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### Pemphigus pathogenesis

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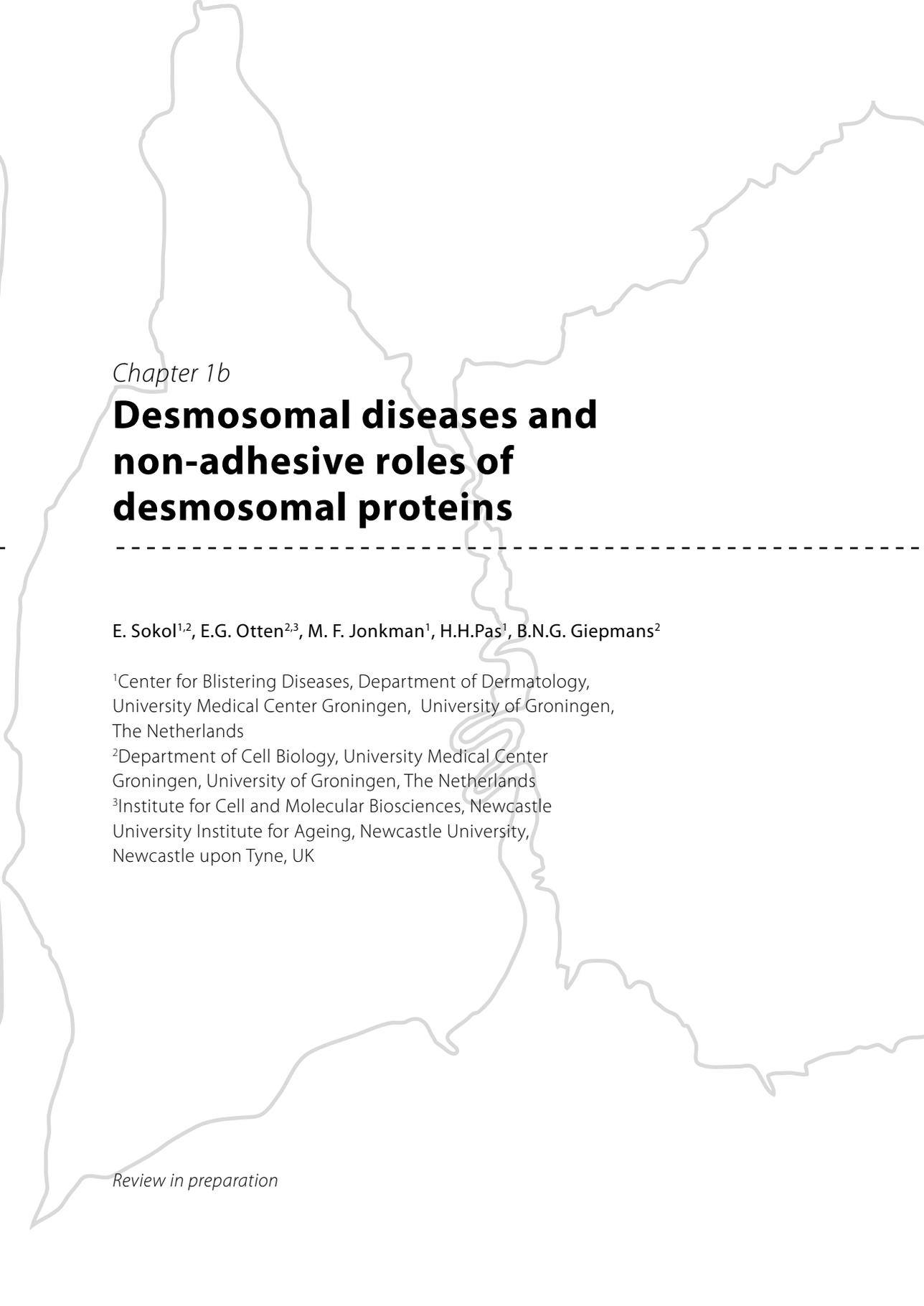
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*Chapter 1b*

# **Desmosomal diseases and non-adhesive roles of desmosomal proteins**

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*Review in preparation*

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## **Abstract**

Desmosomes are complex structures that interconnect intermediate filament networks of neighboring cells and thereby provide strength to tissues. They are built from specialized proteins, of which adhesive functions are well investigated and seemingly crucial as human genetic, autoimmune and infective disorders arise from their malfunction. Growing evidence suggests functions of desmosomal proteins beyond cell-cell adhesion: they are involved in other processes, including tissue differentiation, hair follicle function, cell migration, proliferation and apoptosis. Also the role in embryogenesis, carcinogenesis and cellular signaling pathways are recognized. Here, we review the evidence for non-adhesive roles of the five major protein components of desmosomes and discuss when functions beyond adhesion interdigitate with the classical desmosome function.

## 1. Introduction

Desmosomes (desmos- bond, soma-body) are cell-cell junctions that interconnect neighboring cells by bonding their intermediate filament networks (Delva et al., 2009; Waschke, 2008; Garrod and Chidgey, 2008; Kowalczyk and Green, 2013; Stokes, 2007). Their best characterized function is in providing cell-cell adhesion and tissue strength, and therefore they are abundant in tissue that experience stress, including skin and heart. Desmosomes locate in epithelia, stratified as well as simple, and in non-epithelial tissue as meningeal cells of the arachnoid mater and the follicular dendritic cells of lymph follicles (Waschke, 2008).

Briefly, the five major protein types that make desmosomes are desmogleins (Dsgs), desmocollins (Dscs), desmoplakin (Dp), plakoglobin (Pg) and plakophilins (Pkps) (for an extensive review see: (Garrod and Chidgey, 2008)). They belong to three families of proteins: cadherins (Dsgs and Dscs), armadillo proteins (Pg and Pkps) and plakins (Dp). Various isoforms of Dsgs (1-4), Dscs (1-3, a and b), Dps (I and II) and Pkps (1-3) are known [Table 1]. Autoantibodies or bacterial toxins that affect desmosomal proteins or mutations in one of the genes that code for them can lead to severe human disorders (Brooke et al., 2012). Therefore each component of the complex desmosomal structure is important for tissue stability and function. Moreover, correct expression of isoforms of desmosomal proteins is also important for tissue homeostasis and aberrant expression is found in cancers (Brooke et al., 2012).

Some isoforms are ubiquitous, while others are tissue and differentiation specifically expressed (Harmon and Green, 2013), which may point to distinct roles of the isotypes (Dusek et al., 2007; Green and Simpson, 2007). The pathogenesis of desmosomal targeted disorders cannot always be explained by defects of adhesion only and therefore other functions of are likely involved (Spindler and Waschke, 2014; Broussard et al., 2015). Here we review the data on supra-adhesive roles of the major desmosomal proteins, with focus on possible involvement in disease.

**Table 1. Desmosomal proteins and their structure.** Desmosomal proteins belong to three families: desmosomal cadherins, armadillo proteins and plakins. Desmosomal cadherins are desmogleins (Dsgs) and desmocollins (Dscs). There are four isoforms of Dsgs which structurally differ in the number of RUD domains. There are three isoforms of Dscs and two splicing variants Dsc a and Dsc b form. Desmosomal cadherins are composed of extracellular region that contains five extracellular domains (EC1-5). Transmembrane domain TM is located in the plasma membrane. Intracellular part of desmosomal cadherins starts with intracellular anchor (IA) that is followed in case of Dsgs and Dsc a by intracellular cadherin like sequence (ICS). Dsgs contain additional intracellular proline-rich linker (IPL), a variable number of repeat unit domains (RUD) and desmoglein terminal domain (DTD). Armadillo proteins are cytoplasmic proteins and in desmosomes are plakoglobin (Pg) and plakophilins (Pkp). Pg is composed of 12 arm repeats and distinct amino and carboxy terminal domains. There are three isoforms of Pkps of which Pkp1 and 2 have two splicing variants a and b. Pkps contain 9 arm repeats with an insert between fifth and

Protein family	Proteins	Isoforms	Splicing variants	
Desmosomal cadherins	Desmogleins Dsgs	Dsg1		
		Dsg2		
Dsg3				
Dsg4				
Desmosollins Dscs	Dsc1 Dsc2 Dsc3	Dsc a		
		Dsc b		
Armadillo proteins	Plakoglobin Pg	Pg		
	Plakophilins Pkp	Pkp 1	Pkp a	
		Pkp 2 Pkp 3	Pkp b	
Plakins	Desmoplakin Dp	Dp I		
		Dp II		

sixth arm repeat that introduces a band to an overall structure. Pkp b variants have introduction of certain unit between third and fourth arm repeat in case of Pkp1b and fourth and fifth arm repeat in case of Pkp2b. Desmoplakin (Dp) belongs to plakin family of proteins and has two splicing variants DpI and DpII which differ in the length of Rod domain. Dp is composed of globular head and tail with coiled-coiled rod region in between (rod). Tail contains three plakophilin repeat domains (A, B, C). GSR is glycine serine rich domain.

## 2. Desmosomal cadherins

Desmosomal cadherins, Dsgs and Dscs [Table 1] are the transmembrane calcium dependent cell-cell adhesion proteins that form the adhesive interface of desmosomes (Garrod et al., 2002). There are four isoforms of Dsg and three isoforms of Dscs. Every isoform of Dsc furthermore exists in a longer 'a' form and a shorter 'b' form as a result of alternative splicing. How these specific isoforms may be involved in supra-adhesive functions is discussed per protein below and listed in table 2.

Protein	Suggested non-adhesive function	Associated mechanism	References
<b>Dsg1</b>	Epidermal differentiation	EGFR and ERK1/2 EphA2 Erbin/SHOC2/Ras Bcr/MAL/SRF	Getsios et al. 2009 Lin et al. 2010 Harmon et al. 2013 Dubash et al. 2013 Dusek et al. 2006
	Elimination of damaged keratinocytes	Cleavage by caspase 3	Dusek et al. 2006
	Desquamation of epidermis	Cleavage by kallikreins	Borgono et al. 2007
<b>Dsg2</b>	Embryotic development	Embryotic stem cell proliferation and survival	Eshkind et al. 2002
	Proliferation, anchorage independent cell survival, development of skin tumors	PI 3-kinase/AKT, MEK-MAPK, STAT3 and NF-kappaB, Hedgehog signaling	Brennan et al. 2007, 2015
	Cell proliferation	EGFR and Erk1/2	Kamekura et al. 2014
	Regulation of apoptosis	Cleavage by cysteine proteases	Nava et al. 2007
	Receptor for adenoviruses	Intercellular junctional weakness and increase access to receptors	Wang et al. 2011
<b>Dsg3</b>	Regulation of actin dynamics and formation of filopodia	GTA bound Rac1, Cdc42 and RhoA	Tsang et al. 2010, 2012
	Cancer growth	Pg, c-myc, cyclin D1 and MMP-7	Chen et al. 2013
	Cell proliferation	Secondary to cell-cell adhesion	Mannan et al. 2011
<b>Dsc2</b>	Regulation of cell proliferation	Akt/ $\beta$ catenin	Kolegraff et al. 2011
	Cell migration	Actin	Roberts et al. 2011
	Tumor invasion	miR-25/ $\beta$ catenin	Fang et al. 2013
<b>Dsc3</b>	Early embryotic development	Pre-implantation development	Den et al. 2006
	Suppression of cancerogenesis	EGFR/ERK pathway	Cui et al. 2012

**Table 2. Non-adhesive functions of desmosomal cadherins: desmogleins and desmocollins.** Suggested non-adhesive function with its associated signaling pathway/mechanism. Dsg4 and Dsc1 non-adhesive functions are less studied.

### **2.1. Desmoglein 1 in tissue differentiation, apoptosis and desquamation of epidermis**

The 165 kDa Dsg1 is specifically found in stratified epithelia and involved in genetic, autoimmune and infective disorders. In the cornified stratified squamous epithelium, the epidermis of the skin, its expression increases from the basal to the upper epidermal layers (Mahoney et al., 2006). In the stratified epithelium of the mucosa its expression is lower and completely absent in the basal layer (Mahoney et al., 2006; Donetti et al., 2005). In cell cultures of normal human keratinocytes Dsg1 becomes expressed when cells differentiate from the monolayer (Denning et al., 1998).

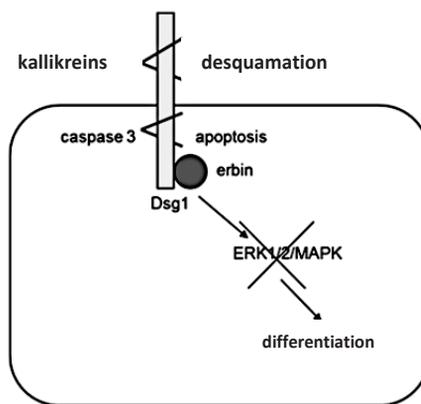
Dsg1 is a target antigen in the autoimmune bullous diseases pemphigus foliaceus and mucocutaneous pemphigus vulgaris (Amagai and Stanley, 2012). Mutations of Dsg1 gene can give striate palmoplantar keratoderma (SPPK) (Hershkovitz et al., 2009; Has et al., 2015) and SAM syndrome (severe dermatitis, multiple allergies and metabolic wasting) (Samuelov et al., 2013). Dsg1 is also a substrate for exfoliative toxins of *Staphylococcus aureus* and when cleaved results in staphylococcal scalded skin syndrome (SSSS) that leads similar to pemphigus foliaceus to subcorneal blisters (Stanley and Amagai, 2006; Hanakawa and Stanley, 2004). In Barrett's disease, metaplasia of the lower portion of the esophagus and risk factor for esophageal adenocarcinoma, Dsg1 is one of the strongest reduced differentiating markers. In vitro experiments suggest that this is caused by chronic exposure of esophageal cells to bile acid by inhibiting genes driving epithelial differentiation (Reveiller et al., 2012).

There is also growing evidence that Dsg1 promotes epidermal differentiation via various signaling pathways (Harmon et al., 2013; Getsios et al., 2009; Dubash et al., 2013). Interestingly, this might contribute to the multiple layers of crusted scaling typical seen in pemphigus foliaceus. Further suggestions are that Dsg1 is involved in the apoptosis of the keratinocytes (Dusek et al., 2006) and desquamation of the epidermis (Borgono et al., 2007) [Figure 1].

In mice knockdown of the Dsg1 transcription factor Grh11 leads to defects in both adhesion and differentiation of keratinocytes suggesting that both functions of this protein are important for normal strength and function of the epidermis (Mlacki et al., 2014; Wilanowski et al., 2008). The adhesive strength and terminal differentiation of keratinocytes via Dsg1 can be upregulated by ligand targeting of the receptor tyrosine kinase EphA2 and activation of this receptor is required for down regulation of extracellular signal regulated kinase 1/2 (Erk1/2)-mitogen activated kinase (MAPK) signaling. Adding the ligand to a monolayer of keratinocytes increases their differentiation (Lin et al., 2010). Getsios et al. demonstrated in the three dimensional human epidermal raft model how Dsg1 promotes differentiation. Dsg1 inhibits epidermal growth factor receptor (EGFR)-extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, allowing for the progression of keratinocytes toward a more

differentiated phenotype. For this function the cytoplasmic tail of Dsg1 is responsible, since the cleavage of Dsg1 did not result in impaired epidermal morphogenesis, but knock down of Dsg1 did (Getsios et al., 2009). From both studies emerged that downregulation of Erk1/2 is required for differentiation of keratinocytes via Dsg1. Harmon and Simpson et al. investigated which binding partner of the cytoplasmic tail of Dsg1 is involved in the epidermal differentiation. From work of Getsios et al. it became clear that plakoglobin (PG) which binds Dsg1 is not involved in this function, since Dsg1 mutant with no binding site for PG was capable to induce differentiation (Harmon et al., 2013). Instead Dsg1, interacts with the extracellular signal regulated kinase (ERK) regulator Erbin, which suppresses ERK activation to support epidermal differentiation (Getsios et al., 2009).

Besides epidermal differentiation, Dusek *et al.* also suggested a potential function for Dsg1 in the elimination of damaged keratinocytes. UV radiation induced apoptosis of cultured keratinocytes, caused cleavage of both endogenous and exogenous Dsg1, most likely due to the activation of caspase 3. In vitro caspase 3 cleaved the intracellular domain of Dsg1 generating products of 17kDa and 78 kDa (Dusek et al., 2006). A caspase-3 consensus site (DXXD), DLRD, is present at amino acids 885-888 and *in vitro* it was shown that Dsg1 indeed is cleaved at Asp<sup>888</sup>. Caspase-3 is known to be present in human skin (Takahashi et al., 1998).



**Figure 1. Non-adhesive roles of desmoglein 1.** Dsg1 promotes epidermal differentiation and apoptosis and is involved in desquamation of the epidermis. Extracellular domain of Dsg1 is cleaved by kallikreins (KLK) and in this way Dsg1 is involved in desquamation of epidermis. Intracellular domain is cleaved by caspase 3 and promotes apoptosis in cultured cells. Dsg1 interacts with Erbin and inhibits ERK do drive epidermal differentiation.

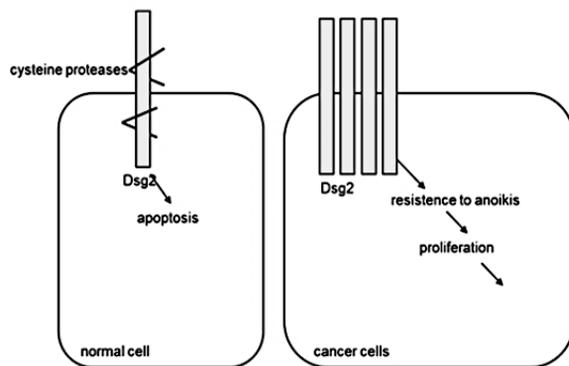
Dsg1 is also an important molecule of desmosomes in corneal layer of the epidermis-corneodesmosomes and degradation of Dsg1, together with Dsc1 and corneodesmin is a prerequisite for desquamation of the epidermis. Borgono et al. demonstrated *in vitro* that kallikreins (KLK), among them KLK1, KLK5, KLK6, and KLK14, cleave Dsg1 and identified a number of putative cleavage sites (Borgono et al., 2007). In Netherton syndrome where mutations in serine protease inhibitor of Kazal type 5 (*SPINK*) lead to decreased KLK inhibition the skin has a disrupted barrier function (Hovnanian, 2013).

## 2.2. Desmoglein 2 in cancer, apoptosis and embryotic development

The 116 kDa Dsg2 is the most widespread desmoglein isoform. It is present in almost all tissues possessing desmosomes including all simple epithelial tissue, myocardium, lymph node follicles and it is commonly found in the desmosomes of cell lines (Schafer et al., 1994; Schafer et al., 1996). Under normal conditions Dsg2 is expressed in the basal cells of the hair follicle and sweat glands but not in the basal keratinocytes of the epidermis or mucosa. It therefore may be seen as a marker of less differentiated cells (Wu et al., 2003). Surprisingly in lesional and perilesional pemphigus skin Dsg2 is also found in the lower layers of the skin (Iwatsuki et al., 1999).

It seems that proper isoform expression of Dsgs is important for normal epidermal morphology and function. Transgenic overexpression of Dsg2 in the epidermis of mice resulted in epidermal hyperplasia, higher risk for papilloma development and increased epidermal cell proliferation (Brennan et al., 2007). Keratinocytes that were cultured from this transgenic skin showed increase resistance against anoikis, apoptosis induced by lack of correct cell-extracellular matrix attachment, compared to wild-type keratinocytes. Dsg2 expressing mice were also more prone to carcinogen-induced tumor development (Brennan et al., 2007). Tumor development is shown to be induced by modulation of Hedgehog signaling via Dsg2 (Brennan-Crispi et al., 2015).

It thus seems that proper expression of Dsg2 is important for epidermal homeostasis and that miss expression can lead to malignancies [Figure 2]. Indeed in human malignant skin carcinomas as well as in colonic adenocarcinomas Dsg2 is overexpressed (Brennan and Mahoney, 2009). Colon cancer cell lines expressing Dsg2 promote proliferation and tumor growth while Dsg2 deficient colon tumor cells failed to grow in vivo (Kamekura et al., 2014).



**Figure 2. Non-adhesive roles of desmoglein 2.** Dsg2 overexpression promotes cancerogenesis, while in colon Dsg2 is involved in regulation of apoptosis. a) Dsg2 is cleaved by cysteine proteases and in this way involved in regulation of apoptosis. b) Overexpression of Dsg2 promotes resistance of cells to anoikis and proliferation which drives cancerogenesis.

Abnormal expression of Dsg2 is also found in epithelial malignancies such as gastric cancer (Biedermann et al., 2005) and squamous cell carcinomas (Kurzen et al., 2003). Recently it has been reported that the cancer stem cell marker CD113 required for the tumorigenicity of the cancer stem cell line from clear cell carcinoma of the ovary

regulates Dsg2 protein levels (Koyama-Nasu et al., 2013) and that knock down of CD113 decreased Dsg2 protein levels. Thus, crosstalk between CD113 and Dsg2 is recognized.

In contrast to the resistance that transgenic Dsg2 provided to anoikis in mouse keratinocytes, Dsg2 was identified as a potential regulator of apoptosis in intestinal epithelium where it is the only isoform expressed (Nava et al., 2007). During the onset of apoptosis in epithelial intestinal cells Dsg2 is cleaved by cysteine proteases and down regulation of Dsg2 protects the cells from apoptosis [Figure 2]. In native human colon and colonic epithelial cell lines, several DSG2 photolytic products were identified (Kolegraff et al., 2011).

Loss of Dsg2 in mice leads to embryonic lethality and this is independent of mature desmosome formation, suggesting a non-adhesive role of Dsg2 during embryonic development (Eshkind et al., 2002). Mutations in the gene that encode these ubiquitous desmosomal cadherin target heart and are found in arrhythmogenic right ventricular cardiomyopathy (ARVC) (Pilichou et al., 2006; Syrris et al., 2007; Rasmussen et al., 2013). Very recently Dsg2 was shown to be the primary high-affinity receptor used by adenoviruses Ad3, Ad7, Ad11 and Ad14 that are the key causes for respiratory and urinary tract infections (Wang et al., 2007).

It seems that this ubiquitous protein has recognized functions in tumor development when miss expressed, apoptosis in intestinal epithelium, not yet identified function in embryonic development and function as a receptor in respiratory and urinary tract infection.

### ***2.3. Desmoglein 3 in epidermal integrity, hair development, actin dynamics, cell migration and proliferation***

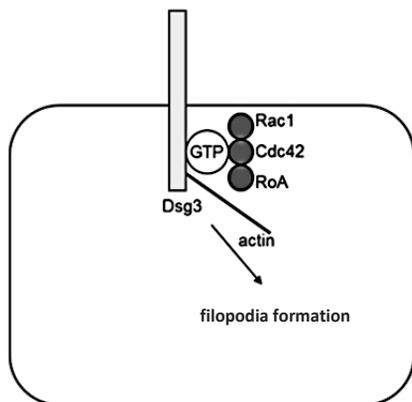
First identified as the pemphigus vulgaris antigen (Amagai et al., 1991) the 130 kDa Dsg3 is expressed in the stratified squamous epithelia. In the skin expression of Dsg3 is limited to the lower cell layers, while in the stratified epithelium of the oral mucosa it is expressed within all cell layers (Mahoney et al., 2006). In cultured keratinocyte cell lines the Dsg3 decreases as keratinocytes undergo stratification. Dsg3 is the major antigen in autoimmune blistering disease mucosal pemphigus vulgaris (PV) and together with Dsg1 in mucocutaneous PV (Amagai and Stanley, 2012). The antibodies alone are capable of causing loss of cell-cell adhesion. Genetic disorders with mutations in the *Dsg3* gene are not known.

In a transgenic mouse model expression of N-terminally truncated Dsg3 resulted in skin abnormalities. It led to a reduction in the number of desmosomes, to widening of the intercellular space between cells and to hyperproliferation of the spinous and granular layer (Allen et al., 1996). Dsg3 knockout mice suffer from suprabasal epidermal loss of cell-cell adhesion, oral erosions and hair loss. Thus Dsg3 is not only important for epidermal integrity, but as well for normal function and development of the hair (Koch

et al., 1998). Hair loss and oral lesions are also observed in *sqk* mice that express a hypomorphic Dsg3 protein with a 69 amino acids deletion in the EC2 domain. The pathology observed in these hypomorphic Dsg3 expressing mice was even worse than that of Dsg3 knockout mice suggesting that apart from disturbed desmosome formation also processes are affected that are insensitive to complete DSG3 deficiency (Kountikov et al., 2015).

Transgenic expression of Dsg3 under the involucrin promoter gives a Dsg3 expression pattern throughout the complete epidermis that resembles that of mucosa (Elias et al., 2001). The stratum corneum of these mice however is abnormal and excessive transepidermal water loss is present leading to death shortly after birth. Electron microscopy showed that desmosomes start to dissolve at the interface of the granular and cornified layer, and also loss of cohesion of corneocytes. This demonstrates that altered Dsg isoform expression may lead to severe defects. However, other transgenic experiments demonstrate that such results should be interpreted with care. If Dsg3 was brought under the keratin 1 promoter the epidermis became abnormal in differentiation and proliferation but the stratum corneum of the mice stayed intact (Merritt et al., 2002). When PV patient anti-Dsg3 IgG was added to cultured HaCaT cells, it was observed that desmosomal remodeling was accompanied with reorganization of the actin cytoskeleton (Gliem et al., 2010). Actin cytoskeleton is connected to adherens junctions, which are another type of cell-cell junctions with E cadherin as transmembrane protein. Overexpression of Dsg3 resulted in the reduction of the level of E-cadherin, while knock down of Dsg3 reversed the E-cadherin levels and partially blocked phosphorylation of Src, a non-receptor kinase that is involved in formation of filopodia used for migration (Tsang et al., 2010).

In a follow up study it was shown that non-junctional Dsg3 can form a complex with E cadherin and directly regulated Src signaling with E cadherin complex during the process of cell-cell junction formation (Tsang et al., 2012b). Dsg3 is thus important for proper formation of adherens junctions. Dsg3 interacts with actin and forms a complex with GTP bound Rac1, Cdc42 and RhoA. Through interaction with these GTPases, which regulate actin dynamics the formation of actin-based membrane structures as filopodia are promoted (Tsang et al., 2012a) [Figure 3]. Apart from cell migration Dsg3 is also important for cell proliferation and silencing Dsg3 in human keratinocytes results in reduced cell proliferation (Mannan et al., 2011). Dsg3 is shown to be overexpressed in head and neck cancers where it can function as an oncogene for cancer growth (Chen et al., 2007; Chen et al., 2013).



**Figure 3. Non-adhesive role of desmoglein 3.** *Dsg3 is involved in actin dynamics and filopodia formation. Dsg3 interacts with actin and forms GTP bound Rac1, Cdc42 and RhoA which regulate actin dynamics and formation of filopodia.*

#### **2.4. Desmoglein 4 in hair follicle function and epidermal homeostasis**

The 180 kDa Dsg4 is extensively expressed throughout the matrix, precortex, and inner root sheet of the hair follicle and it is detected in the suprabasal layers of the epidermis (Kljuic et al., 2003; Whittock and Bower, 2003). Mutations in Dsg4 are associated with localized autosomal recessive hypotrichosis characterized by reduction or loss of hair (Kljuic et al., 2003).

Dsg4, being the only hair follicle desmoglein in the more differentiated layers of the hair follicle, is therefore suggested to be not only important for cell-cell adhesion of hair follicle keratinocytes, but also for their transition from proliferation to differentiation and differentiation itself as loss of Dsg4 in mutant mice led not only to defective cell adhesion but also to epidermal hyperproliferation (Kljuic et al., 2003). The epidermis of these mice had ectopic expression of epidermal growth factor (EGFR) and  $\beta$ -1 integrin in the superbasal layers of epidermis, as well as increased number of positive proliferating cells in the basal and suprabasal layers. The authors hypothesised that the kinetic behavior of the mutant keratinocytes are mediated by genes downstream of EGFR.

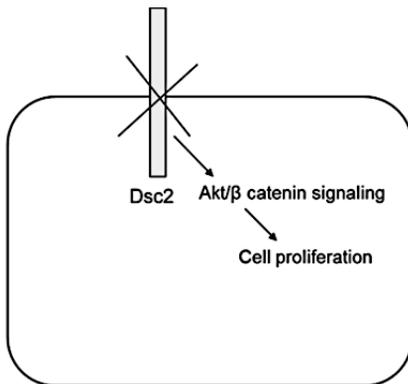
When the TGF $\beta$ /Activin/BMP signaling mediator Smad4 is deleted from keratinocytes Dsg4 becomes also downregulated. This might explain loss of hair seen with Smad4 deletion. It was shown that the expression of DSG4 indeed depends on Smad4 and that Dsg4 is a direct transcriptional target as Smad4 binds to the Smad Binding Element (SBE) of the Dsg4 promoter (Owens et al., 2008).

### 2.5. *Desmocollin 1 in epidermal integrity and differentiation*

Dsc1 is expressed in all suprabasal layers of the epidermis (Theis et al., 1993; King et al., 1993) and in cell lines when stratification starts (Nuber et al., 1995). Misexpression of Dsc1 in the epidermal basal layers of the transgenic mice did not result in abnormalities, neither in the skin and hair follicle histology and desmosome morphology at the ultrastructural levels (Henkler et al., 2001). However in Dsc1 knockout mice skin barrier function and epidermal adhesion are impaired and epidermal differentiation is abnormal, indicating that both adhesive and non-adhesive functions of Dsc1 play an important role in maintenance of epidermal tissue (Chidgey et al., 2001). In comparison to the other desmosomal cadherins, studies on Dsc1 are limited and further investigations are required.

### 2.6. *Desmocollin 2 in cancer and cell migration*

Dsc2 like Dsg2 is expressed in all desmosome possessing tissue, including simple epithelia, myocardium (Schafer et al., 1994; Schafer et al., 1996) and in the basal layers of the stratified epithelia (Theis et al., 1993). In several tissues and cell lines Dsc2 is the only isoform of desmocollins present. Mutations in Dsc2 are found in arrhythmogenic right ventricular cardiomyopathy (ARVC) which indicates importance of Dsc2 in normal cardiac desmosome formation and cardiac function (Heuser et al., 2006; Gehmlich et al., 2011). Proper expression of Dsc2 is required for normal tissue homeostasis and reduced levels of Dsc2 are found in colorectal carcinoma. *In vitro* down regulation of Dsc2 in colonic epithelial cells resulted in increased cell proliferation through activation of Akt/ $\beta$ -catenin signaling (Kolegraff et al., 2011) [Figure 4]. Dsc2 does not interact with  $\beta$



**Figure 4. Non-adhesive role of desmocollin 2.** Downregulation of Dsc2 activates Akt/ $\beta$  catenin signaling to promote cell proliferation. Possible mechanism involved in cancerogenesis.

catenin. However, regulation of Dsc2 enhanced transcription of  $\beta$ -catenin. Akt activity in these cells was enhanced as well as levels of 3,4,5 triphosphate (PIP3) which is known to recruit Akt to the membrane. Akt enhanced activation of  $\beta$ -catenin by promoting its nuclear localization. Knock down of both Dsc2 and  $\beta$ -catenin prevented cell proliferation supporting that cell proliferation through down regulation of Dsc2 is dependent on  $\beta$ -catenin. This is a possible mechanism by which Dsc2 is involved in cancerogenesis (Kolegraff et al., 2011).

Colorectal cancer is not the only cancer associated with reduced levels of Dsc2. In

esophageal squamous cell carcinoma (ESCC) decreased expression of Dsc2 is associated with bad prognosis (Fang et al., 2010). *In vitro* down regulation of Dsc2 in ESCC cell lines increased cell motility through redistribution of adherens junctions and activation of  $\beta$ -catenin signaling (Fang et al., 2013) indicating that Dsc2 regulation of  $\beta$  catenin plays an important role for controlled cell proliferation and motility, and that miss regulation leads to uncontrolled proliferation.

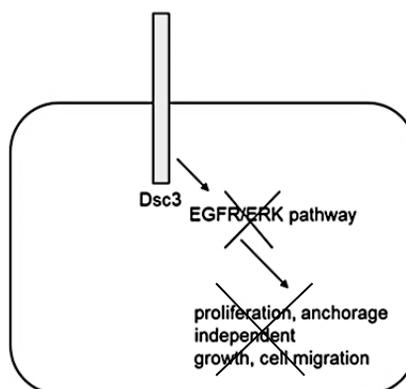
Dsc2 involvement in motility was demonstrated in the wound healing assay experiments where Dsc2 was found to be present in the leading edge of migrating cells. While in non-migrating cells Dsc2 colocalizes with keratin, in migrating cells it colocalizes with actin in the leading edges (Roberts et al., 2011).

### 2.7. *Desmocollin 3 in cancer and epidermal and hair integrity*

Dsc3 is a characteristic desmocollin for stratified epithelia, it is found in the basal layer of the epidermis, in the hair follicle and in other stratified epithelia of the human respiratory and urogenital tract (Nuber et al., 1995). Mutations in the *Dsc3* gene result in sparse and fragile hair and large skin vesicles filled with watery fluid (Ayub et al., 2009).

Knocking out Dsc3 in mice is embryonic lethal and at E2.5, before desmosome formation, most embryos have already disintegrated. Thus, Dsc3 is essential for embryonic development (Den et al., 2006). In conditional Dsc3 null mouse loss of cell-cell adhesion and hair in the epidermis was observed (Chen et al., 2008). Transgenic expression of Dsc3 in suprabasal layers in mice epidermis results in abnormalities, but only in the adulthood. These mice develop hair defects, altered epidermal proliferation and early differentiation of the keratinocytes in the epidermis. Furthermore, levels of  $\beta$ -catenin become upregulated in transgenic skin, suggesting that Dsc3 regulates  $\beta$ -catenin (Hardman et al., 2005).

Altered expression of Dsc3 is found in several cancers, including lung squamous cell carcinoma (Wang et al., 2007). In a mouse skin tumor model it was shown that loss of Dsc3 increases tumor incidence (Chen et al., 2008) and in lung cancer cell lines the *Dsc3* gene



**Figure 5. Non-adhesive role of desmocollin 3.** *Dsc3* suppresses cancerogenesis via inhibition of ERK pathway.

appears to be hypermethylated and thus downregulated. Ectopic expression of Dsc3 in these cell lines inhibited cell proliferation, anchorage-independent growth, migration, as well as invasion, and led to reduced phosphorylation levels of extracellular signal-regulated kinase1/2 (Erk1/2). It seems that Dsc3 suppresses carcinogenesis through inhibition of EGFR/ERK pathway (Cui et al., 2012) [Figure 5].

### 3. Desmosomal armadillo proteins

The proteins of armadillo family are named after a characteristic repeat of around 40 amino acids (called armadillo repeat) and are important linker proteins in the desmosomal structure. Armadillo proteins in the desmosomes are plakoglobin and plakophilins 1-3 (Delva et al., 2009). Overview of non-adhesive function of desmosomal armadillo proteins is listed in table 3.

<b>Protein</b>	<b>Suggested-non adhesive function</b>	<b>Associated mechanism</b>	<b>References</b>
<b>Pg</b>	Activation of Lymphoid enhancer-binding factor/transcription factor	Wnt/ $\beta$ catenin signaling pathway	Yin et al. 2005
	Suppression of cell motility	Src kinase dependent signaling	Yin et al. 2005 Franzen et al. 2012
	Regulation of apoptosis	Wnt signaling pathway and Src activity	Dusek et al. 2007
	Cell adhesion via regulation of keratin organization	P38MAPK	Spindler et al. 2014
	Maintenance of epidermal stem cells and controlled differentiation	cMyc suppression	Williamson et al. 2006
<b>Pkp1</b>	Shifting desmosomal adhesion to calcium independent	Increased incorporation of Dsg3 into desmosomes	Tucker et al. 2014
	Cell proliferation	Translation via recruitment of eIF4A1 insulin/Akt	Wolf et al. 2010, 2013
	Cell survival	Binding to DNA	Sobolik-Delmaire et al. 2010
<b>Pkp2</b>	Proliferation/migration/tumor development	EGFR	Arimoto et al. 2014
	Regulation of actin cytoskeleton	RhoA- and PKC-dependent pathways	Godsel et al. 2010
<b>Pkp3</b>	Desmosome and adherens junctions assembly	Rap 1 GTPase	Todorovic et al. 2014
	Cancer progression/metastasis	Keratin 8 phosphorylation	Khapare et al. 2012

**Table 3. Non-adhesive functions of desmosomal armadillo proteins: plakoglobin and plakophilins. Suggested non-adhesive function and its associated mechanisms.**

### **3.1. Plakoglobin in Wnt/ $\beta$ signaling, cell motility and apoptosis**

The 82 kDa plakoglobin (also called  $\gamma$ -catenin) is an intracytoplasmic plaque protein not found only in desmosomes, but also in another type of cell-cell junction, the adherens junction (Cowin et al., 1986). Pg binds to desmogleins and desmocollins and connects them with desmoplakin (Trojanovsky et al., 1994; Chitaev et al., 1996). Pg comprises 13 central repeat domains of which repeat domains 1-4 are involved in binding to Dsg1 (Witcher et al., 1996). Mutations in the Pg gene are associated with Naxons disease which is characterized by arrhythmogenic right ventricular cardiomyopathy, palmoplantar keratoderma and woolly hair, and to a lethal congenital form of epidermolysis bullosa (McKoy et al., 2000; Pigors et al., 2011). In pemphigus foliaceus (PF), Pg together with the targeted Dsg1 and pathogenic IgG becomes widely redistributed and clustered (Oktarina et al., 2011).

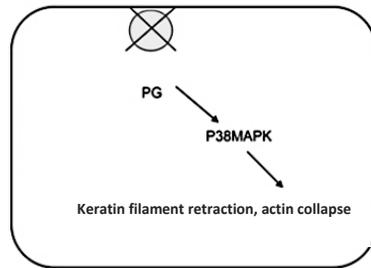
That Pg is essential for the adhesive desmosomal function was demonstrated in Pg null mice, which died due to the skin blistering and heart defects during embryogenesis or just after birth (Bierkamp et al., 1996; Ruiz et al., 1996). In these mice, the number of desmosomes was reduced together with alterations of their structure. So far one case of lethal congenital epidermolysis bullosa was described, which was caused by a nonsense mutation in the JUP gene that encodes Pg. Here similar aberrations were seen (Pigors et al., 2011).

The closest homolog of Pg is  $\beta$ -catenin that normally localizes to the adherens junctions, but became localized to the desmosomes in Pg mutant null mice. This could be a compensatory mechanism, even though it did not rescue completely (Bierkamp et al., 1999). Pg mutations in the epidermis of the transgenic mice led to clinical phenotype of human palmoplantar keratoderma with increased thickening of the epidermis, increased cell apoptosis and cell proliferation, as well as increased levels of  $\beta$ -catenin (Li et al., 2012).

Pg as the closest homologue of  $\beta$ -catenin is also indirectly involved in the canonical Wnt signaling pathway (Wnt/ $\beta$  catenin signaling) by competing with the active  $\beta$ -catenin pool in the cytosol and thereby affecting  $\beta$ -catenin localization and function (Simcha et al., 1998). Pg can bind to Lymphoid enhancer-binding factor/ transcription factor (LEF/TCF), promoting its transcription. However, the interaction of  $\beta$ -catenin with LEF is stronger than the interaction of Pg with LEF, hence it is proposed that Pg activates LEF/TCF transcription by elevating the level of endogenous  $\beta$ -catenin (Zhurinsky et al., 2000). Pg is shown to suppress cell motility through regulation of Src kinase dependent signaling. This effect is independent of cell-cell adhesion, since both C- and N-terminally PG deletion mutants had proper adhesion, but only the C deletion mutant was able to suppress single cell motility. In addition, active Src antagonized the inhibitory effect of Pg on cell motility in Pg depleted cells (Yin et al., 2005). This inhibitory effect of PG on

cell motility was also demonstrated in prostate cancer cell lines. PG inhibits motility and invasion in prostate cancer cell lines through inhibition of Src signaling (Franzen et al., 2012). Downregulation of PG results in loss of cell-cell adhesion, upregulation of the extracellular matrix protein vitronectin and activation of Src signaling, what in turn leads to increased prostate cancer cell motility (Franzen et al., 2012).

Pg has a role in controlling cell adhesion via p38MAPK-dependent regulation of keratin filament organization (Spindler et al., 2014). Silencing of Pg caused activation of p38MAPK-dependent keratin filament collapse and cell dissociation [Figure 6]. Another study showed that PG is a key suppressor of c-Myc in keratinocytes and has a role in pathogenesis of PV (Williamson et al., 2006).



**Figure 6. Non-adhesive role of plakoglobin.** Silencing of Pg leads to activation of P38 MAPK and keratin filament retraction.

### **3.2. Plakophilin 1 in cell migration, translation and cell survival**

Pkp1 is a desmosomal plaque protein found in various stratified and complex epithelia (Heid et al., 1994), where it is present in desmosomes, cytoplasm and nucleus. It exists in two isoform variants Pk1a and Pk1b where PKP1b is 21 amino acids longer than Pk1a and found exclusively in the cell nucleus (Schmidt et al., 1997). Mutations of the Pkp1 gene in humans are associated with ectodermal dysplasia- skin fragility syndrome, which is characterized by skin thickening, loss of cell-cell adhesion and reduction in size and number of desmosomes in the epidermis (McGrath and Mellerio, 2010). Overexpression of Pkp1 enhances the number and size of cell-cell contacts in cultured cells (Bornslaeger et al., 2001; South, 2004), which correlates with the expression of Pkp in the lower regions of epidermis inhabited by larger desmosomes. Overexpression not only leads to larger desmosomes, but also increases desmosomal adhesion by shifting desmosomes from the calcium-dependent to the calcium-independent hyperadhesive state (Tucker et al., 2014). In line with this, Pkp1 deficient keratinocytes have less calcium-independent desmosomes and in wound healing assays these cells migrate faster (South et al., 2003). Also in cancers loss of Pkp1 is observed and its decrease seems to correlate with an aggressive phenotype (Breuninger et al., 2010).

Under environmental stress Pkp1 is found in stress granules (granules containing translation pre-initiation RNA/protein complexes that due to stress failed translation) together with plakophilin3 (PKP3), eukaryotic translation initiation factor 4E (eIF4E) and the ribosomal protein S6 suggesting a role for Pkp1s in translation and RNA metabolism (Hofmann et al., 2006). Eukaryotic initiation factor 4A1 (eIF4A1) is a direct binding

partner of the head domain of Pkp1. This binding is important for the regulation of translation via recruitment of eIF4A1 to the initiation complex and eIF4A1 activation (Wolf et al., 2010). This leads to an overall up regulation of translation in Pkp1 overexpressing cells, where the knockdown reduced protein biosynthesis. By enhancing translation rates Pkp1 can increase cell proliferation. This proliferation-promoting function is activated by growth factor signaling (Wolf et al., 2013).

Pkp1 through its head domain can also bind single stranded DNA. During DNA damage and DNA replication levels of single stranded DNA are increased, and induction of DNA damage resulted in a partial redistribution of Pkp1 to the nucleus. Depletion of Pkp1 resulted in increased cell survival in response to DNA damage. Thus Pkp1 through interaction with DNA seem to be able to influence cell survival (Sobolik-Delmaire et al., 2010).

### ***3.3. Plakophilin 2 role in nucleus, maintance of cell migration and proliferation, formation of desmosomes and actin dynamics***

Pkp2 is the most widely distributed isoform among PKPs and it is present both in desmosomes and the nucleus (Mertens et al., 1996). In the epidermis its expression is restricted to the basal layer (Mertens et al., 1999). Mutations in Pkp2 are common in arrhythmogenic right ventricular cardiomyopathy (ARVC) (Gerull et al., 2004). Lack of Pkp2 in mice resulted in alterations of cardiac muscle and cardiac function, indicating an important role in heart tissue (Grossmann et al., 2004). Down regulation of Pkp2 in cells resulted in accelerated migration and increased proliferation but also in adiposis (Matthes et al., 2011), which could explain that in ARVC myocardial tissue is replaced by fibrous and adipose tissue.

In the nucleus, Pkp2 has been detected in specific complexes containing the largest subunit of RNA polymerase III (RPC155), the pol III subunit of 39 kDa and transcription factor TFIIB and also in distinct nucleoplasmic granules (Mertens et al., 2001). This interaction may provide a clue to the nuclear function of Pkp2, although RNA polymerase III has a very general role in DNA transcription. Pkp2 was found to be a substrate of Cdc25C-associated kinase 1 (C-TAK1). C-TAK1 phosphorylates Pkp2 and thereby generates a 14-3-3 binding site. A mutation in the 14-3-3-binding site results in an increased nuclear localization of Pkp2, suggesting that 14-3-3 binding is likely to be involved in the nuclear shuttling of Pkp2 (Muller et al., 2003).

Pkp2 seems to have an important role in proper assembly of desmosomes. There is some evidence that PKP2 governs desmoplakin assembly dynamics by scaffolding a Dp-P2-protein kinase C $\alpha$  (PKC $\alpha$ ) complex, which is disrupted by Pkp2 knockdown. Bass-Zubek et al. proposed a model whereby cell-cell contact triggers a signal, and Pkp2, either directly or in conjunction with other scaffolding proteins, is required for communicating this signal by recruiting activated PKC to Dp, which in turn is

phosphorylated. The phosphorylation of DP allows for assembly into cell-cell junctions (Bass-Zubek et al., 2008).

The possible relation between Pkp2 and actin dynamics to promote desmosome assembly was studied by Godsel et al (Godsel et al., 2010). They show that a pool of Pkp2 appears at newly forming cell-cell junctions, together with E-cadherin and before Dp assembly into desmosomes during the early stages of actin rearrangements. Loss of Pkp2 results in structural defects in cortical actin remodeling, accompanied by a defect of RhoA to concentrate at cell-cell interfaces, and an overall elevation of cellular RhoA and downstream indicators of contractile signaling. Activation of RhoA accelerated DP assembly into desmosomes. Together with the earlier finding of Bass-Zubek et al., these data suggest that Pkp2 may functionally link RhoA- and PKC-dependent pathways to drive actin reorganization. Godsel and al. claim that RhoA and PKC cannot function in the same pathway, since RhoA activity was not affected either by silencing or elevating PKC $\alpha$  activity.

Pkp2 was shown to specifically interact with EGFR via its N-terminal head domain and enhances EGF-dependent and EGF-independent EGFR dimerization and phosphorylation, resulting in EGFR activation, which is shown to be implicated in tumor development (Arimoto et al., 2014).

#### ***3.4. Plakophilin 3 function in hair, skin, neural tracts, cell migration and proliferation***

Pkp3 is equally distributed among all the epidermal layers and is found in most simple and stratified epithelia. In the cell Pkp3, as Pkp1, localizes to the desmosome, cytosol (stress granules) and nucleus (Hofmann et al., 2006; Bonne et al., 1999). Human disorders due to mutations in the *Pkp3* gene are not known. It is important in morphogenesis of the hair follicle, since Pkp3 knockout mice showed impaired hair development. The epidermis of the mice thickened, suggesting increased proliferation, but no differences in differentiation of keratinocytes were observed (Sklyarova et al., 2008). Another interesting finding was that Pkp3 knockout mice were prone to dermatitis what may indicate that Pkp3 is involved in limitation of inflammatory reactions in skin.

A possible role of Pkp3 in cell proliferation was demonstrated in a colorectal cancer cell line (Khapare et al., 2012). Loss of Pkp3 resulted in an increased neoplastic progression and metastasis, which was associated with increased levels of keratin-8 (K8). Inhibition of K8 expression in the Pkp3 knockdown clone resulted in less cell migration during a scratch wound assay. Altered cell migration is often accompanied by altered actin dynamics and the formation of actin dependent structures such as lamellipodia. Inhibition of K8 expression in the Pkp3 knockdown clone resulted in decreased

lamellipodia formation. K8 Pkp3 double knockdown clones showed decreased colony formation, tumorigenesis and metastasis in nude mice (Khapare et al., 2012). These results indicate that Pkp3 is very important for cell migration and metastasis, and that stabilization of K8 filaments may be one mechanism by which Pkp3 loss leads to tumor progression and metastasis.

It has been demonstrated that PKP3 mediates desmosome and adherens junction assembly by maturation of Rap1 GTPase (Todorovic et al., 2014).

#### 4. Plakins

The plakin family functions as a structural protein in desmosomes and links the intermediate filament to the desmosomal plaque. The most important plakin found in all desmosomes is thought to be desmoplakin, which has recognized non-adhesive roles [Table 4].

Protein	Suggested non-adhesive function	Associated mechanism	References
Dp	Inhibition of cell proliferation	MAPK/PI3K	Wan et al. 2007
	Regulation of actin network	-	Bornslaeger et al. 1996
	Microtubule regulation	Ninetin	Lechler et al. 2007

**Table 4. Non-adhesive functions of desmoplakin.** Suggested non-adhesive function with its associated signaling pathway/mechanism. *Dsg4* and *Dsc1* non-adhesive functions are less studied.

##### 4.1. Desmoplakin in actin and microtubule organization and cell proliferation

Desmoplakin (DP), a plakin family protein which exists in two variants DPI (322kDa) and DPII (259kDa), is ubiquitously expressed in all cells and tissue that possess desmosomes (Delva et al., 2009; Garrod and Chidgey, 2008; Hatsell and Cowin, 2001). Genetic mutations in the gene that encodes DP are found in the following disorders: arrhythmogenic right ventricular cardiomyopathy (Rampazzo et al., 2002), striate palmoplantar keratoderma (Armstrong et al., 1999), skin fragility/woolly hair (Whittock et al., 2002) and lethal acantholytic epidermolysis bullosa (Jonkman et al., 2005).

Its importance in anchoring intermediate filaments to desmosomes was demonstrated in DP knockout mice in which the keratin filament network was distracted and DP null embryos died at the early embryotic stage just after implantation (Gallicano et al., 1998). When DP function was rescued in extra embryotic tissue of DP knock out and wild type embryos, which died after gastrulation, defects were found in heart, epidermis, neuroepithelium, but also in the microvasculature tissue, which does not possess desmosomes, suggesting its importance outside desmosomes. A LAEB patient, who had a mutation in DP, resulting in the deletion of the IF binding domains, died a

few days after gestation, indicating that the other domains are essential for fetal development (Jonkman et al., 2005).

In skin DP is essential in epidermal sheet formation (Vasioukhin et al., 2001). Cell culture studies revealed a role of DP in assembly of adherens junctions, since cell lines expressing DP, lacking the keratin filament binding domain, showed redistribution of adherens junction components (Bornslaeger et al., 1996). Silencing DP in a human keratinocytes cell line resulted in increased cell proliferation and activation of the MAPK and PI3K signaling pathway (Wan et al., 2007). How DP interacts with these pathways is not known. In an epidermal specific DP knock down mice, less adherens junctions and reorganization of actin filaments was observed in the epidermis (Vasioukhin et al., 2001). In intestine of intestine specific DP knockout mice actin organization and microvillus structure was impaired, but cell adhesion and keratin organization was not affected (Sumigray and Lechler, 2012). These findings show the importance of DP in actin filament organization both in epidermis and intestinal epithelium. Since DP does not contain an actin binding domain, the question remains how DP affects actin organization and assembly of adherens junctions. In addition to actin regulation, DP also plays a role in microtubule organization. Ninetin, the centrosomal protein required for microtubule anchoring is shown to be recruited by DP in differentiation-related reorganization of microtubules. In mice embryos with epidermal-specific loss-of-function mutations in DP, microtubules had a cytoplasmic concentration instead of junctional in the basal layer of the epidermis (Lechler and Fuchs, 2007). Besides ninetin, other centrosomal proteins among them Lis1, are recruited to the cell cortex by DP upon epidermal differentiation (Sumigray and Lechler, 2012).

## **5. Concluding remarks**

Adhesion molecules of the desmosome have more functions than mere adhesion. An important observation is that up and down regulation of components can have major effects on cells as major signaling pathways become affected. A critical remark to make is however that the majority of the suggestive functions originate from cell models and that confirmation from human tissue is inevitably needed. This thesis addresses the histopathology of pemphigus skin at the ultrastructural level. Conclusions from experiments described here have also heavily influenced pemphigus research over the past fifteen years.

