Bars, 10 μm.

The lack of proliferative defects in cadherin-deficient skin contrasted markedly with *α-catenin* cKO skin, where suprabasal mitoses and enhanced Ki67 labeling were prevalent (Vasioukhin et al., 2001a). These results also differed from *p120-catenin* cKO epidermis, where hyperproliferation was prevalent, although proliferative cells were still confined to the basal layer (Perez-Moreno et al., 2006). An additional perturbation arising throughout the skins of *α-catenin* and *p-120 catenin* cKO mice (Kobielak and Fuchs, 2006; Perez-Moreno et al., 2006), was a striking inflammatory cell infiltrate and epidermal NF-κB activation. Surprisingly, however, no differences were observed in inflammatory cell recruitment or NF-κB activation in cadherin deficient epidermis (Fig. 3.15, D-F). Thus, although junctional defects and increased apoptosis appeared to be features associated with loss of α-catenin and cadherins, defects in proliferation and inflammation appeared to be features unique to loss of catenins and not cadherins.

## Discussion

### Cadherin-mediated control of adhesion complexes

*In vitro*, E-cadherin inhibition either through blocking antibodies, expression of dominant negative cadherins or shRNA expression disrupts formation of the entire epithelial junctional complex (Gumbiner et al., 1988; Wheelock and Jensen, 1992; Amagai et al., 1995; Troxell et al., 1999; Capaldo and Macara, 2007). An interesting outcome in many of these studies, however, is that junction formation is frequently delayed, rather than blocked. It has been surmised that junction recovery is due to the reappearance of E-cadherin at sites of cell-cell contact (Gumbiner et al., 1988). However, since most epithelial cells express multiple classical cadherins (Geiger et al., 1990; Nicholson et al., 1991; Stewart et al., 2000), it is also possible that differential degrees of cadherin impairment could account, at least in part, for the transient defects observed. Most importantly, inhibition of cadherin function through these means may not necessarily reflect the in vitro and/or in vivo response to cadherin loss. For example, upregulation of P-cadherin was not observed in keratinocytes treated with blocking antibody or expressing a dominant negative E-cadherin mutant (Wheelock and Jensen, 1992; Amagai et al., 1995).

Our studies provide several new insights into these issues. In our *in vitro* studies on 1°MKs derived from *E-cadherin* cKO mice, we observed delayed kinetics of AJ formation, actin remodeling and barrier function acquisition during early stages of cell junction initiation, despite an upregulation of P-cadherin.

While the junctional defects we observed resemble those from a recent study of MDCK cells treated with an *E-cadherin* shRNA (Capaldo and Macara, 2007), other classical cadherins were not upregulated in that system. This raised several questions: To what extent do functional differences between E-cadherin and other cadherins, i.e. P-cadherin and cadherin 6, account for these similar phenotypic abnormalities? To what extent do they reflect an inability of compensatory cadherins to reach critical cadherin threshold levels for efficient junction formation?

Although support for both possibilities can be found within the existing literature, our findings provide compelling evidence that overall cadherin level is the physiologically significant factor that defines whether intercellular adhesion and barrier function is established in cultured 1°MKs and intact in the epidermis. Thus, junctional defects in keratinocytes lacking E-cadherin are overcome only when P-cadherin is expressed at a sufficiently high level, while depletion of both E and P-cadherins results in a sustained block in junction formation and barrier acquisition. Significantly, only when cadherin levels are effectively depleted in vivo are intercellular adhesion defects manifested in the epidermis. These quantitative differences may also account for the apparent normal epidermal morphology and integrity in mice lacking p120 in the epidermis, in which cadherin levels are decreased but still present (Perez-Moreno et al., 2006).

Several *in vitro* studies suggest that E-cadherin is dispensable for maintaining intercellular junctions once they have been established (Gumbiner et

al., 1988; Troxell et al., 1999; Capaldo and Macara, 2007). By removing cadherins *in vivo* subsequent to intercellular junction formation, we have uncovered a dependency of TJs on classical cadherins, while Dm formation and structure appeared unaffected in the absence of cadherin function. By contrast, the ability of the Dm-keratin filament network to provide mechanical integrity to the epidermis appeared to be compromised, and ultrastructurally, signs of a collapse in epidermal architecture rather than a disruption in intermembrane sealing appeared to be at the root of the defects observed in cadherin-deficient epidermis.

While the interdependency of junctional complexes on cadherins is likely to be complex, it was notable that the cortical actin belt normally underlying the epidermal membrane was markedly perturbed when cadherins were absent. It is tempting to speculate that this early consequence of the perturbations in the AJ-cortical actin cytoskeleton results in a loss in membrane stability, which in turn contributes to the alterations in cellular shape and collapse of the overall architecture of the epidermis. Alterations in actin dynamics may also be responsible for the failure of cadherin-deficient cultured keratinocytes to assemble intercellular junctions de novo.

**Cadherins and Catenins: Shared Versus Distinct Functions**

In vertebrates, embryos lacking either E-cadherin or $\alpha$-catenin arrest at the blastula stage due to intercellular adhesion defects (Heasman et al., 1994a;

Larue et al., 1994; Riethmacher et al., 1995; Torres et al., 1997; De Vries et al., 2004). Recent evidence however suggests that α-catenin's functions extend beyond its supportive role in E-cadherin-mediated cell adhesion. *In vitro* binding experiments with purified proteins indicate that mutually exclusive from its interaction with E-cadherin-β-catenin, α-catenin associates with F-actin, where it appears to suppress Arp2/3-mediated actin polymerization (Drees et al., 2005; Yamada et al., 2005). Additionally, in either epidermis or CNS, loss of α-catenin results in a striking increase in progenitor cell proliferation, and while the underlying mechanisms appear to be distinct, the proliferative defects appear to be largely independent from alterations in intercellular adhesion (Vasioukhin et al., 2001a; Kobielak and Fuchs, 2006; Lien et al., 2006).

Despite the extensive genetics conducted on AJ components in skin, the consequence of complete disruption of classical cadherin function has remained elusive. Our ability to achieve quantitative loss of classical cadherins in epidermis has revealed important distinctions in cadherin and catenin function. Additionally, conditional targeting of a specific shRNA *in vivo* represents an important technological advance that should be broadly applicable in the future. Most importantly, the loss of both E and P-cadherin unveiled a loss of intercellular adhesion and epidermal integrity that was not seen in skin lacking only one of these cadherins, but which was strikingly similar to that seen in the absence of α-catenin (Vasioukhin et al., 2001a). While the two knockouts differed markedly in whether they localized cadherin-β-catenin complexes at cell borders,

they shared loss of cortical α-catenin, pointing to this feature as the one most critical in effective intercellular adhesion and maintenance of tissue integrity within the epidermis.

While cadherin-inhibited and *α-catenin* cKO mice exhibited marked similarities in their epidermal adhesive and apoptotic defects, clear differences in their proliferative and inflammatory responses were observed. Thus, although epidermal hyperthickening was a feature shared in cadherin-inhibited and α-catenin deficient epidermis, the hyperthickening arising from cadherin inhibition was not accompanied by significant changes in proliferation or MAPK activation. Additionally, while intercellular adhesion was compromised in both cases, only the skin of newborn mice lacking α-catenin displayed an inflammatory cell infiltrate and enhanced epidermal NF-κB activation (Kobielak and Fuchs, 2006). Together, our *in vivo* studies indicate that the proliferative and proinflammatory defects arising from loss of α-catenin within epidermis are independent of cadherin-mediated adhesion, and provide strong support for our prior *in vitro* analyses (Vasioukhin et al., 2001a; Kobielak and Fuchs, 2006).

In summary, we have identified a novel function for E and P-cadherins in mediating effective intercellular junction formation and barrier acquisition in keratinocytes *in vitro* and in maintaining epidermal tissue integrity *in vivo*. Although further studies will be necessary to fully appreciate the functional relation between cadherins and α-catenin, our results show that epidermal cadherins and α-catenin provide overlapping functions to mediate keratinocyte

adhesion, yet differ in their ability to influence proliferative and inflammatory responses in skin. These observations are interesting in light of the many cancers involving alterations in the expression of both cadherins and catenins, and suggest that the loss of one may not functionally equate to the loss of the other.

## Materials and Methods

### Separation of epidermis, keratinocyte isolation and culture, and retroviral production

Backskin from P0 mice was digested overnight at $4^0$C with dispase. Epidermis was then separated and digested with trypsin. Freshly isolated mouse $1^0$MKs were plated on 10 μg/ml fibronectin-coated coverslips or dishes and cultured in E-media supplemented with 15% serum with a final $Ca^{2+}$ concentration of 0.3 mM $Ca^{2+}$ for 12 hours (Blanpain et al., 2004). Cells were then washed extensively and cultured in media containing 0.05mM (low) $Ca^{2+}$ for at least 24 hours before raising $Ca^{2+}$ concentration to 1.5mM (high) for defined times. Cells were then fixed for immunofluorescence or lysed for immunoblot analysis.

1°MKs were co-cultured with mitomycin C-treated 3T3 fibroblast feeder cells and grown in E-media. 1°MKs were then infected with retroviral supernatant supplemented with FBS and 4 μg/ml polybrene and centrifugation at 500 x *g* for 30 minutes. Cells were then washed and cultured in E-media. 2 days post-infection, 1°MKs expressing desired construct were selected by addition of either

5 μg/ml puromycin or 56 μg/ml hygromycin typically for one week. Following confirmation of construct expression, antibiotic was removed and MKs were cultured in standard E-media. Retroviral supernatants were produced by Fugene 6-mediated transfection of Phoenix cells with retroviral DNA constructs. Supernatants were collected, filtered through 0.45 μm filters, snap frozen in liquid nitrogen, and stored at -80°C.

**Protein analysis**

1°MKs and P0 epidermis previously flash frozen in liquid nitrogen and crushed were lysed directly in Laemmli buffer with 5% β-mercaptoethanol or in Cadherin Extraction Buffer (CEB): 50mM Tris pH 7.4, 150mM NaCl, 5mM $CaCl_2$, 5 mM $MgCl_2$, 1% NP-40 and 1% Triton-X supplemented with complete protease inhibitor cocktail (Roche) and 1mM PMSF. CEB lysates were then sonicated briefly and centrifuged at 14,000 x *g* for 10 minutes. Soluble extract was removed and total protein concentration was determined by BCA protein assay (Pierce). Lysates were boiled for 10 minutes and proteins were separated by SDS/PAGE with 4-12% gradient and 7% standard gels (Invitrogen), transferred to nitrocellulose membrane, and subjected to immuoblotting. Membranes were blocked with 5% nonfat milk with 0.1% Tween-20 for 1 hour. Primary antibodies were generally used at 1:1000 dilution and secondary-HRP antibodies were used at 1:5,000. Immunoblots were detected using ECL (GE Healthcare) according to manufacture's recommendations.

**Paracellular diffusion of nonionic molecular tracers**

Measurement of paracellular diffusion of fluorescently labeled molecular tracers was essentially done as described (Jou et al., 1998; Mertens et al., 2005). Briefly, 1°MKs were plated on 10 μg/ml fibronectin coated Transwell™ 0.4 μm filters (Costar) to create a confluent monolayer. Following $Ca^{2+}$ addition for indicated times, 0.2 mg/ml FITC-conjugate dextran (mol wt 3,000 g/mol) or 0.5 mg/ml Texas Red-conjugated dextran (mol wt 40,000 g/mol) (Invitrogen) was added to media within the apical compartment. After 2.5 hours the fluorescence within media of the basal compartment was measured using a fluorometer (Polarstar Optima, BMG Labtechnologies). The amount of fluorescently labeled dextran was calculated from a titration curve of known concentration of the tracer. P-cadherin function was blocked with the addition of 60 μg/ml PCD-1 antibody (Nose and Takeichi, 1986) to KO 1°MKs 30 min prior to $Ca^2$ switch (equal concentration of control rat IgG was used to treat WT 1°MKs). PCD-1 antibody was purified from hybridoma supernatant essentially as described (Nose and Takeichi, 1986) with the additional step of affinity purification using HiTrap™ Protein G HP columns (GE healthcare).

**shRNA and constructs**

shRNA sequences were designed by J. Silva and cloned into pMLP vector (a kind gift from J. Silva, Cold Spring Harbor) containing micro-RNA 30 adaptor

sequences and a puromycin-IRES-GFP cassette. For some experiments, the sequence encoding GFP was removed from the vector through AgeI/ClaI digestion, followed by blunt-end ligation. The P-cadherin hairpin oligo 5' TGCTGTTGACAGTGAGCGCG**CCCTACCGAGCGGGCTTCA**TTAGTGAAGCC ACAGATGTAA**TGAAGCCCGCTCGGTAGGG**CTTGCCTACTGCCTCGGA 3' was used as template in a PCR reaction using 5'-mir30-PCR-xhoI-F and 3'-mir30-PCR-ecorI-R primers (Silva et al., 2005). The PCR product and pMLP vector were digested with XhoI/EcoRI and ligated. E-cadherin-GFP (A. Vaezi, U. Pittsburgh) generated previously in the Fuchs lab was amplified by PCR and cloned into retroviral vector pMSCV-Puro (Invitrogen). P-cadherin cDNA (a kind gift from M. Takiechi) was amplified by PCR and cloned into pEGFP-N1 (Invitrogen) and subsequently subcloned into pMSCVpuro. 4 base pair changes were introduced in P-cadherin cDNA to generate silent mutations within the region targeted by P-cadherin RNAi through Quikchange® site directed mutagenesis kit (Stratagene). This was PCR amplified and cloned into a modified retroviral vector pMSCVhyg (Clontech) expressing the cDNA of cherry fluorescent protein (a kind gift from R. Tsien, UCSD) to generate a C-terminal tagged mutant P-cadherin-cherry protein. All constructs were confirmed by DNA sequencing.

**Generation of Mice**

A BglII/SalI-digested fragment of the pMLP-P-cadherin-shRNA vector encoding a shRNA against *P-cadherin* flanked by micro-RNA 30 adaptor sequences was cloned into a BamHI/XhoI-digested vector under the control of the K14 epidermal keratin promoter (Vasioukhin et al., 1999). This gene was removed from the vector and linearized by digestion with SacI/FspI and transgenic mice were generated. 3 independent PcadRNAi transgenic lines were maintained and mated to *E-cadherin* cKO mice (Tinkle et al., 2004) to generate epidermal-specific cadherin deficient mice: cKO/Tg. K14-GFPactin transgenic mice (Vaezi et al., 2002), $\alpha$*-catenin* cKO mice (Vasioukhin et al., 2001a) and *desmoplakin* cKO mice (Vasioukhin et al., 2001a) have been previously described. *P-cadherin* $^{-/-}$ mice were a generous gift (G. Radice, U. Penn) and have been described (Radice et al., 1997a).

**Histology, Immunofluorescence and Antibodies**

PO backskin and tail skin was embedded in OCT compound, frozen on dry ice and sectioned (10 $\mu$m). Cells and tissue were fixed in 4% formaldehyde in PBS and blocked with PBS, 0.2% Triton-X-100, 1% BSA, 2% gelatin, 2.5% normal goat serum and 2.5% normal donkey serum or subjected to hematoxylin and eosin staining. In the case of anti-occludin immunofluorescence, tissue was fixed in 100% ethanol at 4°C for 30 min. followed by 2 min. in acetone at -20°C. In the case of anti-BrdU immunofluorescence, an additional 30 min incubation in 1 N

HCL at 37°C after fixation was used to expose BrdU epitopes. When mouse monoclonal antibodies were used on tissue, the MOM basic kit (Vector Labs) was used. For immunohistochemistry, samples were fixed overnight in 4% formaldehyde at 4°C and embedded in paraffin.

Primary antibodies and dilutions used were: E (ECCD-2) and P-cadherin (PCD-1) (rat, 1:50 P-cad; hybridomas producing both antibodies were kindly provided by M. Takiechi; Riken, Kobe), P-cadherin (goat, 1:300; R&D systems), R-cadherin, processed for immunofluorescence as described (Matsunami and Takeichi, 1995) (rat, kindly provided by M. Takiechi), N-cadherin (mouse, Invitrogen), pan-cadherin (mouse; Sigma-Aldrich), α-catenin (rabbit, 1:200; Sigma-Aldrich), β-catenin (mouse, 1:200; Sigma-Aldrich), p120 (mouse; Invitrogen), nectin-2 (rat; Abcam), l/s-afadin (rabbit; Sigma-Aldrich), plakoglobin (mouse, 1:50; Invitrogen), pan-desmoglein (rabbit, 1:300; Fitzgerald Industries), desmocollin 2 (rabbit, 1:300; Fitzgerald Industries), desmoplakin (rabbit, 1:300; Fitzgerald Industries), occludin (mouse; Invitrogen), claudin 1 (rabbit; Invitrogen), ZO-1 (rabbit; Invitrogen), VASP (rabbit; Alexis Biochemicals), mena (mouse, BD Biosciences), vinculin (mouse; Sigma-Aldrich), zyxin (rabbit; Sigma-Aldrich), ajuba (rabbit, 1:50; Cell Signaling), Par3 (rabbit; Upstate), aPKCλ (rabbit, Santa Cruz Biotechnology), Scribble (rabbit, Santa Cruz Biotechnology), GFP (mouse, Roche), RFP (rabbit, MBL International), β4 integrin (rat; BD Biosciences), K1 (rabbit, 1:300; Fuchs lab), K5 (rabbit and guinea pig, 1:300; Fuchs lab), K6 (rabbit, 1:500; Fuchs lab), phospho-histone H3 (rabbit, 1:300; Millipore), BrdU

(rat, 1:300; Abcam), active caspase 3 (rabbit, 1:300; R&D systems), loricrin (rabbit, 1:300; Fuchs lab), filaggrin (rabbit, 1:300; Covance), CD3 (rat; Chemicon), CD11b (rat, 1:25; BD Biosciences), Gr1 (rat, 1:25; R&D systems), total MAPK (rabbit; Cell Signaling), active MAPK (mouse, Sigma-Aldrich), total NF-κB (rabbit, Santa Cruz Biotechnology), phospho-NF-κB (rabbit, Cell Signaling), β-tubulin (rat, 1:1,000; Serotec). Unless otherwise stated, primary antibodies were used at 1:100. Fluorescent-conjugated secondary antibodies and dilutions used were: Alexa Fluor 488 (1:1,000; Invitrogen), rhodamine RedX (1:200; Jackson Labs), cy5 (1:100; Jackson Labs). For immunohistochemistry, biotin-conjugated secondary antibodies (1:100) were used and developed using the Vectastain® ABC kit and DAB substrate (Vector Labs). Additional reagents used were TRITC/FITC/Alexa Fluor 647-Phalloidin (1:1,000; Sigma-Aldrich and Invitrogen) to decorate F-actin, ApopTag® fluorescein direct in situ apoptosis detection kit (used according to manufacturer's recommendations; Chemicon), and 4'6'-diamidino-2-phenylindole (DAPI) to label nuclei.

**Image acquisition and manipulation**

Gross images were obtained using a MZFLIII (Leica) dissection scope equipped with an Axiocam digital camera (Carl Zeiss MicroImaging, Inc.) driven by Axiovision (Zeiss) software. Light and fluorescent microscopy images were taken by Axioskop 2 and Axioplan 2 microscopes (Zeiss) equipped with a slider Spot RT (Diagnostic Instruments) and an Orca-ER (Hamamatsu Photonics) digital

camera, respectively. The objectives used were 40X/1.3 NA oil plan Apochromat and 63X/1.4 NA oil plan Neofluor (Zeiss), and images were collected with MetaMorph 6 (Molecular Devices) and AxioVision software (Zeiss), respectively. All images were taken at room temperature in antifade for immunofluorescent images and Permount (Fisher Scientific) for hematoxylin and eosin images. Immunoblot images were acquired with a FluorChem 8900™ AlphaImager (Alpha Innotec). Adobe Photoshop 8 software was used for brightness and contrast adjustments of images.

**Barrier function assays**

Outside-in barrier function was assessed essentially as described (Segre et al., 1999). Briefly, newborn mice were submerged for 12 hours at 37°C in a solution of 1.3mM $MgCl_2$, 100mM $NaPO_4$, 3mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal with a final pH of 4.5. In the absence of an epidermal barrier, the solution penetrates the skin and an endogenous β-galactosidase-like activity results in the formation of a blue precipitate.

Inside-out barrier function was assessed essentially as described (Furuse et al., 2002). Briefly, newborn mice were injected subcutaneously with 50 μl of 5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in PBS with 1mM $MgCl_2$ and 1mM $CaCl_2$ and after 30 mins were killed and processed for immunofluorescence as described above. FITC-conjugated streptavidin (1:1,000; Invitrogen) was used to detect the extent of biotin labeling of suprabasal keratinocyte membranes.

**Electron Microscopy**

Tissues were fixed for >1 hr in 2% gluteraldehyde, 4% formaldehyde, 2mM $CaCl_2$ in 0.05 M sodium cacodylate buffer, and processed for embedding as described (Alonso et al., 2005). Samples were visualized with a Tecnai G2-12 FEI transmission electron microscope equipped with a XR60 (Advanced Microscopy Techniques, Corp.) digital camera.

# Chapter 4: Summary and Perspectives

During development the skin systematically transforms from a single layer of progenitor cells into a highly structured stratified epithelium. Additionally, specification of epidermal appendages results in complex tubular structures that branch from the basal layer of the epidermis. These spectacular cellular rearrangements rely on the rapid and precise modulation of intercellular adhesion.

To explore how epidermal classical cadherins contribute to these complex cellular rearrangements, the function of E- and P-cadherin was specifically inhibited, both individually and in combination, in the developing mouse epidermis. Additionally, epidermal keratinocytes were isolated from mutant mice and the spatiotemporal dynamics of epidermal sheet formation were analyzed. These studies have revealed important insights into the functional significance of classical cadherins in the formation of cell adhesion complexes in keratinocytes and maintenance of epidermal and hair follicle integrity *in vivo*. They have also enabled us to dissect those functions dependent on cadherins versus those that are unique to individual catenins.

## Classical Cadherin Function in Skin

Previous studies investigating the direct role of cadherins in mammalian epidermis were limited to zygotic loss of P-cadherin, which resulted in viable and fertile mice (Radice et al., 1997a), and transgenic expression of E-cadherin

throughout the epidermis using the K14 promoter (Jamora et al., 2003). Deleterious consequences resulting from the loss of P-cadherin were restricted to the mammary gland, however, and no obvious defects were observed in the epidermis. In contrast, overexpression of E-cadherin blocked hair follicle morphogenesis and resulted in perinatal lethality, the mechanism of which has yet to be clearly elucidated. These results have suggested that P-cadherin is dispensable in epidermal physiology, while modulation of E-cadherin level is required for hair follicle morphogenesis and epidermal homeostasis.

To further explore the function of cadherins in skin, E-cadherin was initially depleted from the developing skin epidermis. These studies revealed important differences in the way tissues reacted to reductions in versus complete loss of cadherin-based adhesion. The epidermis responded by upregulating P-cadherin, and while the integrity of the epidermis was largely unaffected, progressive hyperplasia developed with age. In the hair follicle, which did not appear to upregulate P-cadherin, intercellular adhesion was perturbed and ultimately these structures degenerated.

These findings are interesting in light of the seemingly disparate results from studies of cadherin knockout mice and human tumor samples and cell lines. On the one hand, ablation of cadherin is associated with cell dissociation and tissue degeneration, while on the other, reduced levels and/or activity are associated with tumorigenesis and metastasis (Cavallaro and Christofori, 2004). A striking observation from these studies, however, is that loss of tissue integrity

is typically observed only in tissues with restricted cadherin expression (Larue et al., 1994; Radice et al., 1997b; Boussadia et al., 2002), while tumors frequently show co-expression and/or upregulation of distinct cadherins (Radice et al., 1997b; Agiostratidou et al., 2007). Thus, as our results suggest, complete loss of cadherins may be incompatible with epithelial tissue survival, whereas partial compensation may result in alterations in proliferation.

To directly test the potential compensatory function of P-cadherin and to more clearly assess the contribution of cadherins in skin biology, conditional gene targeting was combined with transgenic RNAi to generate animals deficient in both E- and P- cadherins. Results obtained from this study support our previous conclusions, as *in vivo* inhibition of both cadherins did indeed compromise epidermal cell adhesion and integrity, resulting in increased apoptosis, defects in barrier function, and ultimately perinatal lethality. Furthermore, analysis of the actin and keratin cytoskeletons revealed marked perturbations in the cortical actin belt and irregular aggregates of keratin filaments.

While speculative at this point, defects in adherens junction-cortical actin organization may result in a loss in membrane stability, which in turn may compromise desmosome-keratin integrity. These alterations may set in play a cycle leading to distortions in cellular shape and eventual collapse of the overall epidermal architecture. That said, drastic cell dissociation was not observed like that seen with mutations in epidermal components of desmosomes (Vasioukhin

et al., 2001b), and ultrastructural analysis revealed that desmosome did form and their number was not altered in the absence of cadherins. These results suggest that, despite defects in desmosome-keratin organization, perturbations in intercellular adhesion resulting from loss of cadherins may be dampened in tissues particularly rich in desmosomes, such as the skin.

Epidermal loss of function of both E- and P-cadherin revealed critical differences not observed with single cadherin loss. In addition, the ability of both overexpressed E- and P-cadherin to rescue defects in the initial stages of cell junction formation in E-cadherin null keratinocytes further argued that these proteins exhibit overlapping function both *in vitro* and *in vivo*. So this begs the question, why are these two different cadherins expressed in skin? Given their reciprocal expression pattern during hair follicle morphogenesis, it has been suggested that this “cadherin switch” actively drives the process.

However, two observations suggest that the overall level rather than subtype of cadherin are critical to hair follicle morphogenesis. First, further examination of P-cadherin null mice and P-cadherin RNAi transgenic mice has revealed increased E-cadherin localization in the hair placode, an area that normally exhibits strong downregulation of E-cadherin. However, follicle downgrowth proceeds normally and hair development is unaffected. Second, loss of E-cadherin alone, despite upregulation of P-cadherin throughout the basal layer, does not alter hair follicle morphogenesis. As overexpression of E-

cadherin in the basal layer abrogates follicle downgrowth, it will be interesting to see if overexpression of P-cadherin results in a similar defect.

While epidermal integrity is compromised only with the combined loss of E- and P-cadherins and both cadherins can substitute for one another *in vitro*, further experiments are needed to more fully address the functional overlap of these proteins. It is known that fibroblasts expressing either E- or P-cadherin sort from one another (Nose et al., 1988), however, the quantitative strength of adhesion mediated by these cadherins and whether it differs is not known. Additionally, whether homophilic engagement results in different intracellular signals between these two adhesion receptors is not known. These issues can be addressed using techniques like a dual pipette assay with cell doublets (Chu et al., 2006), and may provide additional insight to their functional similarities and differences.

The ultimate test of functional redundancy, however, lies with *in vivo* analysis and this is now possible with the development of a faithful "knock-in" targeting vector for the *E-cadherin* locus (Kan et al., 2007). By mating E-cadherin *fl/fl* mice with P-cadherin knock-in mice, tissue specific knock-in can be achieved. Mating these mice with K14-Cre mice should reveal to what extent P-cadherin can substitute for E-cadherin function in the epidermis.

**Distinct Functions of Cadherins and Catenins in Skin**

The ability to inhibit both E- and P-cadherins has added an important genetic model in the battery of mouse mutants developed to dissect adherens

junction components in skin. Thus, through a combination of zygotic and conditional gene targeting, the loss of each catenin and the single and combined loss of classical cadherins have now been characterized in the skin. One of the most interesting and quite surprising finding from these studies is the number of distinct intracellular signaling pathways affected by loss of α-, β-, and p120-catenin.

Ablation of β-catenin in the epidermis has demonstrated the fundamental importance of Wnt signaling in the specification of the hair follicle lineage. Total levels of cadherins were not altered, and it has been suggested that epidermal intercellular adhesion is maintained through plakoglobin, a process documented in zygotic loss of β-catenin. Additionally, epidermal integrity was not obviously affected upon loss of epidermal p120, yet this did not appear to be due to upregulation of the closely related p0071 protein and significant reductions in E- and P-cadherin were observed. Instead, cell autonomous activation of the NF-κB pathway appeared to drive the inflammatory and proliferative defects. Intercellular adhesion defects were observed in the epidermis of mice conditionally null for α-catenin, yet constitutive activation of the Ras-MAPK and NF-κB pathways appeared to underlie the striking hyperproliferative changes.

A shared limitation of these studies has been the persistence of cadherin-catenin complexes and their localization at cell borders, and thus, the consequence of complete disruption of classical cadherin function has remained elusive. Additionally, central functions of each catenin have been intimately

linked to the stability, organization, and activity of classical cadherins. Thus it has been difficult to address to what extent perturbations in the above signaling pathways are specific to individual catenins or alterations in cadherin function. Quantitative loss of classical cadherins has now revealed an important distinction between cadherin-mediated adhesion and catenin-dependent intracellular signaling in the epidermis.

Interestingly, loss of epidermal classical cadherin function did not appear to result in dysregulation of MAPK or NF-κB activation. Furthermore, since hair follicle placodes were still initiated and de novo hair follicle morphogenesis was not observed, a phenotype resulting from aberrant β-catenin activation in skin (Gat et al., 1998), it seems less likely that the block in hair follicle morphogenesis in cadherin-deficient mice originates from aberrations in β-catenin-mediated Wnt signaling. In contrast, cadherin-inhibited and α-catenin conditional knockout mice exhibited marked similarities in epidermal adhesion and apoptotic defects. While the localization of cadherin-β-catenin complexes at cell borders differed markedly between the two knockouts, they shared loss of cortical α-catenin, suggesting this feature is critical in mediating effective intercellular adhesion and maintaining tissue integrity within the epidermis.

These results are particularly interesting in light of recent data suggesting that, contrary to the prevailing notion of the past twenty years, α-catenin does not serve as the molecular link between classical cadherins and the actin cytoskeleton (Drees et al., 2005; Yamada et al., 2005). Current experiments,

including live cell imaging of the dynamic organization of actin and cadherin-α-catenin chimeric proteins in mutant keratinocytes, are currently underway in the Fuchs lab and should help resolve these issues. An exciting new approach that allows for *in utero* gene silencing has recently been employed in the lab, and this may provide a more feasible way to analyze compound adherens junction mutants previously difficult to develop through breeding or impossible to produce due to genetic linkage. Finally, grafting of cadherin-deficient skin onto nude mice will help reveal the long term consequences of cadherin inhibition and should allow for a direct comparison to the tumorigenic consequences of long term epidermal loss of α-catenin (Kobielak and Fuchs, 2006).

**Adherens Junctions in Disease**

Many of the morphogenetic processes that occur during development are corrupted during disease states, particularly during tumor development and metastasis. The realization that cadherin-based adhesion and signaling are critical in the regulation of many aspects of morphogenesis, has resulted in a large body of work examining the role of cadherins in tumorigenesis. In most tumors of epithelial origin, E-cadherin expression is lost concomitantly with progression towards malignancy (Cavallaro and Christofori, 2004). Furthermore, inactivating mutations within the E-cadherin gene have been causally linked to a hereditary form of diffuse gastric carcinoma (Guilford et al., 1998) and observed in 50% of lobular breast carcinoma (Kang and Massague, 2004).

Importantly, the level of E-cadherin expression often correlates inversely with the degree of differentiation and stage of the tumor (Kang and Massague, 2004). In many tumor cells there is a direct correlation between the loss of E-cadherin and the 'dedifferentiation' into a mesenchymal phenotype, in which the acquisition of mesenchymal markers, increased cell migration and loss of cell polarity occur (Thiery, 2003). In some cases, partial or complete reversion of the invasive, mesenchymal phenotype is achieved with constitutive expression of E-cadherin (Auersperg et al., 1999; Thiery, 2003), leading to the idea that E-cadherin may represent the critical regulator of the epithelial phenotype.

E-cadherin does not, however, stand alone as the sole component of adherens junction associated with tumor development and metastasis. Given the crucial roles catenins play in cadherin regulation, this is perhaps not too surprising. However, what has become increasingly more evident is that dysfunction in the signaling function of individual catenins plays an important role on the road to cancer development. β-catenin's central role in mediating Wnt growth factor signaling lies at the heart of a number of human malignancies, and mutations in components of the Wnt/β-catenin pathway have been detected in ~90% of colorectal tumors (Brembeck et al., 2006).

While the role of α-catenin and p120-catenin in malignancies is less clear, the recent *in vivo* analyses of α-catenin and p120-catenin ablation in skin and other tissues has revealed striking proliferative and inflammatory alterations, and in some cases signs of neoplasia (Vasioukhin et al., 2001a; Davis and Reynolds,

2006; Kobielak and Fuchs, 2006; Perez-Moreno et al., 2006). This has prompted extensive analysis of $\alpha$-catenin and p120-catenin status in human tumors, and results from many of these studies suggest that both catenins are prognostic factors in cancer progression (Benjamin and Nelson, 2008; van Hengel et al., 2008).

Because E-cadherin serves as the nexus of cytoplasmic catenins, the question of the chicken or the egg, i.e. does E-cadherin loss result in catenin-dependent signaling or does altered catenin signaling promote downregulation of E-cadherin, is an important one when trying to understand the role adherens junction components in cancer. Central to our ability to unravel this problem is the ability to dissociate cadherin and catenin functions and to identify how and where catenin signaling is initiated. The generation of skin-specific conditional cadherin and catenin-deficient animals and the ability to study the long-term consequence of these mutations through grafting, should provide an ideal system to further tackle these problems.

Cadherin dysfunction is not limited to cancer, however, and in 2001, a homozygous mutation in the *P-cadherin* gene was identified in individuals with hypotrichosis with juvenile macular dystrophy, a rare autosomal recessive disorder (Sprecher et al., 2001). Mutations in *P-cadherin* have also been linked to the related syndrome, ectodermal dysplasia, ectrodactyly, macular dystrophy (EEM) (Kjaer et al., 2005). The common features of both diseases are sparse hair and retinal degeneration leading to blindness. Interestingly, all but one

mutation analyzed by these and other studies map to the extracellular domain of P-cadherin, resulting in truncations or disruptions in the calcium binding domain (Shimomura et al., 2008). While the mechanism by which mutations in P-cadherin result in these diseases is currently unknown, it is interesting that discrepancies in the functional significance of P-cadherin between humans and mice appear to exist. While there are many possible explanations, these differences may result from the presence of modifier genes and/or genetic background of the mouse model.

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