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DIPLOMARBEIT

Titel der Diplomarbeit

„Studies on the functions of calpain as a plectin isoform 1a-degrading protease involved in keratinocyte differentiation and Epidermolysis Bullosa Simplex Ogna“

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Table of contents

Abbreviations

ABD	actin-binding domain
AP	alkaline phosphatise
ARP complex	actin related protein complex
ATP	adenosine tri phosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
BPAG1	bullous pemphigoid antigen 1
BSA	bovine serum albumin
CH, CH1, CH2	calponin homology (domain 1,2)
DMF	dimethyl-formamide
DTT	dithiotreitole
EBS-PA	epidermolysis bullosa simplex –pyloric atresia
EBS-MD	epidermolysis bullosa simplex- muscular dystrophy
EBS-CMS	epidermolysis bullosa simplex-congenital myasthenia
EBS-Ogna	epidermolysis bullosa simplex-Ogna
ECM	extracellular matrix
EDTA	ethylene-diamine tetra-acetic acid
EGTA	ethylene glycol-bis (β-aminoethylether)-N,N,N’,N’-tetra-acetic acid
EPU	epidermal proliferative unit
GAR	Gas2 homology region
GSR repeats	glycine-serine-arginine repeats
IF	intermediate filament
IFBD	intermediate filament binding domain
IFE	inter follicular epidermis
IMK	immortalized mouse keratinocytes
MAP	microtubule associated protein
MT	microtubule
MTOC	microtubule organizing centre
HD	hemidesmosome

HPC	hemidesmosome enriched protein complexes
PBS	phosphate-buffered saline
PKC	protein kinase C
PMK	primary mouse keratinocytes
PMSF	phenyl-methyl-sulfonyl-flourid
PRD	plakin-repeat domain
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH3	Src homology 3
TA cells	transit amplifying cells
TEWL	transepidermal water loss

INTRODUCTION

THE CYTOSKELETON

A single living cell is the very foundation of every organism and enables with its function life itself. Every cell has a tightly regulated internal skeleton, referred to as the cytoskeleton, in order to provide strength to withstand mechanical forces, change shape, to be able to move and to rearrange internal structures for cell division. All of these processes require a network of three distinct cytoskeletal filament systems: the actin filament network, the microtubule (MT) network and the intermediate filament (IF) network. These filament systems are characterized by distinct mechanical properties, dynamics and biological functions. The focus of this work is set on the IF network and its associated cytolinker protein plectin.

Intermediate filaments

Intermediate filaments (IFs) represent the most heterogeneous type of cytoskeletal filaments. They are assembled into ropelike structures with a diameter of 10-12 nm and share a common domain organisation. (Strelkov et al., 2003). IFs are subgrouped into five types according to their amino acid sequence homology (Table 1) (Fuchs and Weber, 1994; Herrmann et al., 2007).

Table 1: Types of mammalian intermediate filaments

Type I/ II[1]	**Type III**[2]	**Type IV**[3]	**Type V**[4]	**Type VI**[5]
Keratins	Desmin	Neurofilaments	Lamins	Bfsp1
	Vimentin	α-Internexin		Bfsp2
	GFAP	Nestin		
	Synemin	Syncoilin		
	Peripherin			
	Paranemin			

[1] **Type I/II:** Acidic (type I) and basic (type II) keratins form the IF network of simple and stratified epithelia.
[2] **Type III:** Desmin and synemin are abundantly expressed in skeletal muscle. Vimentin is the principal IF component of cells of mesenchymal origin, and GFAP of neuroglial cells. Peripherin is expressed in the peripheral nervous system and is thought to play a role in neurite elongation during development and axonal regeneration after injury. Paranemin supports the formation of an extended desmin network.
[3] **Type IV:** Neurofilaments, alpha-internexin and nestin fulfill numerous functions in neurons. Syncoilin links desmin IFs to the dystrophin-associated protein complex.
[4] **Type V**: Lamins are the major component of the nuclear lamina.
[5] **Type VI:** Bfsp1 and Bfsp2 are lens specific components of beaded filaments, a unique cytoskeletal element of the vertebrate lens. Modified from Herrmann et al., JCI, 2009; Eriksson et al., 2009.

A large family of genes encodes IF-proteins. Three lamin genes contribute to the nuclear family of IF proteins and 67 genes encode the cytoplasmatic IF-proteins (Coulombe and Wong, 2004; Herrmann et al., 2007). All IFs display a tripartite structure (Fig. 1) consisting of a central alpha-helical rod domain that is required for dimerisation of IF proteins and non helical head and tail domains that vary in length, biochemical properties and aminoacid sequence. The head and tail domains are essential for interaction with cytoskeletal components (Herrmann et al., 2007; Herrmann et al., 2009). In contrast to tubulin and actin, the cytoplasmic IF system is not required for survival at the single-cell stage, as it is absent from most bacteria, yeast, and drosophila (Herrmann and Strelkov, 2011).

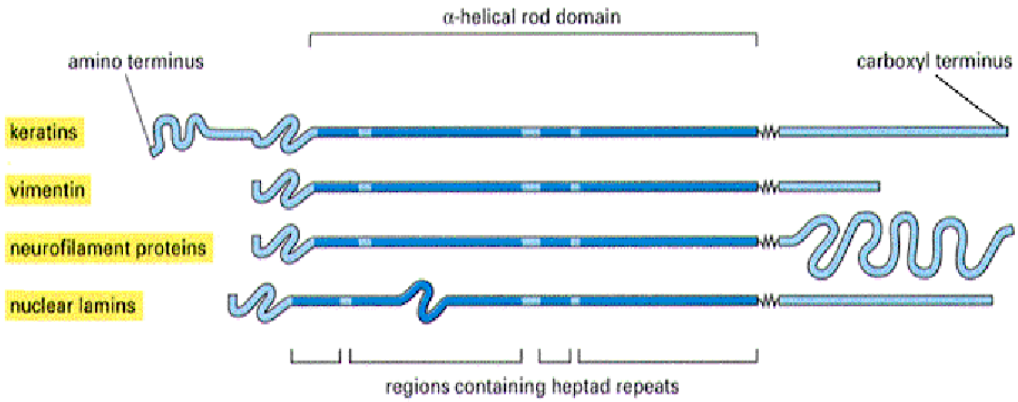

Figure 1: Tripartite structure of the IF monomer. The conserved central alpha-helical rod domain contains heptad repeats, such that every first and fourth amino acid side chain is hydrophobic, generating a hydrophobic seam which is required for coiled-coil formation and thus subunit dimerisation. The head and tail domains of IF monomers have no defined structure and vary in length and sequence. Head and tail domains are crucial for interaction with a variety of cytoskeletal proteins.
Source: http://www.cytochemistry.net/Cell-biology/intermediate_filaments.htm

Another striking difference to actin filaments and MTs is that IFs do not bind and metabolize nucleotides for their assembly and disassembly. No defined nucleator has so far been found for IF assembly. Type I/II IFs (keratins) tend to assemble near actin rich focal contacts, whereas some other IF- proteins such as the type III IF protein peripherin are transcribed and assembled along MTs in a process called dynamic co-translation (Chang et al., 2006; Windoffer et al., 2006). Cytoplasmic IF-proteins dimerize in a parallel fashion via their α-helical rod domains; these very stable dimers then associate laterally in an anti-parallel half-staggered fashion to tetramers. Eight tetramers form a structure called unit length filament (ULF) (Fig. 2) (Herrmann et al., 2009). ULFs anneal longitudinally to form short

filaments, and filament growth proceeds further by end-to-end association of filaments. Finally, nascent filaments (diameter: ~16 nm) are radially compacted into mature, ~11 nm thick IFs (Goldman et al., 2008; Herrmann et al., 2009). The regulation of IF-assembly still remains largely unclear. For neurofilaments it has been demonstrated that their assembly is regulated temporally. Neurofilament subunit proteins are synthesized in the cell body and are transported down the axon either as assembled IF subunits, what accounts for a fast assembly, or as filament polymers that are transported slowly (Yan and Brown, 2005).

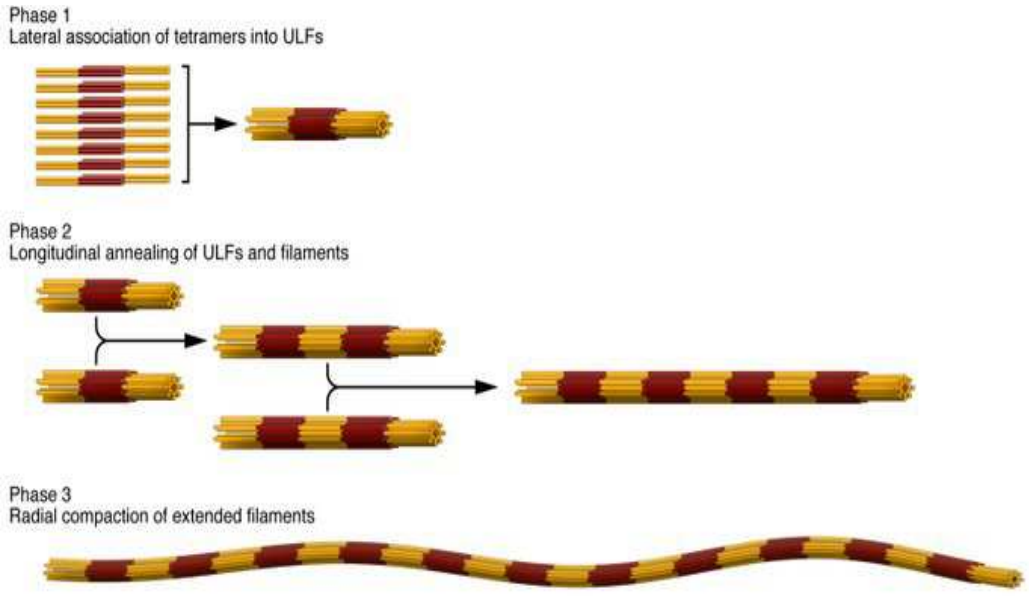

Figure 2: Assembly of intermediate filaments. Phase 1: IF-monomers dimerize in a parallel fashion into dimers via their α-helical rod domains. Dimers form a staggered tetramer which associates into a protofilament consisting of eight tetramers. Protofilaments associate laterally to a ropelike structure called unit length filament (ULF). **Phase 2:** ULFs anneal longitudinally to other ULFs and short filaments. **Phase 3:** In addition the filaments compact radially until a diameter of 11nm is reached. Modified from Herrmann et al, JCI, 2009.

IFs are generally believed to form stable structures but the filaments display dynamic properties depending on the subcellular context. For some types of IFs a dynamic mechanism similar to actin treadmilling has been reported (Stewart and Roberts, 2005), but the mechanism of their steady state dynamics remains unclear. Furthermore it has been demonstrated that IF proteins can be turned over rapidly in a proteasome-dependent way through phosphorylation or ubiquitination (Ku and Omary, 2000). Moreover, phosphorylation of ULFs can prevent the lateral

assembly of the filaments. But it remains unclear whether the subunits disassemble at the end or from within the rod domain (Sihag et al., 2007).

IFs are cross-linked to MTs and actin filament networks, but also to adhesive complexes including desmosomes and hemidesmosomes (HDs) (see below). The subcellular distribution and cytoarchitecture of IFs varies according to cell type and tissue. In keratinocytes of the epidermis, IFs are organised as a dense network spanning the cytoplasm and are anchored at cell-matrix and cell-cell adhesions (Svitkina et al., 1996; Fuchs and Cleveland, 1998; Jefferson et al., 2004). In polarized simple epithelial linings, the IF-network is more sparse and located predominantly at the apical pole of the cell (Coulombe and Omary, 2002). In the myofibers of skeletal muscle, the IF-network is concentrated at the Z-lines and thus interlinks adjacent sarcomers (Lazarides, 1980; Ball and Singer, 1981; Clark et al., 2002). In fibroblasts, the filaments span the whole cytoplasm in close proximity to MTs (Ball and Singer, 1981). Finally, in myelinated CNS neurons, IFs form parallel arrays in the axioplasm outnumbering MTs. In this case IFs are required for radial growth of axons (Cleveland and Hoffman, 1991).

All types of IFs contribute to the structural support of the tissue. Recent works however, demonstrate more versatile functions of IFs. For instance, in epidermal keratinocytes the IF network is required for the mechanical stability of the tissue, as demonstrated by the fact that mutations in the genes encoding keratin 5 (K5) or keratin 14 (K14) cause the skin blistering disease Epidermolysis Bullosa Simplex (EBS) (Bonifas et al., 1991; Coulombe et al., 1991; Lane et al., 1992). However, the epidermal keratins K6, K16 and K17 are induced in the epidermis of the ectoderm and adult skin upon wounding and other stressors and play a major role in epidermal wound healing (Takahashi and Coulombe, 1997; Mazzalupo et al., 2003; Lessard and Coulombe, 2012). Moreover, keratins are involved in regulation of protein synthesis and cell growth as they associate with a number of regulatory factors. K17 interacts with 14-3-3 proteins thereby regulating mTOR signalling and consequently protein synthesis (Bertram et al., 1998; Kim et al., 2006). Other binding partners of type I and II IF proteins involved in translational control are the eukaryotic elongation factor 1Bγ (eEF1Bγ), eukaryotic initiation factor 3 (eIF3), and the tumour suppressor protein tuberous sclerosis complex 1 (Tsc1) (Bousquet et al., 2001; Lin et al., 2001; Haddad et al., 2002). Furthermore,

the type III IF protein vimentin is required for regulation of the ERK pathway (Kumar et al., 2007). Finally, IFs have been reported to be involved in death receptor-mediated apoptosis (Inada et al., 2001; Oshima, 2002; Yoneda et al., 2004; Tong and Coulombe, 2006), protein targeting (Ameen et al., 2001), vesicle transport (Styers et al., 2004), cell adhesion (Kowalczyk et al., 1999), and formation of the nuclear lamina (Shimi et al., 2010; Worman, 2012).

The Plakin Protein Family

The plakin protein family (Janda et al., 2001) consists of large modular proteins which are also referred to as cytolinkers (Leung et al., 2002; Sonnenberg and Liem, 2007). They crosslink the various cytoskeletal networks to each other and to membrane-associated adhesion junctions (desmosomes and HDs) (Leung et al., 2001). A set of several domains is variably arranged to form these large cytoskeletal linker proteins (Fig. 3) (Sonnenberg and Liem, 2007). The plakin domain contains α-helical bundles, so called spectrin repeats, and is common to all protein members except for epiplakin (Sonnenberg and Liem, 2007). This domain mediates direct or indirect binding to adhesion receptors (Rezniczek et al., 1998; Koster et al., 2003; Koster et al., 2004; Al-Jassar et al., 2011). The actin-binding domain (ABD), located in the N terminus displays differences in amino acid sequence among the members of the plakin family (Leung et al., 2001). The coiled-coil domain contains heptad repeats (first and fourth amino acid side chains are hydrophobic) and mediates dimerization of plakin molecules (Sonnenberg and Liem, 2007). The plakin repeat domain (PRD) consists of a varying number of repeating subdomains termed A, B and C and enables interaction with IFs (Leung et al., 2002). The spectrin repeat (SR) domain found in the plakin protein family (Roper et al., 2002; Sonnenberg et al., 2007) is believed to form a flexible rod-like structure that separates the distinct functional domains within the plakin molecule (Roper et al., 2002; Sonnenberg and Liem, 2007). Furthermore, in some plakins, two speclialized domains, the Gas2 homology domain (GAR) and the Gly-Ser-Arg repeat domain (GSR), can directly bind to MTs (Leung et al., 2001). Several isoforms are generated through alternative splicing of all members of the plakin family except of envoplakin, periplakin and epiplakin.

MACF1 is the vertebrate homologue of the ancestral gene for plakin, the *short-stop/kakapo* gene, in *D. Melanogaster and C. Elegans* (Leung et al., 2001). It contains a MT-binding GAR domain and an ABD and thus facilitates interaction of the actin and MT network at the periphery. Furthermore it anchors MTs to cell-cell junctions (Karakesisoglou et al., 2000).

BPAG1 is expressed in various isoforms (Roper et al., 2002). The major isoform BPAG-1a is expressed in the neurons and interlinks the NFs to the actin network (Leung et al., 2001). BPAG1-e is an epithelial isoform that anchors keratin IFs to HDs and is required for epidermal integrity (Yang et al., 1996; Liu et al., 2012). BPAG-1b is expressed in muscle and has the potential to interact with all cytoskeletal structures (Steiner-Champliaud et al., 2010).

Desmoplakin is expressed as two isoforms (Green et al., 1990). The larger isoform is located at desmosomes (which are formed in epithelial and cardiac muscle cells) where it anchors IFs (Norgett et al., 2000; Getsios et al., 2004; Yin and Green, 2004). The smaller isoform contains a shortened rod domain and shows a more restricted tissue distribution (Angst et al., 1990; Green et al., 1990).

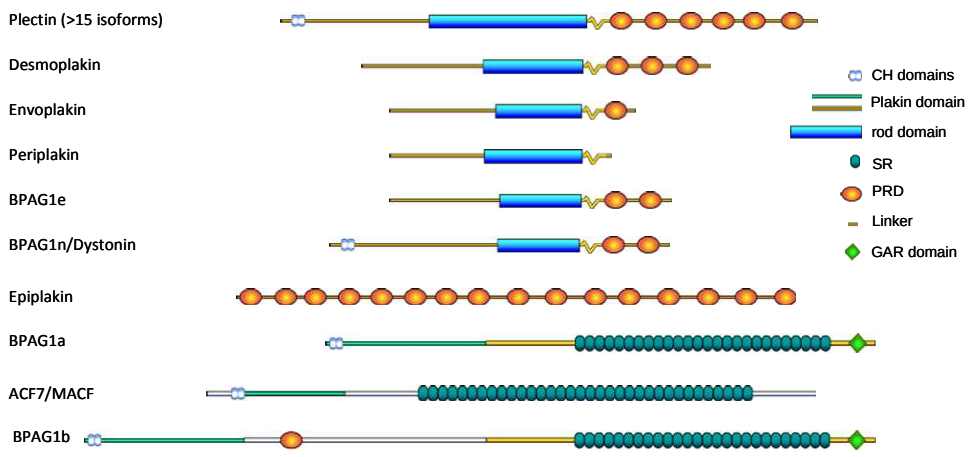

Figure 3: The plakin family. Common to all members of the plakin protein family except for epiplakin is the plakin domain. The plakin domain consists of up to nine pairs of spectrin repeats interrupted by a Src homology 3 domain (SH3). The highly conserved actin-binding domain (ABD) is shared by plectin, BPAG-1a, BPAG-1b and both MACF isoforms. It consists of two calponin homology domains (CH1 and CH2). All members except of BPAG and MACF isoforms contain a coiled-coil rod domain, which is required for dimerization of plakin molecules. The Gas 2 homology domain and the Gly-Ser-Arg repeats domain (GSR) mediate interaction with MTs in both MACF isoforms and BPAG1a and BPAG1b. The spectrin repeat domain (SR) forms a long rod-like structure which structurally separates the functional domains of the plakin molecule. The plakin repeat domain PRD is built up of varying numbers of repeating unit subdomains, which are termed A, B or C, depending on their degree of similarity to each other. This domain is expressed in both desmoplakins, plectin, BPAG1e, envoplakin and epiplakin. Modified from Rezniczek, unpublished data.

Envoplakin and Periplakin are highly homologuous to each other and function as a complex, which is associated with desmosomes and keratinocyte cornified envelopes (Ruhrberg et al., 1996; Ruhrberg et al., 1997; DiColandrea et al., 2000). Periplakin has been shown to interact with vimentin and K18 (Choi et al., 2002; Karashima and Watt, 2002).

Epiplakin, which is widely expressed in epidermal keratinocytes, liver, and pancreas, interacts predominantly with keratin IFs (Fujiwara et al., 2001; Spazierer et al., 2003; Jang et al., 2005; Spazierer et al., 2006). Epiplakin has been proposed to act as a platform in signalling pathways, similar to plectin, but this has yet to be determined (Spazierer et al., 2006; Yoshida et al., 2008; Matsuo et al., 2011).

Plectin

Plectin is the best studied and most versatile member of the plakin family. Depending on the isoform, plectin has a molecular mass between 499-533 kDa (Foisner and Wiche, 1987; Rezniczek et al., 2003). Plectin isoforms are expressed in all mammalian cell types except for certain neurons (Wiche, 1998). Plectin displays a tripartite structure consisting of a central ~200 nm long rod domain flanked by two large globular domains (Fig. 4) (Wiche, 1998).

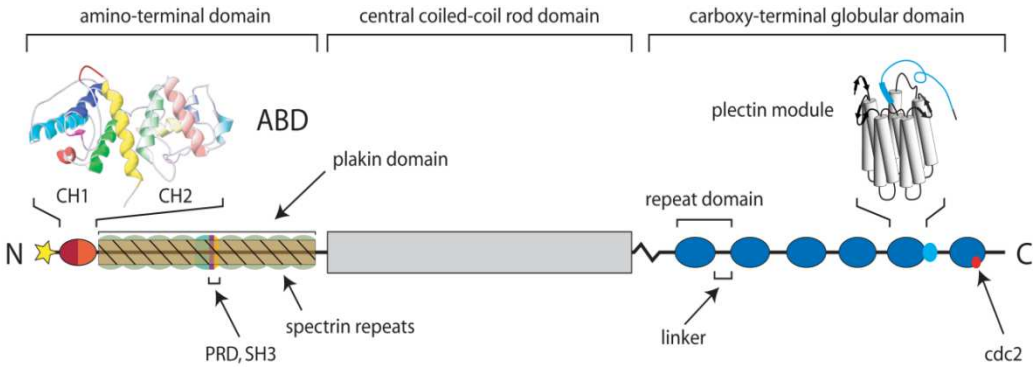

Figure 4: Schematic structure of plectin. Plectin displays a tripartite structure that consists of a central rod flanked by N-terminal and C-terminal globular domains. Differences in the N terminus, generated through alternative splicing, determine subcellular localization of plectin. The N-terminal part harbors an actin-binding domain (ABD, red) comprised of two calponin homology domains and a plakin domain (brown). The ABD mediates binding to ITGβ4, vimentin and the EF-ZZ domain of dystrophin and utrophin. Plectin isoforms with non coding first exons lack the first CH domain and thus binding to ITGβ4. The plakin domain contains nine plakin repeats (PRD) and a central SH3 domain. The C-terminal domain consists of six highly homologous plakin repeat domains that comprise plectin modules (blue ellipses) and a linker domain. The linker domain between plectin repeats 5 and 6 harbors an IF-binding site (small blue circle). In the sixth domain a unique protein kinase $p34^{cdc2}$ phosphorylation site is indicated. Modified from Rezniczek et al, Dermatol Clin, 2010.

The C-terminal globular (CG) domain comprises six highly homologous repeat regions (Janda et al., 2001). An IF-binding domain is located between repeat 5 and 6, and repeat 6 harbors a binding site for protein kinase $p34^{cdc2}$ (Foisner et al., 1996; Nikolic et al., 1996; Wiche, 1998). Additionally, the β4 subunit of integrins binds between repeat 2 and 6 of the CG domain (Rezniczek et al., 1998). The N-terminal domain harbours a plakin domain (Sonnenberg et al., 2007; Ortega et al., 2011) and an actin-binding domain (ABD) (Andrä et al., 1998), which is built up of two calponin homology domains (CH1 and CH2) (Sevcik et al., 2004). The N-terminal part of the molecule is required for its targeting to adhesion complexes at the membrane, such as desmosomes and HDs (Rezniczek et al., 1998).

Plectin is encoded by the PLEC gene that is located in humans on chromosome 8 (q24) (Liu et al., 1996) and on chromosome 15 in mouse (Fuchs et al., 1999). Several isoforms of plectin are generated through alternative splicing. Eleven exons (1-1j) are spliced into a common exon 2, three exons are spliced upstream of exon 1c (-1, 0a, 0) and two exons are optionally spliced within the ABD-enconding exons (2α, 3α) (Fuchs et al., 1999; Rezniczek et al., 2003). The resulting isoforms display characteristic expression patterns in different cells and tissues (Fuchs et al., 1999; Andra et al., 2003; Rezniczek et al., 2003; Rezniczek et al., 2007; Hijikata et al., 2008; Kostan et al., 2009; Burgstaller et al., 2010). P1a is expressed in epithelial tissues, such as skin, lung and small intestine (Fuchs et al., 1999; Andra et al., 2003). P1d is expressed in skeletal and heart muscle (Fuchs et al., 1999; Rezniczek et al., 2007; Konieczny and Wiche, 2008). P2α-containing isoforms were reported in skeletal and heart muscle as well as in brain, whereas P3α-containing isoforms were exclusively found in brain (Fuchs et al., 1999; Fuchs et al., 2005). Moreover, the different isoforms display specific subcellular localizations. P1a for example is targeted to HD-like structures in keratinocytes, P1b to mitochondria and P1f to mature focal adhesions (Andra et al., 2003; Rezniczek et al., 2003; Rezniczek et al., 2007; Winter et al., 2008; Burgstaller et al., 2010; Wiche and Winter, 2011).

Plectin contains multiple interaction domains for various cytoskeletal proteins enabling it to bind to and interlink all of the cytoskeletal filament networks. With its IF-binding domain at the C terminus, plectin interacts with vimentin, GFAP, cytokeratins, the neurofilament triplet proteins, desmin and lamin B

(Pytela and Wiche, 1980; Foisner et al., 1988; Foisner et al., 1991; Tian et al., 2006). Binding to IF is tightly regulated by phosphorylation of plectin involving protein kinases such as cAMP-dependent protein kinase A (PKA), Ca^{2+}/phosphatidyl-dependent kinase C (PKC) (Foisner et al., 1991) and M-phase activated protein kinase $p34^{cdc2}$ (Foisner et al., 1996). Furthermore, plectin can interact with MAPs and was postulated to interlink the MT system with the IF network (Herrmann and Wiche, 1987; Foisner et al., 1995). The highly conserved ABD at the N- terminus allows binding to actin filaments (Andrä et al., 1998; Fontao et al., 2001). In this context, plectin has been shown to interact preferentially with fibronectin fibril-aligned fibrillar adhesions and with actin stress- fibers in focal contacts (Burgstaller et al., 2010). Moreover, plectin associates with fodrin and α-spectrin (Herrmann and Wiche, 1987), proteins of the cortical cytoskeleton that may play a major role in maintaining cell polarity as shown for fibroblasts (Burgstaller et al., 2010). Finally, plectin was shown to interact with the desmosomal protein desmoplakin (Eger et al., 1997). Likewise, it connects HDs to the keratin-IF network as it directly binds to ITGβ4 via the ABD and the plakin domain of P1a (Rezniczek et al., 1998; Kostan et al., 2009). As a major structural component of HDs, plectin mechanically stabilizes basal epidermal keratinocytes (Andrä et al., 1997; Ackerl et al., 2007). Furthermore plectin is crucial for the regulation of actin stress fiber dynamics (Andra et al., 1998), and also acts as docking platform for signalling complexes in several cell types (Lunter and Wiche, 2002; Osmanagic-Myers and Wiche, 2004; Gregor et al., 2006; Takawira et al., 2011). In muscle several isoforms of plectin are required to maintain the integrity of myofibrils (Andrä et al., 1997). P1f anchors desmin-IFs to the dystroglycan complex (DGC) of the membrane and P1d attaches desmin-IFs to the Z-disks of the sarcomere (Rezniczek et al., 2007; Konieczny et al., 2008, Winter et al., 2008). In addition, P1 links desmin-IFs with the nuclear membrane and the ER and P1b connects the sarcomere with mitochondria (Konieczny et al., 2008, Winter et al., 2008).

This work is focused on P1a, the plectin isoform predominantly expressed in skin. Therefore, what follows is an introduction into the biology of the skin and its substructures.

Biology of the Skin

The skin functions as a barrier between the organism and the environment. It protects the organism from external factors such as microbes, harmful chemical substances and UV-radiation, regulates temperature of the body and controls loss of water to the environment. Three major tissue layers, the subcutis, the dermis and the epidermis, built up the skin and enable its vital function (Fig. 5) (Schiebler and Korf, 2007).

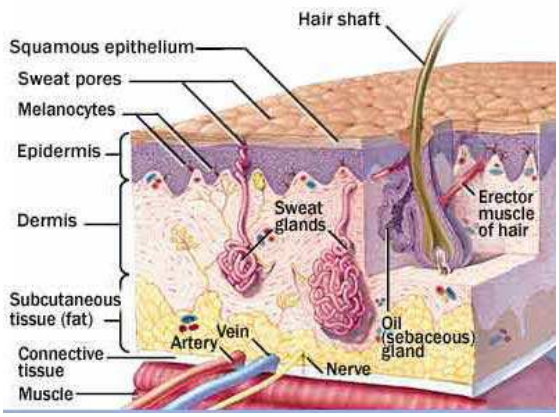

Figure 5: Tissues of the skin. The skin is composed of three tissue layers. The subcutis consists of collagens and fat cells. It harbors blood vessels and neurons and regulates body temperature. The major components of the dermis are fibroblasts and collagen bundles. In addition, blood and lymph vessels transport nutrients to the epidermis. Hair follicles, lymph vessels, sensory axons and sweat glands are also found in the dermis. The epidermis is the outermost layer of the skin and consists of keratinocytes, melanocytes, Langerhans cells and Merkel cells. Source: http://www.mayoclinic.com/health/medical/IM00941

The deepest layer of the skin, the subcutis, consists of a dense network of collagens and fat cells. It conserves body heat and contributes to wound healing of the skin (Schiebler and Korf, 2007). The dermis lies in between the subcutis and the epidermis and contains blood and lymph vessels, hair follicles, sweat glands, collagen bundles, fibroblasts and nerve endings (mechano- und thermoreceptors) (Schiebler and Korf, 2007). Through the dermis nutrients are supplied to the epidermis and elasticity is provided to the skin. Nerve cell endings in the stratum papillare of the dermis mediate sensory functions.

The outermost skin layer, the epidermis, is subdivided into distinct zones depending on the differentiation state of the keratinocytes (Fig. 6), the major cell type of the epidermis (Schiebler and Korf, 2007). The outermost zone, the stratum corneum is characterized by flat anucleated keratinocytes adjacent to a nucleated epidermis consisting of stratum granulosum, stratum spinosum and stratum basale. The stratum basale is the basal keratinocyte cell layer of the epidermis that is

in contact with the basal lamina. It contains a population of epidermal stem cells. Other types of cells found within the stratum basale are melanocytes, Langerhans cells, and Merkel cells (Habif, 2010). The stratum corneum is the principal barrier against percutaneous penetration of chemicals and microbes and withstands mechanical forces. Furthermore, it regulates the trans-epidermal water loss (TEWL) (Madison, 2003). The stratum corneum is composed of corneocytes and a hydrophobic intercellular matrix consisting of lamellar lipid layers (Candi et al., 2005). Corneocytes represent terminally differentiated keratinocytes which are organised in condensed layers of flattened cells through keratin- filament- bundling proteins such as filaggrin (Candi et al., 2005). Dysfuction of filaggrin leads to impaired epidermal barrier homeostasis (Presland et al., 2000; Presland et al., 2004; Palmer et al., 2006; Smith et al., 2006). Corneocytes are characterized by the presence of the cornified envelope, which replaces the plasma membrane of differentiated keratinocytes. It consists of keratins that are enclosed within an insoluble amalgam of proteins, which are cross-linked by transglutaminases and surrounded by a lipid envelope (Candi et al., 2005). Together, keratins and filaggrin constitute 80-90% of the protein mass of the epidermis (Candi et al., 2005). The cornified envelope proteins, including involucrin, loricrin and trichohyalin, are crucial for TEWL and barrier repair (Nemes and Steinert, 1999; Egberts et al., 2004; Candi et al., 2005; Elias et al., 2010).

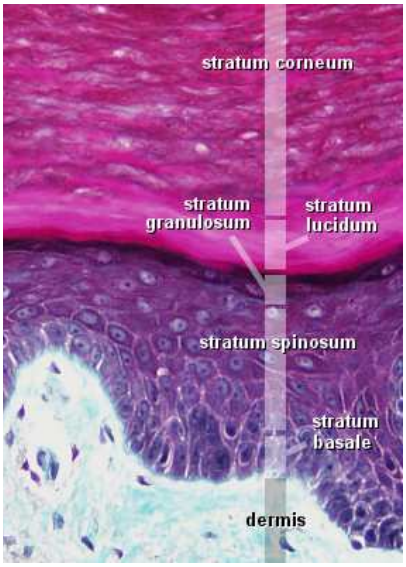

Figure 6: Structure of the epidermis. The epidermis is build up from keratinocytes. The stratum basale is a single keratinocyte cell layer that is in contact with the basal lamina and contains a population of stem cells. The basal lamina separates the epidermis from the underlying dermis. Differentiating keratinocytes migrate up towards the epidermal surface, thereby forming the stratum spinosum. In the stratum spinosum the cells are attached to each other via desmosomes. The stratum granulosum consists of several layers of flattened keratinocytes, which contain lamellar granules. These granules consist of proteins and lipids that are crucial for the barrier function of the skin. The stratum lucidum is exclusively found in thick skin and is composed of several layers of flattened dead cells. The outermost layer of the epidermis is the stratum corneum. The cells of the stratum corneum are completely filled with keratin filaments which are embedded in a dense intercellular matrix of lipids.
Source: http://www.lab.anhb.uwa.edu.au/mb140/corepages/integumentary/integum.htm

The nucleated epidermis is also significant for barrier function as it assists in preventing extensive water loss by forming a second barrier against harmful substances. Several substructures that stabilize or interconnect keratinocytes are crucial for the barrier function.

Keratins form the IF network of the epidermis and thus are the major structural proteins expressed in keratinocytes (Alonso and Fuchs, 2003; Gu and Coulombe, 2007; Uitto et al., 2007). They span the whole cytoplasm and terminate at desmosomes and HDs (Gu and Coulombe, 2007; Uitto et al., 2007; Coulombe et al., 2009). Keratins play a major role for mechanical stability of keratinocytes (Gu and Coulombe, 2007; Coulombe et al., 2009). A unique set of keratins is expressed in keratinocytes depending on the skin- type and differentiation state of the skin (Fig. 7).

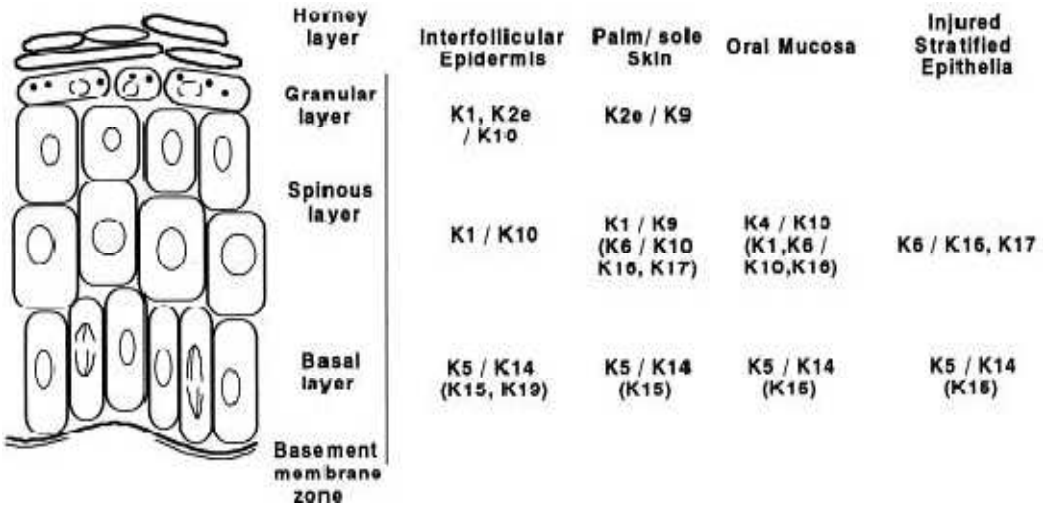

Figure 7: Tissue-specific expression of keratins. In proliferating basal keratinocytes K5 and K14 are the major IF components. Upon terminal differentiation expression of K 5 and K14 is downregulated and K1 and K10 are upregulated. K2e expression is restricted to the spinous and granular layer of the epidermis. K9 is found exclusively in the granular layer of thick skin (palm or soles), whereas K4 and K13 are restricted to the spinous layer of oral mucosa. K6, K16 and K17 are wound inducible genes that are expressed during inflammation or wound healing.

K5 and K14 are expressed in basal keratinocytes attached to the basal membrane, whereas K1 and K10 are exclusively expressed in differentiating cells (Coulombe et al., 2009; Alonso and Fuchs, 2003). K6, K16 and K17 mark hyper-proliferating keratinocytes upon inflammation or wound healing (Uitto et al., 2007; Gu and Coulombe, 2008). Additionally, K9 is exclusively expressed in the palmoplantar suprabasal keratinocyte layers and K2e is expressed in the upper spinous and

granular cells of adult epidermis (Candi et al., 2005; Gu and Coulombe, 2009). Defects in the keratin network give rise to trauma-induced skin blistering diseases, and in some cases also result in impaired barrier repair, and extensive water loss (Gu and Coulombe, 2007; Schweizer et al., 2007; Coulombe et al., 2009; McLean and Moore, 2011).

Tight junctions are cell-cell connections that control paracellular transport of molecules and separate the apical and basolateral domains of epithelial cells (Brandner et al., 2006; Kirschner et al., 2010). Occludin, claudin, JAM-1, zonula occludens protein 1 and MUPP-1 represent tight junction proteins in the nucleated epidermis (Kirschner et al., 2010; Brandner, 2006). The synthesis of these proteins precedes formation of the stratum corneum and knockout of tight junction proteins is lethal due to extensive water loss. Consequently, these proteins were proposed to function as a rescue system in case of an impaired stratum corneum and assist in basic barrier function (Ohnemus et al., 2008; Kirschner et al., 2010).

Adherens junctions and desmosomes play an important role in barrier function as they provide stable intercellular adhesion (Lai-Cheong et al., 2007; Niessen, 2007; Jonca et al., 2011). Consequently, their dysfunction compromises epidermal barrier function and tissue integrity (Ishii and Green, 2001; Lai-Cheong et al., 2007).

Gap junctions control the communication between cells and are expressed under hyper-proliferative conditions such as occurring during wound healing (Coutinho et al., 2003). Mutations in gap junction proteins have also been implicated in epidermal barrier diseases (Lai-Cheong et al., 2007).

Additional, very important junctional complexes of skin cells are the HDs. As they play a major role in this thesis their structure and function will be discussed in more detail below.

Hemidesmosomes (HDs) and hemidesmosome-enriched protein complexes (HPCs)

HDs are trans- membrane adhesion complexes that mediate stable attachment of basal keratinocytes to the underlying basal lamina through connecting keratin IFs to the extracellular matrix (Nievers et al., 1999; Pulkkinen et al., 1999; Litjens et

al., 2006; Margadant et al., 2008). They are mainly expressed in epithelial tissues exposed to mechanical stress such as the epidermis. Furthermore, HDs are involved in wound healing, carcinoma invasion and were recently proposed to function as signalling platform (Mercurio et al., 2001; Lipscomb and Mercurio, 2005). The hemidesmosomal attachment apparatus (Fig. 8) consists of a set of unique proteins. The transmembrane laminin receptor ITGα6β4 connects the basal membrane to the ECM through direct interaction with laminin322. The cytolinker proteins plectin and BPAG1e interlink the basal membrane with the keratin IF network of basal keratinocytes (K5 and K14) through direct binding to ITGβ4 (Rezniczek et al., 1998; de Pereda et al., 2009). The association of lamin-322 with ITGα6β4 is crucial for the formation of HDs (Koster et al., 2004), whereas the mechanical stability is contributed through the elastic properties of the rod like structure of the plakin domains within plectin and BPAG1e (de Pereda et al., 2009).

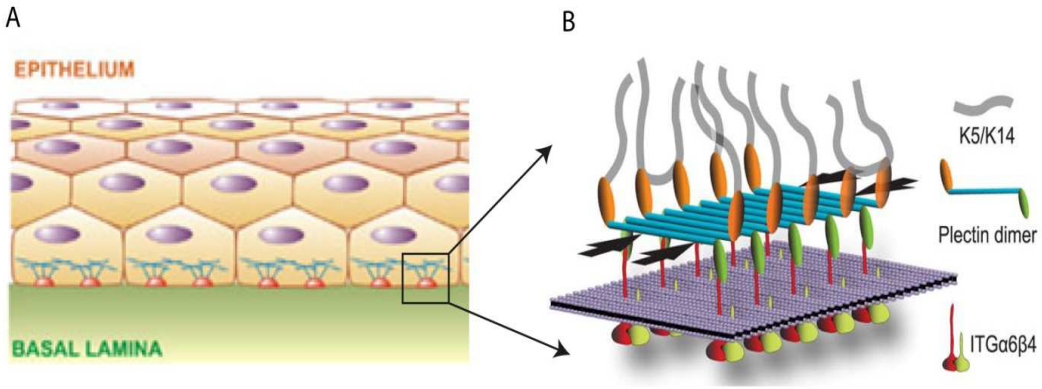

Figure 8: Tissue localization and protein composition of HDs. (A) HDs link the basal epithelial cell layer to the basal membrane. (B) A HD is a multiprotein complex, which core proteins are α6β4 integrin receptor and the cytolinker protein plectin. The K5/K14 filaments are linked to laminin-322, a component of the extracellular matrix. The integrity of HDs depends on a vertical force component which is comprised of P1a dimer interaction with ITGb4 and K5/K14. In addition, the lateral association of multiple P1a dimers is likely to generate a horizontal force component (arrows), parallel to the membrane (violet sheet), which supports the stability of HDs.
Modified from: Alberts, Molecular Biology of the cell, 5th edition and Walko et al., 2011.

Although HDs provide mechanical stability they appear also as dynamic structures at least when visualized in cultured keratinocytes. Most cultured cells fail to assemble tissue-like HDs *ex vivo* but cultured keratinocytes possess hemidesmosome-enriched protein complexes (HPCs) (Carter et al., 1990; Ozawa et al., 2010). HPCs show dynamic properties during cell migration and cell division (Geuijen and Sonnenberg, 2002; Tsuruta et al., 2003; Ozawa et al., 2010). HPCs,

enriched in ITGβ4 and BP180, have been shown to be assembled and disassembled in a relatively short time frame, and to move in the plane of the membrane (Geuijen and Sonnenberg, 2002).

The turnover of HPCs is tightly regulated through posttranslational modifications of HD proteins (Santoro et al., 2003; Rabinovitz et al., 2004; Wilhelmsen et al., 2007; Kariya and Gu, 2011; Mizutani et al., 2011; Yu et al., 2012). A key step in HD assembly and disassembly is the regulation of the plectin-ITGα6β4 association (Santoro et al, 2003; Rabinovitz et al., 2004; Wilhelmsen et al., 2007). Plectin targeting to developing HDs requires binding to ITGα6β4 (Fig. 8B). This binding is mediated through the ABD and plakin domain of P1a with the FnIII-1/2-domain of the ITGβ4 subunit. It is weakened through phosphorylation of the cytoplasmic CS domain of the ITGβ4 subunit (Rezniczek et al., 1998; de Pereda et al., 2009; Kostan et al., 2009; Koster et al., 2004). The Ca^{2+}-binding protein calmodulin can compete with the ITGβ4 subunit for association with the ABD of P1a. This process has been implicated in the initial stages of HPC disassembly during Ca2+-induced keratinocyte differentiation (Kostan et al., 2009). Furthermore, it has been shown in *C.elegans* that minor fluctuations in the quantity of HD proteins greatly affect the stability of HDs (Zahreddine et al., 2010). Taken together regulatory processes such as posttranslational modifications and regulation of HD components, but also competition events and cellular trafficking are required to fine tune the turnover of HDs and HPCs (Zhang and Labouesse, 2010).

Keratinocyte stem cells and epidermal homeostasis

Mammalian epidermis consists of a multilayered sheet of keratinocytes, interspersed with hair follicles, sebaceous glands, and sweat glands (Fuchs, 2007; Jones et al., 2007; Blanpain and Fuchs, 2009; Fuchs, 2009). All compartments of the skin are turned over throughout adult life. In the interfollicular epidermis (IFE), proliferation is confined to keratinocyte cells in the basal cell layer that adhere to the underlying basement membrane. On commitment to terminal differentiation, basal keratinocytes lose their attachment to the basement membrane and move into the suprabasal cell layers, where they reach their final stage of differen-

tiation as anucleated corneocytes, which are ultimately shed from the cell surface (Jones et al., 2007). This requires the migration of differentiating keratinocytes from the basal layers to the stratum corneum. As a consequence of the constant renewal of the epidermis, there is a continual requirement for proliferating cells to replace cells lost by terminal differentiation (Jones et al., 2007).

Stem cells are defined as individual cells that retain a high capacity of self renewal throughout adult life and are able to produce daughter cells committed to terminal differentiation (Lajtha, 1979). In the epidermis, stem cells have been located in the sebaceous glands, the hair follicle bulge and the inter-follicular epidermis (IFE) (Watt et al., 2006; Jones et al., 2007; Fuchs, 2008; Blanpain and Fuchs, 2009; Fuchs, 2009). In undamaged epidermis hair follicles, IFE, and sebaceous glands are each thought to be maintained by their own discrete stem cell population (Jones et al., 2007; Blanpain and Fuchs, 2009). However, under some circumstances, such as wound healing, any of the three stem cell populations is capable of producing any of the differentiated lineages of the epidermis (Owens and Watt, 2003; Jones et al., 2007). Therefore, it seems likely that lineage selection of the different stem cell progeny is determined by local environmental cues and that lineage plasticity is a response to alterations in environmental signalling after injury, transplantation, or genetic manipulation (Owens and Watt, 2003; Jones et al., 2007).

Several models for epidermal homeostasis have been developed throughout the last decade. The earliest model, the “epidermal proliferative unit” (EPU) model, was based on the existence of transit amplifying (TA) cells. TA cells are a stem cell progeny that are destined to undergo terminal differentiation; however, after leaving the basal lamina the cells undergo a limited number of rounds of divisions before differentiation is initiated (Potten, 1981). The EPU model was based on the observation that in some areas of mouse IFE the cells are arranged in columns with a single cornified cell on top (Mackenzie, 1970; Jones et al., 2007). These columns are attached to each other in a regular array and are based on one single slowly cycling stem cell at the centre of the basal layer that maintains the surrounding TA and differentiating cells (Fig. 9) (Potten and Allen, 1975).

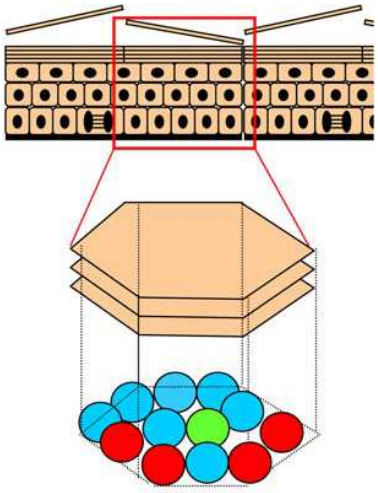

Figure 9: The epidermal proliferative unit (EPU) model as described by Potten (Potten, 1983). The epidermis is organized in stacks (inset) of cornified keratinocytes which are maintained by the basal cells underlying it. A single stem cell (green) produces committed transit amplyfing cells (blue) that undergo several rounds of cell division before entering terminal differentiation (red). The differentiating cells leave the basal cell layer and migrate vertically towards the cell surface through the suprabasl cell layers. Adopted from Jones et al., 2007.

A more recent model of epidermal homeostasis now proposes a single population of dividing cells in the basal cell layer that maintains IFE (Clayton et al., 2007; Jones et al., 2007). In this model, the two daughter cell clones produced by these dividing cells can adopt three different types of fate: i) both cells can remain highly proliferative, ii) both can terminally differentiate, iii) one cell remains proliferative while the other is committed to terminal differentiation (Fig. 10) (Clayton et al., 2007; Jones et al., 2007).

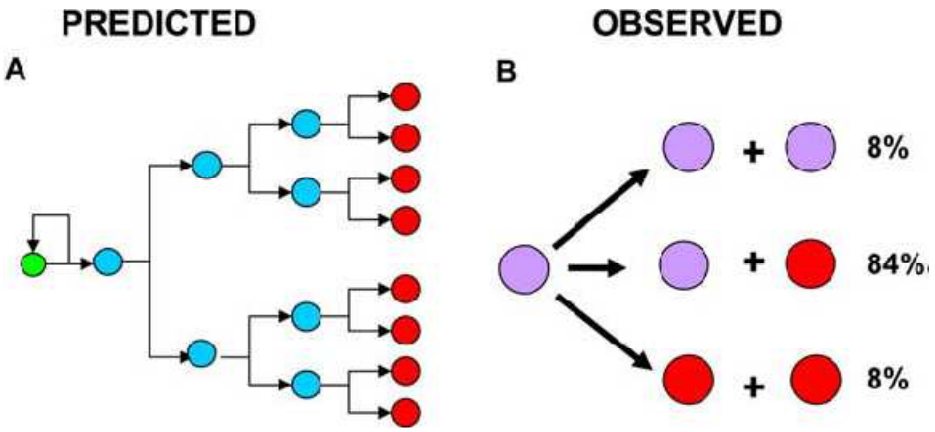

Figure 10: A novel model for epidermal homeostasis. (A) Determined cell fate as proposed in the EPU model. (B) A new empirical quantitative model based on clonal labeling data (Clayton, 2007). Proliferative basal keratinocytes (purple) may produce daughter cells with three different fates: both cells may remain proliferative, both may terminally differentiate (red), or one may remain proliferative and one may become postmitotic. The possibilities of the three types of fates are given in percentages. The choice of fate is a stochastic process and not determined by the number of proliferation rounds.
Adopted from: (Jones and Watt, Cell Stem Cell, 2007)

The clone fate simply depends upon the average cell division rate and the proportion of divisions that result in asymmetric fate (Clayton et al., 2007). To guarantee that the steady-state population of proliferating basal cells is maintained at a con-

stant level, the proportion of cells that create daughter cells with each type of symmetric fate must be equal (Clayton et al., 2007; Jones et al., 2007). This model is however incompatible with the existence of TA cells (Clayton et al., 2007).

Asymetric division is a proposed mechanism of keeping the balance between proliferating and differentiating cells (Fig. 11) (Hall and Watt, 1989; Watt and Hogan, 2000; Blanpain and Fuchs, 2009). The orientation of the mitotic spindle at a right angle to the basal membrane leads to the formation of one proliferating and one differentiating daughter cell. This model was observed in adult human oesophageal epithelium and in the developing mouse epidermis but not in adult epidermis (Koster and Roop, 2005; Clayton et al., 2007; Blanpain and Fuchs, 2009). Another proposed mechanism is populational asymmetry, where the rates of two daughter cells which remain highly proliferative and two daughter cells which differentiate are equal (Hall and Watt, 1989; Watt and Hogan, 2000; Jones et al., 2007).

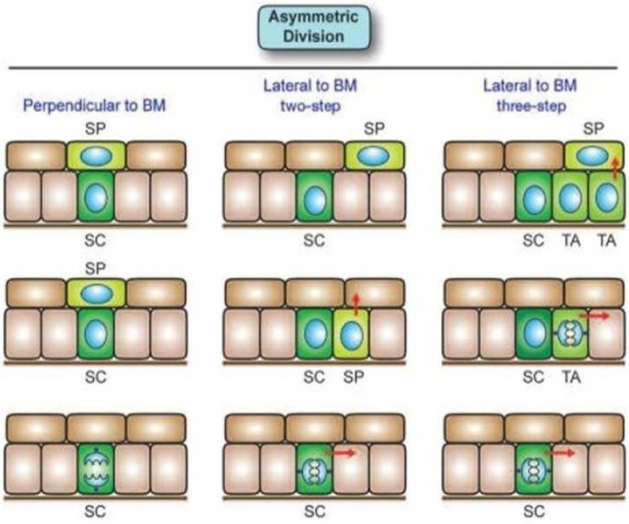

Figure 11: Three models of epidermal homeostasis based on asymetric stem cell division. Organization of the mitotic spindle perpendicular to the basal membrane (BM) leads to a daughter cell that is committed to become a terminally differentiating spinous cell (SP) and a stem cell (SC) that retains the ability of self renewal. Mitotic spindles that are oriented laterally to the BM result in a daughter cell that delaminates and migrates into the suprabasal layer. Another possibility is the generation of transit amplifying (TA) cells that undergo some cell divisions before they differentiate and thus leave the proliferative niche. Adopted from (Fuchs and Novak, 2008).

Under certain conditions the balance between proliferating and differentiating is required to shift. During wound healing the balance has to shift towards proliferating cells. Three different mechanisms have been described for this process: First, stem cells that are quiescent are mobilized to move into the suprabasal layers (Ghazizadeh and Taichman, 2001; Claudinot et al., 2005; Ito et al., 2005; Levy et

al., 2005), second, the proportion of daughter cells that become two proliferating cells is increased, and third the differentiation of committed cells is reversed to generate proliferative cells (Fuller and Spradling, 2007).

The next section is focused on the skin blistering disease Epidermolysis bullosa simplex (EBS) and its subtype EBS-Ogna which is caused by a mutation in the gene coding for P1a.

Epidermolysis Bullosa Simplex

Epidermolysis bullosa (EB) defines a group of rare genetic diseases in which bullous lesions (fluid filled cavities and large blisters) occur in the skin after exposure to mechanical stress (reviewed in Coulombe et al., 2009). Three major forms of EB have been defined using clinical and histological criteria. The dystrophic, junctional and simplex forms of EB are characterized by loss of tissue integrity in the upper dermis, the dermo-epidermal interface, and within the epidermis, respectively (Fine et al., 2000; Sawamura et al., 2010).

This work focuses on the epidermally manifested type of epidermolysis bullosa simplex (EBS). EBS is a clinically and genetically heterogeneous skin disease that affects the basal keratinocyte layer of the epidermis and is characterized by rupture of basal keratinocytes upon exposure to mechanical stress (Coulombe et al., 2009; Sprecher, 2010). With rare exceptions, EBS is inherited in an autosomal dominant fashion (Coulombe et al., 2009; Sprecher, 2010). Several forms of EBS can be distinguished depending on the localization of the blisters as well as on the presence of additional phenotypes. In EBS-generalized (also called Koebner) the whole body is affected by blistering, in the mildest EBS-localized (also called Weber-Cockayne) the blistering occurs in peripheral parts of the body such as hands or feet, and in the severe EBS Dowling-Meara blisters occur on the whole body but in a characteristically clustered pattern (Coulombe et al., 2009). Other forms of EBS are less frequent. EBS-mottled pigmentation (EBS-MP) is characterized by abnormal skin pigmentation, EBS-muscular dystrophy (MD) displays a progressive limb-girdle type of muscular dystrophy, EBS-pyloric atresia (PA) shows gastric abnormalities, and EBS-Ogna exclusively manifests as

skin blistering (Koss-Harnes et al., 2002; Fine et al., 2008; Coulombe et al., 2009; Chiaverini et al., 2010; Sprecher, 2010).

Most forms of EBS result from mutations in the genes encoding K5 or K14 (Coulombe et al., 2009; Sprecher, 2010). These proteins form the heterodimeric substructure of the IF network in keratinocytes (Fuchs et al., 1981; Fuchs, 1995). Thirty six percent of all keratin disease-associated mutations are located within the genes of K5 and K14 (Szeverenyi et al., 2008). Furthermore, some types of EBS are caused by mutations in the genes coding for plectin (EBS-MD, EBS-PA, EBS-Ogna, EBS-limb girdle) and ITGα6β4 (Fine et al., 2008). With the help of transgenic mouse models it was shown that mutations in the K14 rod domain lead to the formation of keratin-positive aggregates of amorphous proteins in the cytoplasm, similar aggregates have been observed in the most severe forms of EBS (Coulombe et al., 1991; Ishida-Yamamoto et al., 1991; Vassar et al., 1991). The symptoms of EBS diseases became apparent shortly after birth due to the embryonic onset of K5/ K14 gene expression (Kopan and Fuchs, 1989; Byrne et al., 1994).

In vitro, EBS-causing mutations in K5 and K14 render individual IFs in solutions less elastic and more prone to breakage upon exposure to shear stress as tested with rheological methods (Ma et al., 2001). These alterations became even more pronounced when the filaments are incorporated into filament networks under in vitro conditions promoting cross bridging (Ma et al., 2001). In addition, the amorphous keratin-positive protein aggregates add to the severity of EBS. The misfolded protein response is activated upon aggregation of mutated keratin but if it fails to eliminate the aggregates the protein homeostasis of the cell is imbalanced leading to elevated cellular stress (Ku and Omary, 2000; Janig et al., 2005). Finally, also inflammation processes were proposed to play a role in EBS pathophysiology (Lugassy et al., 2008; Coulombe et al., 2009).

Epidermolysis Bullosa Simplex Ogna

This rare type of EBS (OMIM:131950) manifests as generalized skin fragility and was first identified in 1973 in a Norwegian family living near the small town of Ogna (Gedde-Dahl, 1971; Koss-Harnes et al., 2002). Since then the disease has

also been described in a German familiy as a result of a de novo mutation (Koss-Harnes et al., 2002). The underlying mutation was mapped to the human PLEC gene on chromosome 8q24 (Liu et al., 1996; Koss-Harnes et al., 2002). Most mutations in the plectin gene are inherited in an autosomal-recessive fashion resulting in EBS-MD (OMIM:226670), EBS-PA (OMIM:612138), and EBS-CMS (EBS with congenital myasthenia (Maselli et al., 2010). In contrast, EBS-Ogna (OMIM:131950) is caused by an autosomal dominant mutation. This heterozygous mutation is located in the amino acid sequence encoding for plectin's rod domain. A C to T transition within exon 31 leads to the substitution of an arginin by a tryptophan (p.Arg2000Trp) (Koss-Harnes et al., 2002).

The EBS-Ogna phenotype manifests with a generalized bruising tendency and blistering of the skin, predominantly on hands and feet. In contrast to keratin mutation-associated forms of EBS the blisters originate in the deepest areas of the basal cell cytoplasm immediately above the HDs (Koss-Harnes et al., 2002; Walko et al., 2011). The keratin network appears unaffected but their insertion into the HD attachment plates is mostly impaired (Koss-Harnes et al., 2002; Walko et al., 2011). In a recently generated mouse model of EBS-Ogna, HD numbers were found to be greatly reduced (Walko et al., 2011). The HD structure appears unaltered in EBS-Ogna skin with the exception of the intracellular IF attachment plate which is generally thinner in EBS-Ogna skin (Koss-Harnes et al., 2002). An important diagnostic feature of EBS-Ogna is that the basal keratinocyte cell layer is lacking anti-plectin immunoreactivity (Koss-Harnes et al., 1997; Walko et al., 2011). As recently shown the HD-associated P1a is dramatically diminished in the epidermis of mice carrying the EBS-Ogna mutation. In these mice, P1a but not isoform P1c was found to be downregulated (Fig. 12), although the expression of mRNA transcripts remained unaltered (Walko et al., 2011). Furthermore, it was shown that the EBS-Ogna mutation sensitized plectin to degradation by epidermis-specific proteases. As EBS-Ogna manifests exclusively in skin without muscular phenotype, in contrast to EBS-MD and EBS-PA, it can be considered as a HD-specific disease (Koss-Harnes et al., 2002; Chiaverini et al., 2010; Natsuga et al., 2010; Walko et al., 2011).

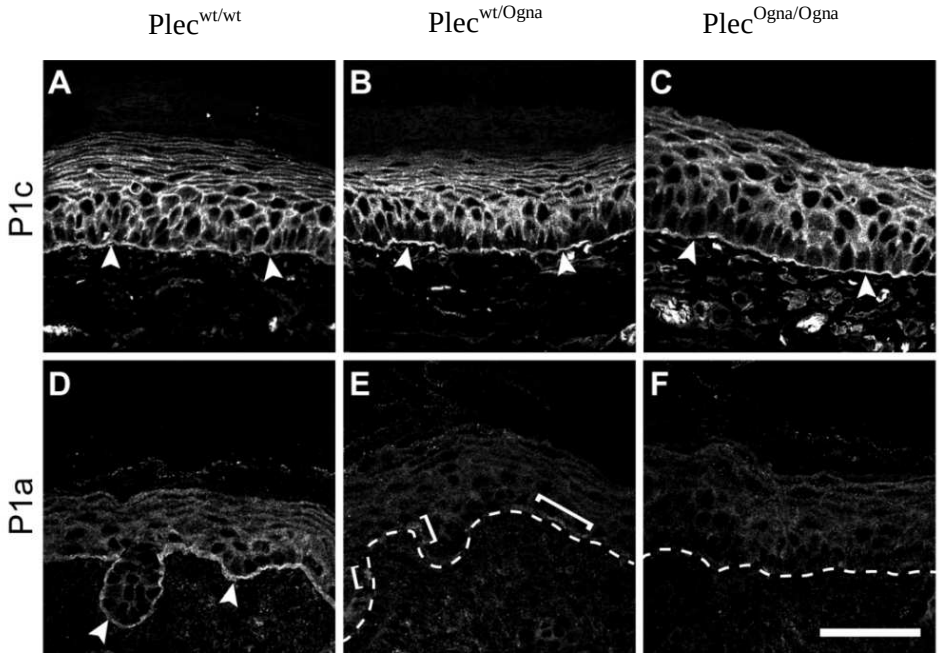

Figure 12: Immunofluorescence microscopy of P1a and P1c of frozen leg skin sections from 1-day-old wild-type and mutant mice. Note that expression and localization of P1c is not altered between any of the three genotypes (D–F). P1a is predominantly expressed at the basal cell membrane of basal keratinocytes in *Plec*$^{wt/wt}$ skin (D, arrowheads), but is diminished in a dose dependent manner in mutant epidermis, except for a few P1a-positive patches (E, brackets) remaining in *Plec*$^{Ogna/+}$ skin. Dashed lines represent the dermo-epidermal border. Scalebar is 50 µm (modified from Walko et al., 2011).

Calpains and their potential role in skin biology

Calpains are neutral cysteine proteases that are activated through calcium and membrane phospholipids (Croall and DeMartino, 1991; Saido et al., 1991; Sorimachi et al., 2010; Storr et al., 2011). These proteases are heterodimers comprising a large catalytic and a small regulatory subunit and they display homology to calmodulin (Saido et al., 1994; Sorimachi et al., 1997; Sorimachi et al., 2010). Calpain I (µ-calpain) and II (m-calpain), which are activated by micro- and millimolar levels of calcium, respectively, are ubiquitously expressed (Ohno et al., 1984; Aoki et al., 1986; Croall and Ersfeld, 2007; Sorimachi et al., 2010). Structurally calpain I and calpain II differ in their large ~80 kDa catalytic subunits (which consist of the four subdomains, DI-IV) but share a common ~30 kDa regulatory subunits (containing two subdomains, DV and VI). DIV of the large subunit harbours an EF-hand structure which interacts with the EF-hand structure of DVI within the small subunit (CPSN). This interaction results in the formation

of the heterodimeric calpains I and II (Croall and DeMartino, 1991; Hosfield et al., 1999; Sorimachi et al., 2010). In contrast, the expression of other calpain isoforms, e.g. calpain 3 (Cpn3) and calpain 8/nCL-2, is restricted to specific tissues, such as skeletal muscle and stomach (Sorimachi et al., 1993a; Sorimachi et al., 1993b). During the past years new insights have been gained into the multi-step activation mechansism of calpains (Croall and DeMartino, 1991; Elce et al., 1997; Cottin et al., 2001; Croall and Ersfeld, 2007). In the cell, calpains are mainly located in the cytosol where they exist as inactive enzymes. They translocate to the membrane in response to increases in extracellular Ca^{2+} levels. Thus, the small amount of calpains cofractionating with membranes might represent the physiologically active enzyme fraction. Biochemical studies have revealed that in a first step of activation, constraints imposed by domain interactions on the conserved DII domain are released by Ca^{2+}-binding leading to the dissociation of the 30 kDa regulatory subunit from the catalytic 80 kDa subunit. In a second step, two Ca^{2+} ions bind to the core protease domain and cause a rearrangement of the active site cleft of DII (Nakagawa et al., 2001; Reverter et al., 2001; Moldoveanu et al., 2002). Furthermore, several mechanisms can promote calpain activation by reducing Ca^{2+} requirements, including phospholipids present at the plasma membrane, and protein kinase C (PKC) or Erk-mediated phosphorylation (Coolican and Hathaway, 1984; Imajoh et al., 1986; Saido et al., 1991; Saido et al., 1992; Melloni et al., 1996; Glading et al., 2001; Shiraha et al., 2002; Xu and Deng, 2004; Xu and Deng, 2006; Leloup et al., 2010). As a consequence of calpain activation by Ca^{2+} the amino-terminal DI domain of the catalytic subunit becomes autoproteolytically cleaved (Suzuki et al., 1981; Cong et al., 1989; Baki et al., 1996; Lametsch et al., 2008). However, calpains can also be activated without undergoing autoproteolytic cleavage (Cong et al., 1989; Molinari et al., 1994; Guttmann et al., 1997). It seems therefore that autoproteolytic cleavage of calpains is indicative of sustained strong activation of calpains (Cong et al., 1989; Baki et al., 1996; Chou et al., 2011). Calpains-I and II are associated with their endogenous inhibitor calpastatin and are released upon phosphorylation of calpastatin and calpains (Melloni et al., 2006). However, the precise mechanism of how calpastatin regulates calpains remains unclear. Calpains are involved in a number of processes, such as integrin-mediated signal transduction (Inomata et al., 1996),

actin-remodelling during cell spreading (Potter et al., 1998), embryonic development (Emori and Saigo, 1994) and apoptosis (Squier et al., 1994).

In skin, calcium regulates the terminal differentiation of keratinocytes and thus homeostasis of the epidermis (Bikle et al., 2004; Tu et al., 2004). Consistent with this, calpains are expressed in the epidermis. Calpain I is expressed in its 80 kDa form, but also in its activated 78 and/or 76 kDa form in all epidermal layers of neonatal and adult human skin (Michel et al., 1999). Calpain I is expressed very early during human development (54 days of estimated gestational age) in the basal layer and the periderm of the embryo (Michel et al., 1999). Cultured keratinocytes from neonatal human foreskin epidermis also display calpain I activation upon transition from subconfluent to postconfluent culture (Michel et al., 1999). In the granular cell layer of the epidermis calpain I is required for the processing of profilaggrin to filaggrin (Resing et al., 1993; Hsu et al., 2011). Interestingly, all components of the calpain system (calpains I and II, and calpastatin) are upregulated during differentiation of keratinocytes ex vivo (Garach-Jehoshua et al., 1998). Moreover, calpain II was shown to be involved in EGF-induced migration of human keratinocytes downstream of Mek1/2 (Satish et al., 2004).

The role of calpains in keratinocyte differentiation has not been defined to date. Calpains could be involved in keratinocyte differentiation through integrin proteolysis (Potts et al., 1994; Tennenbaum et al., 1996), cleavage of cell-cell adhesion molecules such as cadherins (Sato et al., 1995), degradation of the EGF receptor (King and Gates, 1985), downregulation of protein kinase C (Croall and DeMartino, 1991) and filaggrin processing (Resing et al., 1993).

Aims of the thesis

The goal of this thesis was to investigate whether calpains are involved in the turnover of hemidesmosome-enriched protein complexes (HPC). To address this issue, first it had to be assessed whether calpains are active in cultured basal keratinocytes and where they are located. Once these questions were solved, the next goal was to interlink the activation of calpains with HPC turnover at the onset of keratinocyte differentiation. An additional focus was set on the role of calpains in the pathology of the skin blistering disease EBS-Ogna which is characterized by impaired HD formation. Finally, an immortalized keratinocyte cell line carrying the EBS-Ogna mutation should be established in order to study the effects of impaired HPC formation on cell proliferation and signalling.

RESULTS

1 Involvement of Calpains in HD homeostasis assessed in cultured keratinocytes

1.1 Analysis of calpains activity in mouse keratinocytes under normal growth conditions

Calpains were demonstrated to be expressed in normal human foetal as well as adult epidermis, and to be activated during differentiation of human primary keratinocyte cell cultures and HaCaT cell lines (Michel et al., 1999; Garach-Jehoshua et al., 1998). However, in mouse keratinocytes calpains have not been studied so far. To investigate calpain activity in mouse keratinocytes, immunoblotting analysis of keratinocyte cell lysates was performed. Since no autoproteolytic cleavage products of calpains, indicative of strong autoactivation (Baki et al., 1996; Shea, 1997), could be detected in cultured mouse keratinocytes (see below), cellular calpain activity was investigated in live cells using an enzymatic assay (Fig. 13).

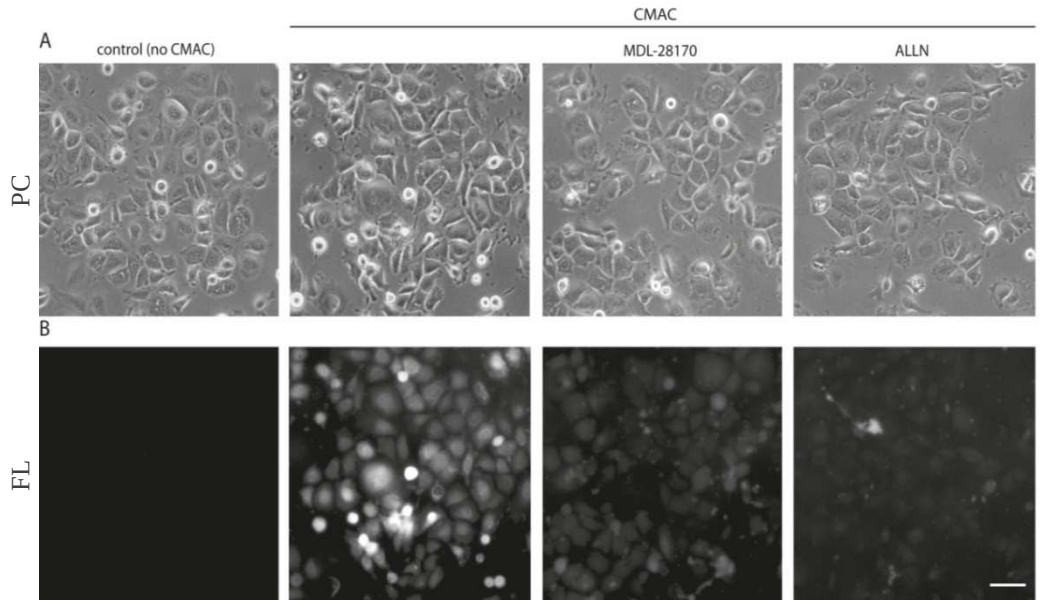

Figure 13: Phase contrast (PC) and fluorescence (FL) microscopy of immortalized wild-type keratinocytes treated with the calpain substrate CMAC. (A,B) Immortalized wild-type keratinocytes were cultured in KGM/0.05 to a confluency of 70% and then incubated with 50 µM CMAC for 20 minutes. Cell morphology and calpain-induced CMAC fluorescence were monitored using phase contrast and fluorescence microscopy. Cells that were not incubated with CMAC were used as negative control. Note strong fluorescence observed when cells were treated with CMAC. For calpain inhibition, the cells were treated with 50 µM MDL-28170 or 10 µM ALLN for 1 hour prior to application of CMAC. Note, upon treatment with MDL-28127, CMAC fluorescence intensity was greatly diminished, and was almost completely abolished when ALLN was used. Scale bar, 100 µm.

This assay was performed with an immortalized (p53-deficient) mouse keratinocyte cell line (immortalized keratinocytes), which displays normal growth and differentiation properties and shows no signs of transformation (Osmanagic-Myers et al., 2006). Immortalized wild-type keratinocytes were cultured to a density of ~70% in regular keratinocyte growth medium containing 0.05 mM Ca^{2+} (KGM/0.05); they were then incubated (20 min.) with the calpain-specific substrate t-BOC-Leu-Met (CMAC), which after cleavage by calpains, produces a blue fluorescence signal (emission maxima of 430 nm). Its fluorescence intensity is therefore indicative of cellular calpain activity (Marzia et al., 2006; Xu and Deng, 2006; Su et al., 2010). Using this assay, I found calpains to be active in keratinocytes grown in KGM/0.05 (Fig. 13).

To demonstrate that the fluorescence signal observed was specific for calpain activity, immortalized keratinocytes were treated with the calpain inhibitors ALLN and MDL-28170 for one hour prior to applying CMAC to the cells. Monitoring fluorescence by microscopy, it was noticed that both calpain inhibitors dramatically diminished fluorescence intensity, with ALLN being more potent than MDL-28170. These results confirmed that calpains in cultured keratinocytes grown under normal conditions (KGM/0.05) were in an active state, and prompted me to investigate the involvement of calpains during terminal differentiation of keratinocytes.

1.2 Analysis of calpain activity in the course of terminal differentiation

The controlled disassembly of HDs is a prerequisite for the detachment of basal epidermal keratinocytes from the basal membrane and subsequent terminal differentiation (Tennenbaum et al., 1996; Frye et al., 2003; Alt et al., 2004; Litjens et al., 2006). The core structure of HDs as well as of their ex vivo equivalents, the HPCs, is formed by the transmembrane laminin receptor protein integrin (ITG) α6β4, which is anchored to the keratin 5/14 (K5/K14) IF network via direct binding of its β4 subunit to plectin isoform 1a (P1a) (Rezniczek et al.,1998; Litjens et al., 2006; Kostan et al., 2009; de Pereda et al., 2009; Walko et al., 2011**)**. At an early stage of Ca^{2+}-induced differentiation of keratinocytes in culture, ITGα6β4 and P1a are relocated from HPCs to the cytosol in a Ca^{2+}-dependent manner resulting in the disassembly of HPCs (Kostan et al., 2009). Activation of calpain-1,

as assessed by its autoproteolytic cleavage, has been shown to increase in cultured human keratinocytes upon reaching confluency, and during terminal differentiation (Michel et al., 1999). Calpains were also found to be involved in the intracellular processing of ITGβ4 in keratinocytes and in the degradation of plectin in liver (Giancotti et al., 1992; Muenchbach et al., 1998), thus opening the possibility that this class of proteases could be involved in regulating HD turnover in keratinocytes.

To investigate whether there are alterations in the protein levels of calpains and HPC proteins during terminal differentiation, primary mouse keratinocytes were grown in KGM/0.05 until they reached confluency. At confluency, terminal differentiation was induced with 1.3 mM Ca^{2+} (KGM/1.3). The cells were then further cultivated for five days and differentiation was monitored by phase contrast microscopy (Fig. 14).

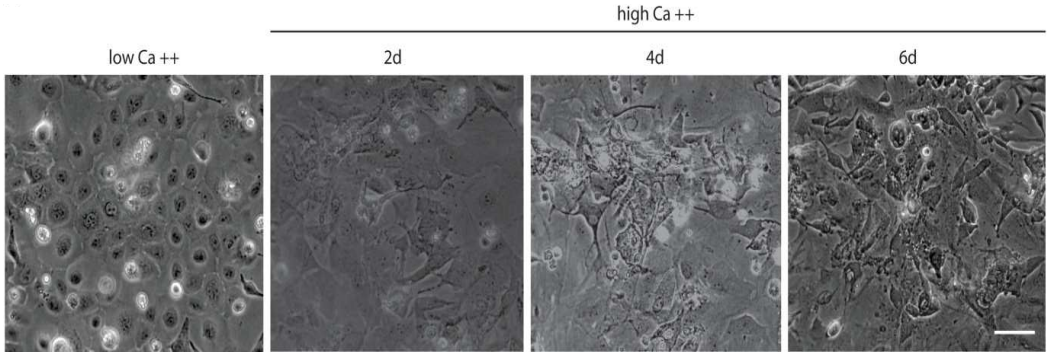

Figure 14: Phase contrast microscopy of differentiating primary wild-type keratinocytes. Primary wild-type mouse keratinocytes were cultivated in KGM/0.05 until they reached 100% confluency. At confluency the medium was changed to KGM/1.3 to induce terminal differentiation. The progress of terminal differentiation was monitored using phase contrast microscopy. Note upon growth in KGM/1.3, primary mouse keratinocytes lost their typical brick like morphology and began to stratify eventually forming a multi-layered cornified epithelium. Scale bar, 100 µm.

Already after two days in KGM/1.3, single cells lost their typical brick like morphology and formed a continuous layer of enlarged, stretched cells that displayed granules in their cytosol, typical signs of keratinocytes undergoing terminal differentiation (Fig. 14) (Watt et al., 1984; Sevilla et al., 2008). After four days, basal keratinocyte had begun to detach from the culture dish and to move over other cells to form a second cell layer (Fig. 14). After six days, a stratified epithelium had formed, consisting of two to three upper cell layers and several compacted cells on top, indicative of cornification and thus marking the final stage of

differentiation (Rice and Green, 1978; Hennings et al., 1980; Hennings et al., 1982) (Fig. 14).

Cell lysates were prepared at each time point and subjected to immunoblotting analysis (Fig. 15), using involucrin as an early differentiation marker (Watt, 1983; Watt et al., 1984; Li et al., 2000). It was found that the expression of involucrin was already upregulated at day one. Involucrin expression subsequently remained constant until day four and slightly decreased again on day five, possibly because involucrin became incorporated into insoluble cornified envelope structures (Groot et al., 2004; Sevilla et al., 2008).

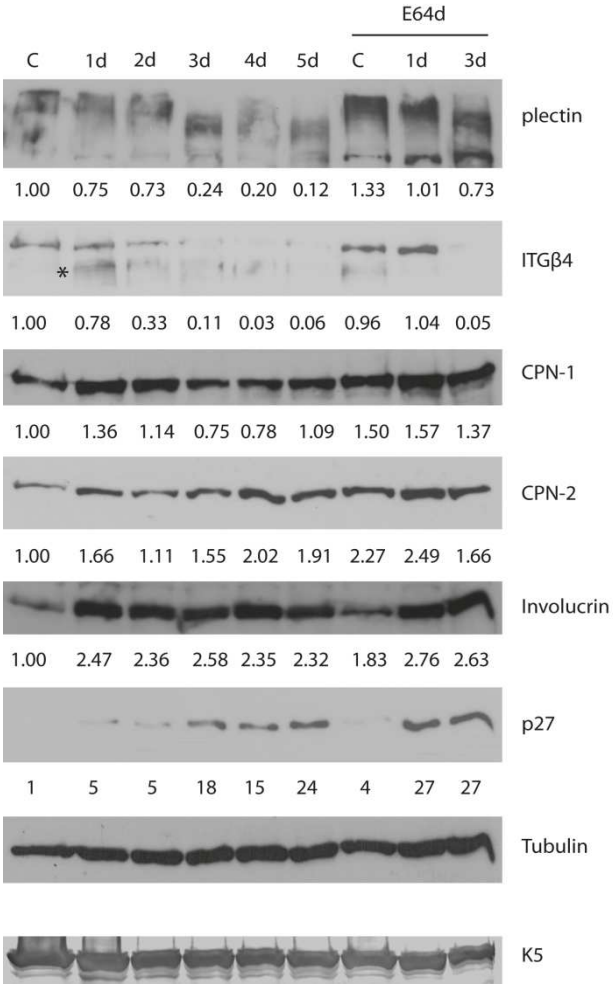

Figure 15: Immunoblotting analysis of differentiating immortalized wild-type keratinocytes. Immortalized wild-type keratinocytes were treated as described in Fig. 16. Cell lysates were prepared from cells grown to 100% confluency in KGM/0.05 (lanes C) and from cells at different time points during Ca^{2+}-induced terminal differentiation (lanes 1d-5d). Proteins were separated by SDS-PAGE on 6% (plectin), 8% (ITGβ4; calpain-1, calpain-2), or 10% (p27, K5, tubulin, involucrin) gels and analyzed by immunoblotting. Bands were scanned and evaluated densitometrically. Numbers below lanes indicate protein ratios relative to an arbitrary level of 1.0 determined by the undifferentiated and untreated control sample (lane C). Note that the protein levels of plectin and ITGβ4 decreased with progression of terminal differentiation. Expression of both hemidesmosomal proteins was stabilized during the first day of differentiation by treatment with E64d. The asterisk indicates a cleavage fragment of ITGβ4, not detected in differentiating cells upon E64d treatment. Protein levels of uncleaved calpain-1 (~80 kDa) increased after onset of differentiation until day two but then decreased slightly during later differentiation stages. In contrast, protein levels of uncleaved calpain-2 (~80 kDa) constantly increased with progressing differentiation. The protein levels of the uncleaved calpain isoforms were increased upon E64d-treatment, indicative of inhibition of calpain activation. The protein levels of the differentiation markers involucrin and p27 increased at the onset of terminal differentiation, but the kinetics of their upregulation differed. Involucrin and p27 expression was increased upon E64d treatment. Tubulin and K5 were used as loading controls.

p27, a cyclin-dependent kinase inhibitor involved in the regulation of terminal keratinocyte differentiation onset (Missero et al., 1996; Hauser et al., 1997; Kolly et al., 2005), was also found to be upregulated, but with slower kinetics than involucrin (Fig. 15). The levels of ITGβ4 and plectin were found to be dramatically diminished after day three of terminal differentiation (Fig. 15) as has previously been reported (Kostan et al., 2009). Expression of the uncleaved (80 kDa) form of calpain-1 was found to slightly increase during the first two days of differentiation and to decrease slightly thereafter (Fig. 15). The decrease of the uncleaved form of calpain-1 during the final stages of ex vivo differentiation could be the consequence of increased autoproteolytic cleavage as a consequence of hyperactivation of the protease (Shea, 1997; Michel et al., 1999) (compare to Fig. 16A). Calpain-2 expression increased over the differentiation period, similar to previous reports from human keratinocytes (Garach-Jehoshua et al., 1998).

To assess whether calpain-1 was involved in the downregulation of ITGβ4 and plectin protein levels and consequently in the destruction of HDs, we monitored the expression of these proteins during Ca^{2+}-induced terminal differentiation in the presence of E64d, a potent, irreversible inhibitor of cysteine proteases including calpains (see Materials and Methods, Table 4). This treatment indeed inhibited proteolytic degradation of ITGβ4 and led to increased levels of intact ITGβ4 protein at day one of differentiation (Fig. 15). However, in the presence of E64d, protein expression of ITGβ4 had almost completely ceased on day three contrary to untreated samples (Fig. 15). Plectin showed a similar tendency (Fig. 15). In confluent keratinocytes grown in KGM/0.05 (Fig. 15) ITGβ4 protein levels were found to be increased contrary to those of plectin. The protein levels of the uncleaved (80 kDa) forms of calpains-1 and 2 were increased upon E64d-treatment, indicative of inhibition of calpain activation (Fig. 15). Surprisingly, E64d treatment appeared to promote the entrance of proliferating keratinocytes into terminal differentiation, since expression levels of p27 and involucrin were upregulated in confluent keratinocytes grown in KGM/0.05 as well as on day one of differentiation (Fig. 15). The above findings provided evidence that calpains were involved in the degradation of HPC proteins at least at the onset of terminal differentiation. These results raised the question whether activated calpains are localized at HPCs and thus can directly regulate HPC disassembly via cleavage of P1a and ITGβ4.

1.3 Hyperactivation and inhibition of calpains in cultured keratinocytes

To investigate the effect of calpain hyperactivation on HPC stability, immortalized keratinocytes were cultured in KGM/0.05 to ~70% confluency, before the medium was switched to KGM/1.8 supplemented with 5 µM ionomycin, a Ca^{2+} ionophore promoting rapid influx of Ca^{2+} into the cytosol. The cells were exposed to Ca^{2+} influx for one, two, or five hours, which leads to autoproteolysis and strong activation of calpains (Croall et al.; Shea et al., 1997;, Sorimachi et al., 2010; Saido et al., 1991). Cell lysates were prepared and subjected to immunoblotting analysis using antibodies specific for the HD proteins P1a and ITGβ4, and calpain-1. As shown in Fig. 16A, the protein levels of P1a and ITGβ4 gradually decreased upon Ca^{2+} influx. This was paralleled by strong activation of calpain-1, manifesting in the appearance of its cleaved (76 kD) form after 1 hour and almost complete loss of the intact (80 kDa) protein after 2 hours (Fig. 16A). By quantifying protein expression levels of P1a and ITGβ4 relative to an untreated control sample it was revealed that the kinetics of P1a degradation were slower compared to that of ITGβ4. This suggested that P1a was more resistant to calpain-mediated degradation than ITGβ4 (Fig. 16A-C). In parallel, immortalized keratinocytes were treated with the potent cell-permeable calpain inhibitor MDL-28170 (see Table 4) for one hour before incubation with ionomycin in KGM/1.8. When cell lysates were subjected to immunoblotting analysis, MDL-28170 was found to efficiently inhibit calpain-1 activity, as the intact (80 kDa) form reappeared as a consequence of the treatment. Inhibition of calpains prevented the ITGβ4 protein from proteolysis (Fig. 16A,C), whereas P1a degradation was only partially inhibited by MDL-28170 after five hours of Ca^{2+} influx (Fig. 16A,B).
As sustained high Ca^{2+} levels eventually induce apoptosis, the failure of MDL-28170 to prevent P1a degradation at the latest time point (5 hours) of Ca^{2+} influx, could be explained by the well known degradation of plectin by apoptotic caspases (Stegh et al., 2000; Aho, 2004; Werner et al., 2007)

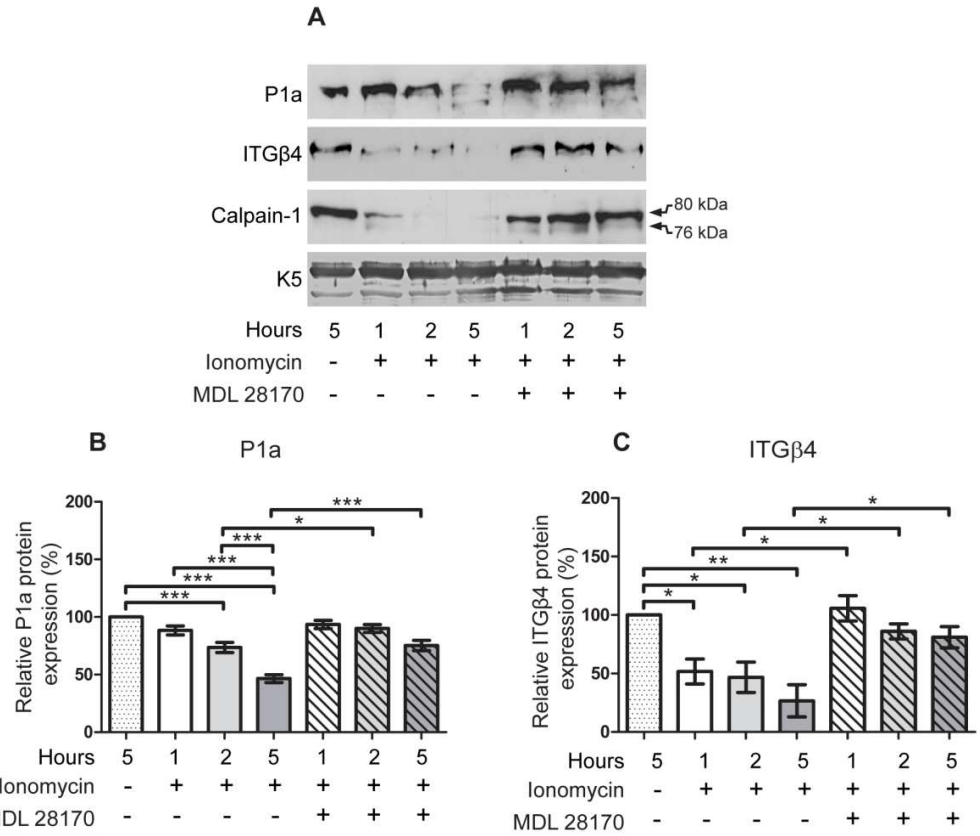

Figure 16: Immunoblotting of wild-type mouse keratinocytes in KGM/1.8 supplemented with ionomycin with and without calpain inhibition. Immortalized keratinocytes were cultured in KGM/0.05 until they reached a confluency of ~70%. Cells were then either left untreated or challenged (for the indicated times) with the Ca^{2+} ionophore ionomycin (5 µM) with or without the addition of the calpain inhibitor MDL-28170 (50 µM). In addition, some cells were pretreated with the calpain inhibitor MDL-28170 (50 µM) for one hour prior to induction of Ca^{2+} influx. (A) Immunoblotting analysis of total cell lysates prepared after one, two, and five hours of Ca^{2+} influx using antibodies specific to P1a, ITGβ4, and calpain-1. (B,C) Densitometric quantifications of P1a (B) and ITGβ4 (C) protein levels relative to that in control samples (100%) using K5 as loading control. Protein expression levels were calculated from quantified immunoblotting signals relative to that of the untreated control. Mean values ±SEM (n = 4) are shown. Statistical significance was demonstrated by one-way ANOVA with Tukey posttest for multiple comparisons (* P<0.05, ** P<0.01, *** P<0.001). Note expression of full length calpain-1 (80 kDa) was lost upon Ca^{2+} influx indicating that the protease was highly activated. Upon treatment with MDL-28170 the full length calpain-1 protein reappeared as a consequence of efficient calpain-1 inhibition. Also note that the amounts of P1a and ITGβ4 decreased gradually during high Ca^{2+} influx, which could be reversed by MDL-28170 treatment. ITGβ4 showed faster degradation kinetics than P1a.

To immunolocalize P1a and ITGα6, cells were methanol-fixed and processed for confocal immunofluorescence microscopy using antibodies to plectin and ITGα6 (Fig. 17). Under standard growth conditions (KGM/0.05, without ionomycin) small keratinocyte cell colonies formed arc-shaped HPCs, typically for keratinocytes in culture (Ozawa et al., 2010; Wilhelmsen et al., 2007; Koster et al., 2004) (Fig. 17). Upon Ca^{2+} influx introduced by ionomycin, HPC stability was affected

already after one hour of treatment (Fig. 17). Disassembly of HPCs was progressive, with HPCs disappearing completely after five hours of Ca^{2+} influx (Fig. 17).

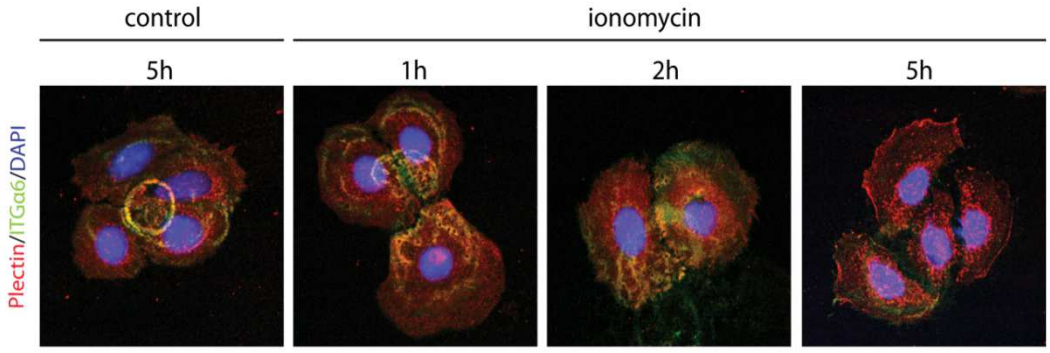

Figure 17: Immunolocalisation (double labeling) of ITGα6 (green) and plectin (red) in immortalized keratinocytes with or without ionomycin treatment for the times indicated. Immortalized keratinocytes were treated as described in Fig. 18. Nuclei were stained with DAPI (blue). Composite images were generated from confocal stacks by maximum intensity projections of the three optical sections closest to the substrate level. Note in untreated keratinocytes (control, 5h), ITGα6 and plectin showed codistribution in densely clustered arch-like HPCs (arrowheads). After 1 hour of ionomycin challenge, HPCs started to become less densely clustered (arrowheads) and ultimately disappeared after 5 hours. Bar 20 µm.

The disassembly of HPCs upon Ca^{2+} influx mediated calpain activation and their reassembly after calpain inhibition confirmed the direct involvement of these proteases in the turnover of HPCs and proposed their direct association with HPCs upon activation.

1.4 Subcellular localisation of calpains

In order to study the subcellular distribution of calpain-1, immortalized keratinocytes cultured in KGM/0.05 to a density of ~70% were lysed and subjected to subcellular fractionation, yielding cytosolic, cytoplasmic membrane, and cytoskeleton fractions. In addition, immortalized keratinocytes were treated in KGM/1.8 supplemented with 5 µM ionomycin for one, two, and five hours prior to subcellular fractionation. The fractions were then subjected to immunoblotting analysis using antibodies to ITGβ4 and calpain-1 (Fig. 18).

Calpain-1 was not only found in the cytosolic but also in the cytoplasmic membrane and cytoskeleton fractions (Fig. 18A). Again, the autoproteolytically cleaved active form of calpain-1 (76 kDa) could not be detected (compare to Fig. 15). One explanation for this observation could be that protein levels contained in the different subcellular fractions were too low to be detectable. As a transmem-

brane protein associated with the cytoskeleton, ITGβ4 was found in both fractions (Fig. 18B), where its levels were dramatically diminished after Ca^{2+} influx into the cell (Fig. 18B).

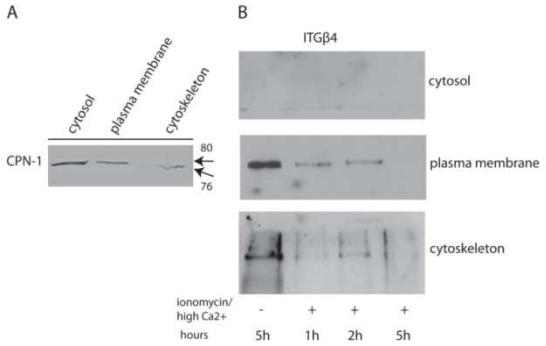

Figure 18: Immunoblotting of calpain-1 in different subcellular fractions and of ITGβ4 after ionomycin treatment. Immortalized wild-type keratinocytes were cultured under standard growth conditions (KGM/0.05) to a confluency of ~70%, and then fractionated into cytosolic, plasma membrane and cytoskeletal fractions. (A) Immunoblotting of fractions using antibodies to calpain-1. Note, intact calpain-1 (80 kDa) was found in all fractions. (B) Keratinocytes cultured in KGM/1.8 were exposed to 5 µM ionomycin (for the times indicated) prior to fractionation and immunoblotting using antibodies to ITGβ4. Note, ITGβ4 was found in membrane and cytoskeleton fractions; ITGβ4 protein levels in membrane fractions were dramatically diminished already after one hour of Ca^{2+} influx and practically nonexistent after five hours.

After five hours ITGβ4 was completely gone from the membrane fraction, whereas residual amounts could still be detected in the cytoskeleton fraction. In conclusion, these results showed that calpain-1 is ubiquitously located at the membrane. For the first time it was demonstrated that a fraction of calpain-1 is also associated with the cytoskeleton in its active form. This observation is surprising as calpain-1 is kept in its inactive form in most tissues. However, the association of calpain-1 in an active form with the cytoskeleton and in an inactive form with the membrane could explain the rapid turnover of ITGβ4 after Ca^{2+} influx.

The results described so far indicated that calpains were involved in HPC disassembly. This raised the question whether active calpains were responsible for the decreased number of HDs observed in the epidermis of EBS Ogna mice.

2 The role of calpains in EBS-Ogna pathology

During differentiation of keratinocytes the tightly controlled degradation of HDs is a necessity for the cells to leave the proliferative niche at the basal membrane.

However, since HDs anchor the epidermis to the basal lamina, loss of HDs has a fatal effect on the mechanostability of the skin (Darling et al., 1997; Chung and Uitto, 2010; Laimer et al., 2010; Sawamura et al., 2010; Shinkuma et al., 2011). In the skin blistering disease EBS-Ogna, an autosomal dominant missense mutation in the plectin gene leads to impaired HD formation (Koss-Harnes et al., 2002). Likewise, in the epidermis of mice harbouring the EBS-Ogna mutation the number of HDs was reported to be dramatically diminished (Walko et al., 2011). This could be attributed to proteolytic degradation of P1a, but not of P1c, the other major isoform expressed in epidermal keratinocytes (Walko et al., 2011). Using recombinant proteins as substrate and enzymatic activities contained in tissue protein extracts, the EBS-Ogna mutation could be shown to render plectin's 190-nm-long coiled-coil rod domain more vulnerable to cleavage by calpains and other proteases activated in the epidermis but not in skeletal muscle (Walko et al., 2011). Since two major calpain isofoms (calpain-1 and calpain-2) are expressed in basal epidermal keratinocytes (Michel et al., 1999; Walko et al., 2011; Kamata et al., 2012) calpains could indeed be involved in the pathogenesis of EBS-Ogna.

2.1 Calpain inhibition in EBS-Ogna keratinocytes

In cell lysates of EBS-Ogna keratinocytes (*Plec*$^{Ogna/Ogna}$) P1a protein levels were found to be reduced to ~35%, compared to wild-type primary keratinocytes (*Plec*$^{wt/wt}$), while in contrast, the protein levels of P1c remained unaffected under these conditions (Walko et al., 2011). Thus to investigate the direct involvement of calpains in the degradation of mutant P1a carrying the EBS-Ogna mutation, I used primary keratinocytes isolated from adult homozygous EBS-Ogna knockin mice (*Plec*$^{Ogna/Ogna}$) as study objects.

Plec$^{Ogna/Ogna}$ primary keratinocytes were exposed for various time periods to different pharmacological inhibitors of calpains (Materials and Methods, Table 4). Afterwards cell lysates were subjected to immunoblotting analysis (Fig. 19A, B).

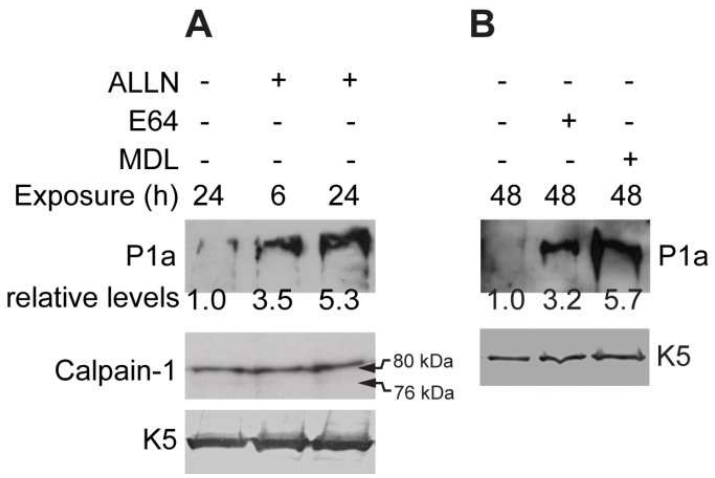

Figure 19: Immunoblotting of P1a expression levels in primary *Plec*$^{Ogna/Ogna}$ keratinocytes treated with calpain inhibitors. (A,B) Primary *Plec*$^{Ogna/Ogna}$ keratinocytes were cultured in KGM/0.05 until they reached a confluency of ~90%, then the Ca^{2+} concentration in the medium was raised to 0.5 mM (KGM/0.5). (A) Cells were then exposed to solvent (DMSO) alone, or 10 µM ALLN for 6 hours and 24 hours, and cell lysates were subjected to immunoblotting analysis. Note samples analyzed contained equal amounts of calpain-1 and K5. Numbers below lanes represent protein ratios relative to an arbitrary level of 1.0 assigned to the control (solvent) sample. Note application of ALLN efficiently increased the protein levels of mutant P1a. After 6 hours of ALLN treatment the mutant P1a protein levels increased to ~3-fold and after 24 hours to ~5-fold compared to untreated cells. (B) Cells were exposed to E64 (20 µM) and MDL-28170 (50 µM) for 48 hours prior to immunoblotting analysis. Note that inhibition of cysteine proteases with E64 for 48 hours increased the P1a protein levels ~3-fold when compared to the protein levels of untreated control cells, while treatment with MDL-28170 led to an ~5-fold increase of the P1a protein levels.

Quantification of P1a immunoblot signals revealed that inhibition of calpains using calpain inhibitor ALLN led to a significant increase of P1a protein levels (Fig. 19A). Already after six hours of drug treatment P1a protein levels were found to be ~3-fold upregulated compared to untreated control cells, while treatment for 24 hours led to even a ~5-fold increase (Fig. 19A).Treatment of primary *Plec*$^{Ogna/Ogna}$ keratinocytes with the irreversible cysteine protease inhibitor E64 for 48 hours showed a lesser effect, leading to a ~3-fold increase in the amount of P1a protein after 48 hours (Fig. 19B). On the other hand, applying the potent, cell permeable and reversible calpain inhibitor MDL-28170 led to a strong response, i. h. a. ~5-fold increase in plectin protein levels (Fig. 19B). The differences in P1a re-expression levels observed with different calpain inhibitors tested can most likely be explained by their different specific IC-50 values, which account for the half minimal (50%) inhibitory concentration (IC) of a substance, and distinct cell permeabilities (see Materials and Methods, Table 4). E.g., E64 is only weakly cell permeable and has a lower inhibitory activity towards calpains than the other inhibitors.

To investigate whether drug induced increases in P1a levels were sufficient to rescue the deficits in HPC formation characteristic of EBS-Ogna keratinocytes (Walko et al., 2011), cultured primary *Plec*$^{Ogna/Ogna}$ keratinocytes were incubated with MDL-28170 for 24 hours, fixed with methanol and processed for immunofluorescence microscopy. In accordance with P1a's upregulation upon inhibition of calpains, I found the number of cells with HPCs to be increased in MDL-28170-treated cultures (Fig. 20A,B). In fact, the number of cells with HPCs increased from ~18% to ~40% upon treatment with MDL-28170 (Fig. 20B).

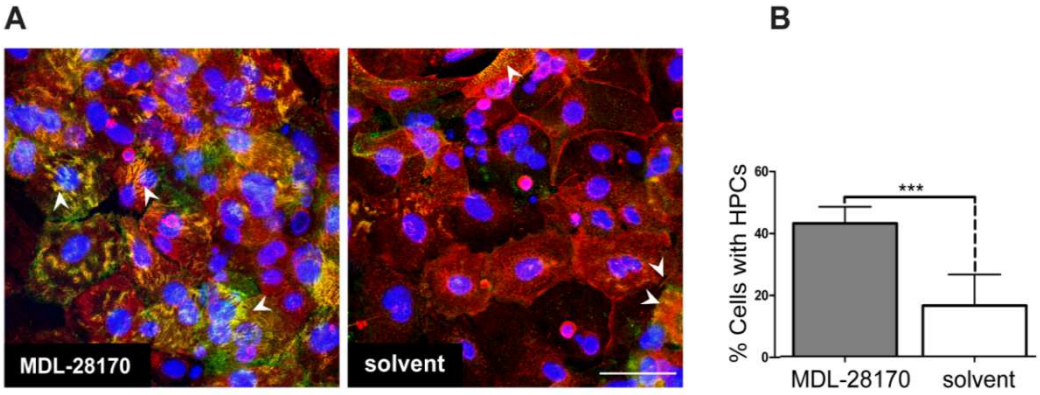

Figure 20: Immunolocalization of HPCs in primary *Plec*$^{Ogna/Ogna}$ keratinocytes treated with calpain inhibitors. (A) Solvent- and MDL-28170-treated primary *Plec*$^{Ogna/Ogna}$ keratinocytes were fixed with methanol and immunolabeled for ITGα6 (red) and plectin (green). Nuclei were stained with Hoechst (blue). Note calpain inhibition with MDL-28170 increased the number of cells with HPCs (arrowheads). Scale bar, 50 µm. (B) The bar diagram shows proportions (%) of cells having formed HPCs. Data shown represent mean values of cell counts (>500/genotype) in randomly chosen optical fields from three independent experiments ±95% CI. *** P<0.001, unpaired Student's t-test.

Primary cultures of *Plec*$^{Ogna/Ogna}$ keratinocytes were also treated with the serine protease inhibitor AEBSF to assess whether serine proteases were involved in degradation of P1a carrying the EBS-Ogna mutation. Quantitative immunoblotting analysis performed with total cell lysates of *Plec*$^{Ogna/Ogna}$ keratinocytes that had been treated with the inhibitor for 24 hours showed no significant increase upon inhibition of serine proteases (Fig. 21). Taken together, these results demonstrated a major role of calpains in the pathology of EBS-Ogna by degradation of mutant P1a.

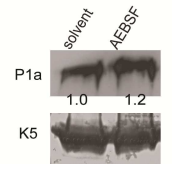

Figure 21: Immunoblotting of P1a expression levels in primary *Plec*$^{Ogna/Ogna}$ keratinocytes treated with inhibitors of serine proteases. Primary *Plec*$^{Ogna/Ogna}$ keratinocytes were grown as described in Fig. 21, and exposed to solvent (dH2O) alone, or to 100 µM AEBSF for 24 hours. Note in contrast to calpain inhibition, the inhibition of serine proteases using the specific inhibitor AEBSF did not dramatically alter the protein levels of P1a.

To lay the groundwork for further studies on the role of calpains in the pathogenesis of EBS-Ogna, a third part of the thesis was devoted to establish immortalized keratinocyte cell lines.

3 Establishment and characterization of immortalized clonal mouse keratinocyte cell lines from mice carrying the EBS-Ogna mutation

More in-depth studies on the functions of calpains in keratinocytes and in particular in the pathogenesis of EBS-Ogna could benefit from the establishment of immortalized keratinocyte cell lines. Establishment of cell lines from clonal origin comprises two steps. First, the immortalization and isolation of primary keratinocytes derived from mice and second, the characterization of immortalized mouse keratinocytes in order to assess the cell-type-specific requirements for growth in vitro. For this purpose, markers specific for keratinocytes and markers to define the differentiation and proliferative state of the cells were used.

3.1 Establishment of immortalized cell lines of clonal origin

Inactivation or loss of p53 facilitates keratinocyte immortalization (Boukamp et al., 1988; Raymond et al., 2005; Raymond et al., 2007), but does usually not cause cell transformation (Sedman et al., 1992), in contrast to, e.g., expression of human papilloma virus E6 and E7 proteins (Sedman et al., 1992), or infection with SV40 virus (Steinberg and Defendi, 1983).

For this purpose, p53 knockout ($p53^{-/-}$) mice were crossed with $Plec^{wt/wt}$, $Plec^{wt/Ogna}$ and $Plec^{Ogna/Ogna}$ *mice*. Primary mouse keratinocytes were then isolated from offspring and genotyped to confirm each type of plectin allele combination ($Plec^{wt/wt}$, $Plec^{wt/Ogna}$ and $Plec^{Ogna/Ogna}$) in the $p53^{-/-}$ background. Afterwards, primary mouse keratinocytes were cultured under standard growth conditions (KGM/0.05) until they reached ~70% confluency. $Plec^{wt/wt}$ *keratinocytes did not enter senescence* and were split 1:10 to obtain single cells for subcloning. The keratinocyte clonal cell lines were then subsequently expanded by subculturing them onto dishes of increasing size. Surprisingly, $Plec^{wt/Ogna}$ and $Plec^{Ogna/Ogna}$ cell

cultures became senescent after isolation and entered apoptosis albeit lacking p53. Consequently, clones originating from single surviving keratinocytes were picked, seeded onto 24-well plates and cultured under standard growth conditions. The keratinocyte clones were then seeded onto 6-well plates and cultured until they reached ~70% confluency. *Plec*$^{wt/Ogna}$ keratinocytes were further trypsinised and seeded (passaged) onto dishes of increasing size. In contrast, *Plec*$^{Ogna/Ogna}$ mouse keratinocytes did not reattach to cell culture dishes after the second round of trypsinisation and consequently died. For this reason it was not possible to establish clonal cell lines of this type. The immortalized *Plec*$^{wt/wt}$ *and Plec*$^{wt/Ogna}$ clonal cell lines were purified from contaminating melanocytes and fibroblasts by selective trypsinisation. Both types of clones could be kept frozen without changes in their properties. A summary of all steps involved in the establishment of keratinocyte cell lines is shown in Fig. 22.

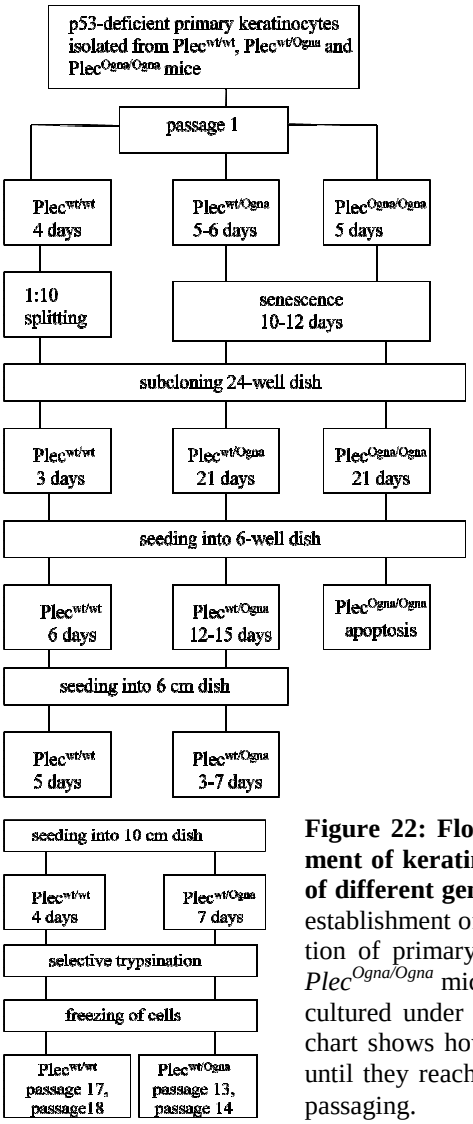

Figure 22: Flow chart of steps involved in the establishment of keratinocyte cell lines and growth characteristics of different genotypes. Depicted are all steps involved in the establishment of keratinocyte cell lines starting with the isolation of primary keratinocytes from *Plec*$^{wt/wt}$, *Plec*$^{wt/Ogna}$ *and Plec*$^{Ogna/Ogna}$ mice, all deficient in p53. The primary cells were cultured under standard growth conditions (passage 1). The chart shows how long the cells remained in the culture dish until they reached a confluency of ~70%, before subsequent passaging.

Of note, cell cultures of distinct phenotypes displayed different proliferation behaviour after isolation (Fig. 22) though they appeared indifferent in cell shape or size as assessed by light microscopy (Fig. 23). First, *Plec*$^{wt/Ogna}$ and *Plec*$^{Ogna/Ogna}$ keratinocytes needed longer until they reached ~70% confluency and even entered senescence after ~10 days in culture, contrary to primary *Plec*$^{wt/wt}$ cells. Furthermore, after subcloning of single surviving cells, *Plec*$^{Ogna/Ogna}$ keratinocytes died after passaging. *Plec*$^{wt/Ogna}$ keratinocytes appeared to grow slower than *Plec*$^{wt/wt}$ clones when monitored over many passages. In particular after subcloning, the cells carrying the EBS-Ogna allele(s) needed ~7-times longer to reach a density of ~70% when compared to the *Plec*$^{wt/wt}$ cells. Consequently, it was crucial to characterise the immortalized clones in the next step to assess whether cell transformation caused the differences in proliferation behaviour.

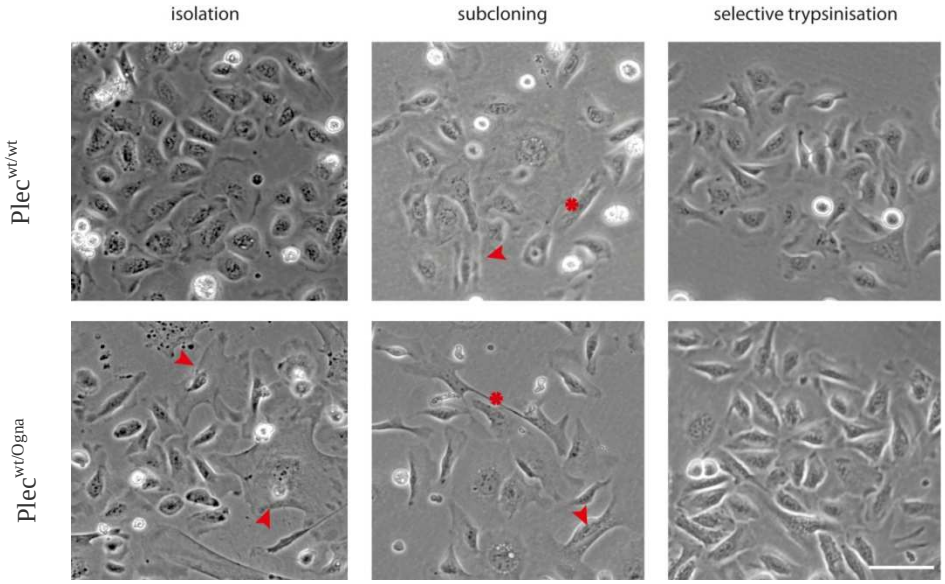

Figure 23: Phase contrast microscopy of mouse keratinocytes at different stages of isolation. Primary *Plec*$^{wt/wt}$ and *Plec*$^{wt/Ogna}$ mouse keratinocytes were isolated, subcloned and purified as schematized in Fig. 22. After initial isolation, primary *Plec*$^{wt/Ogna}$ keratinocyte cultures became senescent as indicated by the appearance of large flattened and often multi-nucleated cells (arrowheads), whereas *Plec*$^{wt/wt}$ cells *showed no signs of senescence*. After subcloning, immortalized clonal keratinocyte cell cultures were still contaminated with other cell types such as melanocytes and fibroblasts (asterisks). After several rounds of selective trypsinisation, immortalized clonal keratinocytes displayed the small triangular or fan shaped morphology typical of this type of cells, and no other contaminating cell types were anymore observed. Scale bar, 100 µm.

3.2 Characterization of immortalized *Plec*$^{wt/wt}$ and *Plec*$^{Ogna/Ogna}$ cell lines

For initial characterisation, cell lysates were prepared from six independent clones of both genotypes (*Plec*$^{wt/wt}$ clones at passage 17, and *Plec*$^{wt/Ogna}$ clones at passage

14) and subjected to immunoblotting analysis along with lysates of primary mouse keratinocytes (pmk), immortalized mouse keratinocytes (imk), a murine fibroblast cell line (3T3), and a protein extract of mouse liver (LE) using a set of antibodies to keratinocyte-, fibroblast-, and simple epithelia-specific marker proteins. As a typical marker of keratinocytes K5 was expressed in all clonal lines from both genotypes and also in the control lysates (imk and pmk) (Boukamp et al., 1988; Paladini and Coulombe, 1999; Troy and Turksen, 1999). Different clones differed however in K5 expression levels (Fig. 24A,B). When, the samples were tested for expression of the type 3 IF protein vimentin, which is commonly expressed in mesenchymal cells such as fibroblasts (Pei et al., 1992; Cano et al., 1996; Taki et al., 2003; Davies et al., 2005), no expression of the protein could be detected in any of the clonal cell lines (Fig. 24A,B); Vimentin was expressed to some extent in primary mouse keratinocyte cultures, which usually contain varying amounts of vimentin-positive melanocytes and also in the fibroblast lysate which served as positive control for this marker.

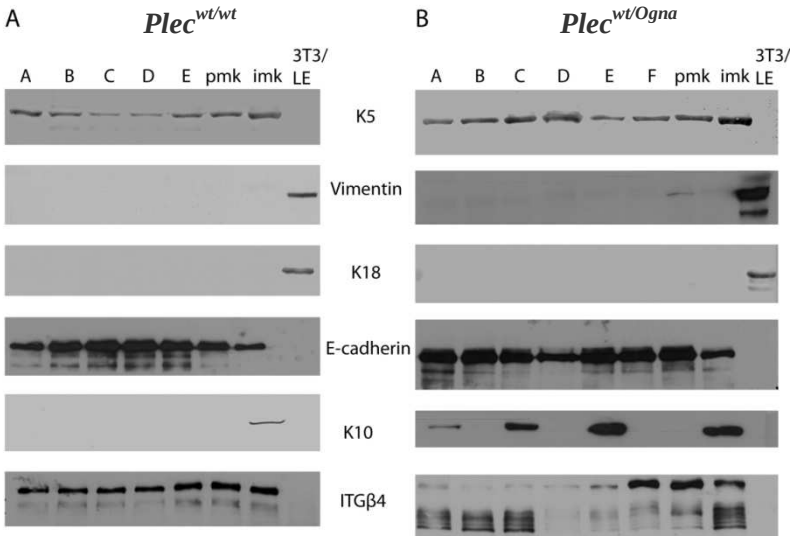

Figure 24: Immunoblotting analysis of immortalized clonal *Plec*$^{wt/wt}$ and *Plec*$^{wt/Ogna}$ keratinocyte cell lines. Cell lysates were prepared from *Plec*$^{wt/wt}$ cells (passage 14) (A) and from *Plec*$^{wt/Ogna}$*/p53* cells (passage 17) (B) at a cell confluency of ~70%. Proteins were separated by electrophoresis on SDS 6% (plectin), 8% (ITGβ4, E-cadherin), or 10% (K5, K10, K18, vimentin) polyacrylamide gels. Different clonal cell lines are indicated with letters A-F. Cell lysates of primary mouse keratinocytes (pmk) and immortalized p53-deficient wild-type mouse keratinocytes (imk) were used as positive controls for expression of keratinocyte-specific proteins. A cell lysate from a mouse fibroblast cell line (3T3) was used as positive control for vimentin expression, and a protein extract of liver (LE) for expression of K18. Immunoblotting was performed using antibodies indicated. Note all wild-type clones displayed comparable expression of the keratinocyte-specific proteins K5 and E-cadherin. K18, vimentin and K10 were not expressed in wild-type clonal cell lines. All wild-type clonal cell lines **expressed comparable amounts of the hemidesmosomal protein** ITGβ4. Also note, the *Plec*$^{wt/Ogna}$ clones displayed expression of the keratinocyte-specific proteins K5 and E-cadherin. K18 and vimentin were not found to be expressed in any of the *Plec*$^{wt/Ogna}$ clones, but clones A, C, and E expressed the early differentiation marker K10. In contrast to the wild-type clones, all *Plec*$^{wt/Ogna}$ clones (except for clone F) expressed reduced amounts of ITGβ4.

In order to exclude malignant transformation of the clones, expression of K18, a keratin typically expressed in simple epithelia and in keratinocytes undergoing malignant transformation was tested (Pei et al., 1992). As K18 is a key protein of the hepatocyte cytoskeleton, it was found to be expressed in murine liver extract but showed no expression in any of the clonal cell lines. Another hallmark of malignant transformation of keratinocytes is the downregulation of cell-cell junction-associated proteins such as E-cadherin (Wilding et al., 1996; Faraldo et al., 1997; Taki et al., 2003), concomitant with the disassembly of cell-cell contacts (Cano et al., 1996) and resistance against calcium-induced differentiation (Kulesz-Martin et al., 1983; Wilding et al., 1996; Raymond et al., 2007). E-cadherin was expressed at similar levels in all cell lines and, most importantly, the expression levels were also similar when compared to the control lysates of primary mouse keratinocytes (pmk) and immortalized mouse keratinocytes (imk) (Fig. 24A,B). Considering unaltered expression of E-cadherin and lack of K18, none of the clones displayed characteristics of malignant transformation.

Another important feature of immortalized cell cultures is to maintain proliferation properties of primary cells. K10 is a keratinocyte-specific protein expressed upon the onset of terminal differentiation (Leigh et al., 1993; Alani et al., 1998; King et al., 2003). To assess whether the immortalized clonal cell lines had not lost the ability to proliferate, I tested K10 expression. In case of the wild-type clonal cell lines, none of the clones expressed K10 (Fig. 24A). Expression of K10 was detectable in primary mouse keratinocyte cultures as a result of different subpopulations of proliferating and differentiating cells being present in such cultures (Fig. 24A,B). However, three of the six *Plec*$^{wt/Ogna}$ clonal cell lines (clones A, C, and E; Fig. 24B) expressed varying amounts of K10, indicating that these cell lines contained an increased proportion of terminally differentiated cells that had lost properties typical of primary cells.

ITGβ4 was used to monitor expression of HPC proteins. All wild-type clones showed similar ITGβ4 expression levels when compared to the cultures of primary mouse keratinocytes and immortalized keratinocytes (Fig. 24A). In contrast, all of the *Plec*$^{wt/Ogna}$ clones displayed significantly reduced ITGβ4 expression levels except for one clone (clone F, Fig. 24B). As ITGβ4 is a component of HDs, the general reduction in ITGβ4 can be explained by the phenotype of EBS-Ogna which manifests with greatly reduced numbers of HDs.

Based on these data three clones of each genotype were cultured in a special growth medium, KGM-Gold (Lonza), promoting both clonal, non malignant growth and high density keratinocyte proliferation for further characterization. As the marker profile among all wild-type clones was equal in this case, the clones with the highest expression of P1a (clones A, B, and E; data not shown) were selected for further characterization. In the case of the *Plec*$^{wt/Ogna}$ cell lines, clones F, A and E, displaying no, low and high K10 expression, respectively, were selected. To assess the potential of the individual clonal cell lines to form HPCs, all clones were cultured to a density of ~70% confluency in KGM-Gold/0.05. The cells were then either fixed with cold methanol and further processed for immunofluorescence microscopy using antibodies to ITGβ4, or cell lysates were prepared for immunoblotting. Alternatively, the cell lines were exposed to 0.5 mM Ca^{2+} (KGM-Gold/0.5) to induce terminal differentiation. After 24 hours, part of the cells were fixed with methanol and processed for immunofluorescence microscopy using antibodies to desmoplakin I and II. The remaining cells were cultured for another two days after reaching confluency and their cell lysates thereafter subjected to immunoblotting analysis. All clones that had been grown in KGM-Gold/0.05 were found to express plectin and ITGβ4 (Fig. 25A), the *Plec*$^{wt/Ogna}$ clones displayed decreased amounts of plectin compared to the *Plec*$^{wt/wt}$ clones.

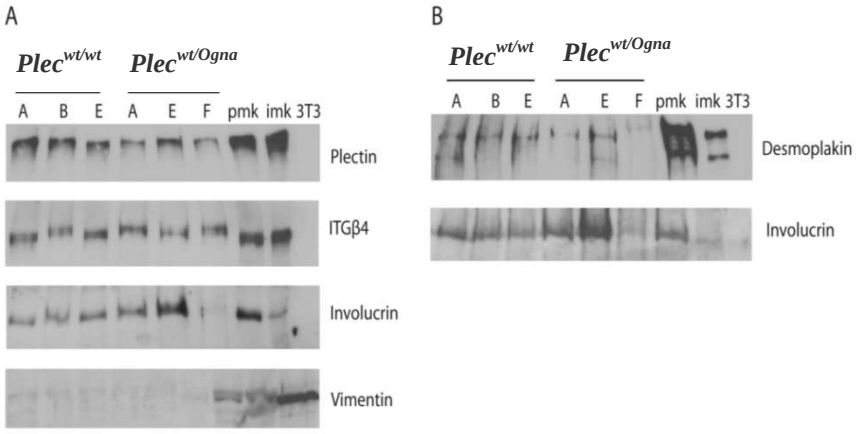

Figure 25: Immunoblotting analysis of selected immortalized clonal cell lines. (A) Clonal cell line A, B and E (*Plec*$^{wt/wt}$) and clonal cell line A, E and F (*Plec*$^{wt/Ogna}$) were grown to a density of ~70% confluency. Cell lysates were then subjected to immunoblotting analysis along with cell lysates of primary mouse keratinocytes (pmk), immortalized wild-type mouse keratinocytes (imk), a lysate of 3T3 mouse fibroblast cells, and a protein extract from liver, using the antibodies indicated. Note that all clonal cell lines expressed the hemidesmosomal proteins plectin and ITGα6, but plectin levels were lower in *Plec*$^{wt/Ogna}$ compared to *Plec*$^{wt/wt}$ clones. Also note that all clonal cell lines except *Plec*$^{wt/Ogna}$ clone F expressed involucrin. Only *Plec*$^{wt/Ogna}$ clone F expressed trace amounts of vimentin. (B) Clonal cell lines were grown until they reached confluency and the medium was then switched to KGM-Gold/0.5. Two days later the cells were lysed and aliquots of lysates were subjected to immunoblotting analysis using the antibodies indicated. Note lesser expression of desmoplakin in clonal cell lines and immortalized keratinocytes cells and almost complete loss of desmoplakin in *Plec*$^{wt/Ogna}$ clone F cells (compared to primary keratinocyte cultures). Also note that the expression of involucrin increased upon terminal differentiation in all clonal cell lines except for *Plec*$^{wt/Ogna}$ clone F.

Thus, in terms of reduced plectin expression, the *Plec*$^{wt/Ogna}$ *cells* showed lower ITGβ4 expression levels compared to the corresponding primary keratinocytes (Walko et al., 2011). Immunofluorescence microscopy using antibodies to ITGβ4 revealed that only one of all *Plec*$^{wt/wt}$ clonal cell lines, namely clone A, was able to form HPCs at significant numbers (arrowheads), whereas only few HPCs could be detected in cells from the other wild-type cell lines (Fig. 26). In the case of the *Plec*$^{wt/Ogna}$ clonal cell lines, few HPCs could be detected in clone A (arrowheads), whereas almost no HPCs were formed by clone E. In cultures of clone F (*Plec*$^{wt/Ogna}$*)*, ITGβ4 was found to be expressed only in rounded and spindle-shaped cells, some of them displaying long protrusions (asterisks).

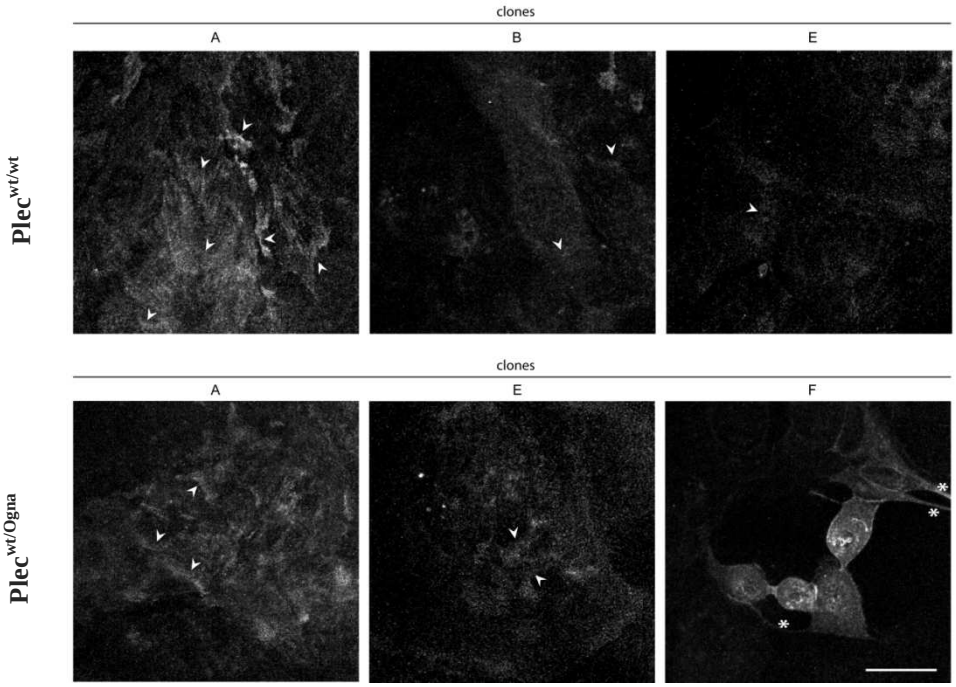

Figure 26: Immunolocalization of ITGα6 in selected immortalized clonal cell lines. Clonal cell line A, B and E (*Plec*$^{wt/wt}$) and clonal cell line A, E and F (*Plec*$^{wt/Ogna}$*)* were cultured to a density of ~70% confluency and fixed with methanol for immunofluorescence microscopy using anti-ITGα6 antibodies. Note clones B and E (*Plec*$^{wt/wt}$*)* displayed decreased formation of HPCs (arrowheads). Note also that among all *Plec*$^{wt/Ogna}$ clonal cell lines only clones A and F displayed HPCs, but in case of clone F they were predominantly located in the cytosol and at the plasma membrane. Moreover, cells of clone F showed an unusual long and stretched shape with long protrusions (asterisks). The scale bar indicates 50 µm.

All clonal cell lines, except for *Plec*$^{wt/Ogna}$ clone F, expressed different levels of the differentiation marker involucrin (Fig. 26A) and formed desmoplakin-positive cell-cell junctions upon confluency in KGM-Gold/0.5 (data not shown) indicating onset of terminal differentiation. Upon prolonged incubation with 0.5 mM Ca^{2+} the expression of involucrin increased in all clonal cell lines except for *Plec*$^{wt/Ogna}$ clone F (Fig. 25B). The same line also expressed greatly reduced amounts of the

desmosomal protein desmoplakin (Fig. 25B). Vimentin was absent in all clones except for *Plec*$^{wt/Ogna}$ clone F, were a faint band could be detected on immunoblots (Fig. 25A). The failure of this clone to induce involucrin expression at the onset of terminal differentiation, along with its expression of vimentin, indicated ongoing malignant transformation.

DISCUSSION

In this diploma thesis I provide evidence that disasssembly of HPCs in cultured mouse keratinocytes upon induction of terminal differentiation is conducted by calpains. Disassembly of HPCs can be triggered by enhancing the activation of calpains through Ca^{2+} influx, and can be blocked by inhibiting the hyperactivation of calpains through peptide inhibitors of calpains. Disassembly of HDs is crucial for many vital processes such as keratinocyte migration during wound healing (Raja et al., 2007; Margadant et al., 2008; Ozawa et al., 2010; Tsuruta et al., 2011) and for promoting terminal differentiation of keratinocytes (Frye et al., 2003; Blanpain and Fuchs, 2006; Blanpain et al., 2006; Gebhardt et al., 2006). This diploma thesis correlates keratinocyte differentiation with the expression of calpain-1 and gives insights into the roles of calpains in the stepwise disassembly of HPCs at the onset of keratinocyte differentiation ex vivo. Furthermore, we found evidence that the EBS-Ogna mutation affects proliferation of keratinocytes lacking the tumour suppressor protein p53.

Calpains as key regulators of HPC turnover?

Several previous observations pointed towards a role of calpains as potential key regulators of HPC/HD turnover. First, calpain-1 was reported to be expressed in normal foetal and neonatal human skin and to undergo activation-associated autoproteolytic cleavage during ex vivo differentiation of primary human keratinocytes and HaCaT cells (Michel et al., 1999; Garach-Jehoshua et al., 1998). Calpains were also reported to be active in proliferating primary cultures of human keratinocytes and in HaCaT cells under normal growth conditions (Satish et al., 2004), as well as in epidermal protein extracts (G. Walko, unpublished data). Second, calpains were demonstrated to be involved in proteolytic

processing of ITGβ4 during the onset of keratinocyte differentiation (Giancotti et al., 1992; Tennenbaum et al., 1996). Moreover, in vitro experiments from our laboratory demonstrated that calpain-1 can degrade plectin (Walko et al., 2011), which together with ITGβ4 builts up the stability axis of HPCs/HDs.

This diplomathesis reports for the first time that, similar to human keratinocytes (Satish et al., 2004), calpains in cultured mouse keratinocytes are active under normal, proliferation-promoting growth conditions (0.05 mM $CaCl_2$). Interestingly, despite clearly detectable calpain-specific proteolytic actvity, no autoproteolytical cleavage of calpains was observed in mouse keratinocytes cultured under normal proliferative growth conditions. This could indicate that either only a small fraction of calpains is active in proliferating keratinocytes, or that calpains display only a low basal enzymatic activity with verly limited amounts of autoproteolytically cleaved calpain beeing generated (Baki et al., 1996; Cong et al., 1989).

In mouse tail skin, calpain-1 was found to partially colocalize with ITGβ4 at the basal cell membrane of basal keratinocytes, thus fulfilling the spatial requirements for a potential HD regulator (Walko et al., 2011). In fact, we could demonstrate that activated calpains can directly regulate the disassembly of HPCs in cultured keratinocytes. Ionophore-mediated influx of Ca^{2+} into keratinocyte cells induced rapid HPC disassembly by calpain-mediated degradation of ITGβ4 and P1a, which could be blocked by treatment with a potent cell permeable peptide inhibitor of calpains. Interestingly, similar Ca^{2+} ionophore treatments have previously been shown to induce differentiation and cornified envelope formation (Ruhrberg et al., 1996; Sarafian et al., 2006). Accordingly, pharmacological inhibiton of calpain activity was able to rescue HPC protein degradation at the onset of terminal differentiation of keratinocytes ex vivo. However, the role of calpains in HPC turnover under normal growth conditions remains elusive, since in proliferating keratinocytes only the protein levels of plectin but not of ITGβ4 were not increased upon treatment with calpain inhibitors. One needs to mention that the specifity of the calpain inhibitors used is limited. The vast majority of available calpain inhibitors also inhibit the enzymatic activity of cysteine proteases and/or the proteasome (see Table 4), thus complicating the interpretation of inhibitor studies.

Another interesting observation from our experiments was that in proliferating keratinocytes, fractions of calpain-1 were found to be located at the plasma membrane and to be also associated with the cytoskeleton. The binding of calpains to the membrane seems to be important for calpain activation (Shao et al., 2006; Leloup et al., 2010). Active calpain attached to the plasma membrane was also reported to be required for the self-sealing repair mechanism of membranes in proliferating fibroblasts (Mellgren et al., 2007). The membrane-associated pool of calpain-1 in keratinocytes could be maintained by EGF and/or the membrane phospholipid PIP_2 (Shaoh et al., 2006; Leloup et al., 2010, Satish et al., 2004). The existence of a membrane-associated fraction of calpain-1 in proliferating keratinocyte cells would also be a prerequisite for the fast and strong calpain-1 activation upon Ca^{2+} influx. When HPC disassembly is required, elevated Ca^{2+} levels could enhance the activation of membrane-associated calpains and in addition promote targeting of cytosolic calpains to the plasma membrane. Consequently, HPC-dependent processes such as keratinocyte differentiation could be triggered through temporal and spatial regulation of calpains. This stepwise mode of regulation may enable a fast and very effective degradation of HPCs.

Insights into the multi-step mechanism of HPC disassembly at the onset of keratinocyte differentiation

Kostan et al. (2009) demonstrated that incubation with elevated extracellular Ca^{2+} levels to induce terminal differentiation of keratinocytes resulted in the rapid dissociation of P1a and ITGβ4. This fundamental process is executed through calmodulin and PKCα. While calmodulin binds to the ABD of P1a, PKCα phosphorylates serine residues in the connecting segment of the cytosolic domain of ITGβ4. Both events lead to the dissociation of P1a from ITGβ4. However, these processes could not explain the subsequent downregulation of both hemidesmosomal proteins during keratinocyte terminal differentiation. The data obtained in this diploma thesis provide evidence that calpains are involved in this process. Interestingly, the expression of calpain-2 during Ca^{2+}-induced terminal differentiation inversely correlated with that of P1a and ITGβ4, whereas that of calpain-1 did not. Albeit calpain enzymatic activity was not assessed in keratinocytes undergoing terminal differentiation, the protein levels of both

calpain isoforms were found to be increased upon treatment of cells with a pharmacological inhibitor of calpains, indicating that a fraction of calpains must have been highly activated during terminal differentiation, resulting in autoproteolytic cleavage and reduced amounts of calpain proteins (Shea, 1997; Neumar et al., 1998; Chou et al., 2011). However, as the activation process of calpains is complex, only the direct assessment of calpain's enzymatic activity could tell us to what extent calpains are active/activated during keratinocyte terminal differentiation.

Expanding a previous model of HPC disassembly in keratinocytes undergoing terminal differentiation (Kostan et al., 2009), our data suggest a stepwise mechanism of HPC disassembly constituting of three distinct phases (Fig. 27).

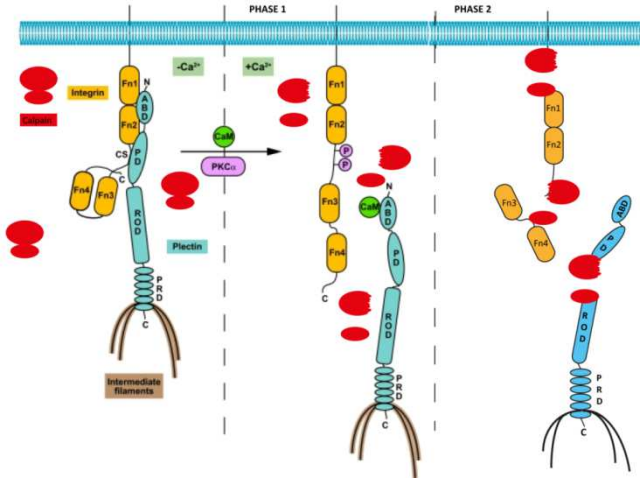

Figure 27: Hypothetical model of HPC disassembly at the onset of Ca^{2+}-induced keratinocyte differentiation. Under low Ca^{2+} concentrations a fraction of calpain (red) is already attached to the membrane but its enzymatic activity is low. The stability of HPCs is mediated by the association of ITGβ4 with P1a via binding of plectin's actin binding domain (ABD) to the first pair of the fibronectin type III (*Fn1* and *Fn2*) domains of ITGβ4 and via interaction between plectin's plakin domain (PD) and the ITGβ4 connecting segment (*CS*). The HPC is connected to the keratin IF network via plectin's C-terminal plakin repat domain (PRD). Phase 1: At the onset of keratinocyte differentiation elevated Ca^{2+} levels activate PKCα (phosphorylating serines at the CS domain of ITGβ4), and calmodulin (CaM), which binds to the ABD of plectin. Both events cause the dissociation of ITGβ4 and P1a. As another consequence of Ca^{2+} influx, heterodimeric calpains consisting of large (domains I-IV) and small subunits (domains V and VI) are strongly activated resulting in autoproteolyis and relase of the small subunit. Phase 2: P1a and ITGβ4 are now exposed to proteolysis and are initially cleaved by active calpains. Phase 3: Once cleaved both proteins are degraded within hours via other proteases (e.g caspases and/or the proteasome). Additionally, ITGβ4 is downregulated at the transcriptional level.

In phase one, the influx of Ca^{2+} upon raising the extracellular Ca^{2+} levels leads to activation of calmodulin and PKCα, which promotes the dissociation of ITGβ4 and P1a and exposes both proteins to subsequent proteolysis. Ca^{2+} activates

membrane-associated calpains and promotes activation of additional calpain molecules from the cytosolic and/or the cytoskeletal pools. In phase two, the now exposed hemidesmosomal proteins ITGβ4 and P1a are initially cleaved by calpains, triggering rapid degradation of both proteins by other proteases. We observed that upon hyperactivation of calpains by ionophore-mediated Ca^{2+} influx, proteolysis of ITGβ4 proceeded faster than proteolysis of P1a, suggesting that P1a was more resistant to calpain-mediated degradation than ITGβ4. This could be explained by ITGβ4-promoted oligomerization of P1a molecules into sheet-like structures of higher molecular order, which would render HD-associated P1a very resistant to calpain-mediated cleavage (Walko et al., 2011). In phase three, as keratinocytes begin to terminally differentiate and leave the basal cell layer, complete downregulation of ITGβ4 would be assured by reduction of mRNA transcripts (Tennenbaum et al., 1996), whereas such a mechanism could not be shown to exist in the case of P1a (Kostan et al., 2009). It is thus more likely that, after initial cleavage by calpains, complete degradation of P1a is mediated by other proteases, e.g. caspases or the proteasome. This would be consistent with the incomplete inhibition of P1a degradation by MDL-28170 upon ionophore-mediated Ca^{2+}-influx and the failure of E64 to prevent downregulation of P1a after day one of Ca^{2+}-induced keratinocyte terminal differentiation.

However, some aspects of the stepwise HPC disassembly mechanism remain elusive. If calpains initially cleave the HD proteins and thus are involved in triggering keratinocyte terminal differentiation why has it then not been possible to block differentiation by inhibition of calpains? Why did the inhibition of calpains even increase the expression of the differentiation markers p27 and involucrin? First of all, the peptide inhibitor used, E64, is not calpain-specific, but inhibits also cysteine proteases, among them several isoforms of cathepsins (see Table 4), a family of lysosomal proteases. Cathepsins B, D, E, H, and L were shown to be upregulated during terminal differention of keratinocytes and cathepsins D, E and L were found to be important positive regulators of keratinocyte terminal differentiation and the formation of the cornified enevelope (Tanabe et al., 1991; Egberts et al., 2004; Reinheckel et al., 2005; Benavides et al., 2011; Kawakubo et al., 2011). Accordingly, inhibition of cathepsins by E64 should have impaired terminal differentiation, rather than promoted it. Interestingly, p27 has been reported as a direct substrate of calpain (Patel and

Lane, 2000). Thus the upregulation of p27 upon E64 treatment could be the direct consequence of calpain inhibition. Since p27 was reported to be involved in regulating the onset of keratinocyte terminal differentiation (Missero et al., 1996; Hauser et al., 1997; Harvat et al., 1998), inhibition of calpains in keratinocytes could have led to its stabilisation, and thus enhancement of terminal differentiation. Calpains were also implicated in the prosurvival activities of both the tumoursuppressor protein p53 and nuclear factor-κB (NF-κB) (reviewed in Storr et al., 2011). Accumulating evidence indicates that calpain is able to cleave wild-type p53, regulating protein stability to prevent p53-dependent apoptosis (Gonen et al., 1997; Kubbutat and Vousden, 1997; Atencio et al., 2000). In addition, calpain can promote survival through activation of NF-κB by cleavage of its inhibitor IκBα. Calpain-mediated IκBα cleavage can occur in response to tumour necrosis factor (TNF) (Han et al., 1999). Thus, inhibition of calpains in keratinocytes could also have triggered apoptosis and thereby have supported terminal differentiation, since this involves some components of the apoptotic machinery (Weil et al., 1999; Janes et al., 2003). It is worth mentioning that light microscopic monitoring of keratinocytes that were treated for prolonged periods with calpain inhibitors revealed signs of cells undergoing apoptosis (unpublished data). Irrespective of the precise mechansim that led to enhanced terminal differentiation of keratinocytes upon E64 treatment, it appears that this mechanism overpowered inhibition of calpain-mediated degradation of HPC proteins and even further accelerated downregulation of P1a and ITGβ4.

As calpains fulfil such a large number of diverse functions the tight regulation of these proteases must be crucial for cell survival. Summarized, the detailed mechanisms contributing to the tight regulation of calpains in the epidermis remain elusive but may help in solving these questions. Thus, further studies on the function and regulation of calpains in keratinocytes are necessary to solve the open questions. First of all, siRNA depletion of either calpain-1 or calpain-2, or both calpains together could help to reveal the specific roles of the two isoforms in HPC disassembly and keratinocyte differentiation without interference with other protease families. Second, studies using conditional skin-specific calpain knock out mice would help to elucidate the hypothized models in vivo.

As in all experiments performed during this diploma thesis cultured keratinocyte cell lines or primary mouse keratinocyte cultures were used it is important to aks the question whether the proposed model of HPC disassembly has true relevance in vivo. Support for a role of calpains for HD turnover in vivo comes from a recent study of a mouse model of the human skin blistering disease EBS-Ogna (Walko et al., 2011). As shown in this diploma thesis, degradation of mutant P1a in basal keratinocytes isolated from EBS-Ogna mice can be blocked by inhibition of calpains, which leads to increased formation of HPCs. Moreover, topical application of the calpain inhibitor MDL-28170 to the tails of EBS-Ogna mice for five days inhibited calpain autoproteolysis and increased P1a levels at the basal cell surface of basal keratinocytes (Walko et al., 2011). Thus, one can conclude that activated calpains must be associated with HDs in basal keratinocytes in vivo, where they are involved the degradation of EBS-Ogna mutant P1a proteins. In fact, zymography and immunoblotting analysis revealed that in epidermal protein extracts calpains exist mostly in the autoproteolytically cleaved form (Walko et al., 2011), indicating that calpains are very active in the epidermis.

Calpains: key proteases in EBS-Ogna pathology

In the pathology of the skin blistering disease EBS-Ogna, reduced numbers of HDs are a consequence of a mutation in the rod domain of P1a, sensitizing it to proteolysis (Walko et al., 2011). A number of proteases capable of plectin cleavage have been described, including caspases 2, 3, 6, 7, and 8 (Stegh et al., 2000; Aho, 2004) , and calpain-1 (Muenchbach et al., 1998). Walko et al. (2011) demonstrated using an in vitro system to detect epidermis-specific protease activities that the plectin rod was degraded via the action of calpains and serine protease activities present in the epidermis. In this diploma thesis this approach was extended to primary mouse keratinocytes carrying the EBS-Ogna mutation. Using pharmalogical inhibitors of calpains, the numbers of cells with HPCs were significantly increased pointing towards calpains as key proteases in the degradation of mutated P1a. Moreover, in vivo inhibitor studies via topical application of the calpain inhibitor MDL-28170 to the tails of mice mimicking EBS-Ogna for five days resulted in increased numbers of HDs in the epidermis of the EBS-Ogna mice (Walko et al., 2011). These studies not only give insights into

the EBS-Ogna pathology but may also highlight a new direction towards prospective future therapies.

HPCs as regulators of cell proliferation?

Throughout the initial establishment of immortalized cell lines, keratinocytes depleted of the tumor suppressor gene p53 and carrying the homozygous EBS-Ogna mutation ($Plec^{Ogna/Ogna}$) displayed defects in cell growth and adhesion. Heterozygous EBS-Ogna keratinocytes appeared also to grow slower when compared to wild-type cells but displayed no impaired adhesion. In contrast, immortalized clones of homozygous EBS-Ogna keratinocytes did not attach after passage two and consequently died. Interestingly, reduced cell adhesion and proliferation has also been described for plectin-deficient immortalized keratinocytes (A. Jörgl, Diplomathesis, 2001). Thus, the P1a dosage-dependent defects observed in EBS-Ogna keratinocytes propose an important role of HPCs in proliferation and adhesion signaling, particularly in a p53-null background.

Reduced adhesion and cell survival have been previously reported for primary and immortalized keratinocytes lacking the laminin α3 or laminin γ2 genes (Ryan et al., 1999; Meng et al., 2003). However, whereas these cells displayed reduced adhesion and survival on plastic dishes, correlating with their inability to deposit laminin-322, these defects could be rescued by growth on exogenous collagen or laminin, indicating that ligation of the collagen receptor ITGα2β1 or the laminin receptors ITGα3β1 and ITGα6β4 was sufficient to rescue cell survival (Meng et al., 2002; Ryan et al., 1999). In cultured keratinocytes, ITGα3β1 is a component of focal adhesions (Raghavan et al., 2003; Ozawa et al., 2010), whereas ITGα6β4 is a component of HPCs. ITGα3β1 was reported to be important for keratinocyte cell adhesion and migration, and for stimulating cell proliferation (Brakebusch et al., 2000; Raghavan et al., 2000; Grose et al., 2002; Manohar et al., 2004), whereas ITGα6β4 appears to play no role in keratinocyte proliferation (Raymond et al., 2005). Moreover, keratinocytes deficient in ITGβ4 can still attach to laminin-322 via ITGα3β1 (Niessen et al., 1996). Thus, the compromised formation of HPCs in EBS-Ogna keratinocytes likely does not account for the observed adhesion and proliferation defects. ITGα6β4 is thought to have distinct functions depending on whether it is incorporated into HDs or associated with the leading edge of the cell during keratinocyte migration. Whereas in HDs/HPCs ITGβ4

promotes cell anchorage to the extracellular matrix, upon release from HDs/HPCs by EGF and PKC signalling or Ron-mediated displacement, it can associate with F-actin at the cellular periphery and with lipid rafts, where it is believed to function as a singnalling transducer protein (Rabinovitz et al., 1999; Gagnoux-Palacios et al., 2003; Santoro et al., 2003; Rabinovitz et al., 2004; Frijns et al., 2010; Yu et al., 2012). Interestingly, ITGα6β4 was shown to inhibit haptotactic migration-related functions of ITGα3β1 via Erb2 and PI3K in a cell-cell contact-dependent fashion (Hintermann et al., 2001). Possibly, ITGα6β4 released from HPCs could act in a similar fashion also on the adhesion and proliferation-related functions of ITGα3β1.

Surprisingly, the adhesion and proliferation defects of immortalized keratinocytes homozygous for the EBS-Ogna mutation are very similar to that observed for keratinocytes lacking or expressing reduced amounts of K14 (Troy and Turksen, 1999; Alam et al., 2011). K14-deficient keratinocytes displayed reduced adhesion, spreading, slow growth and considerable cell lysis when seeded onto collagen upon initial cell isolation, and considerable cell loss was observed during the first few days after replating (Troy and Turksen, 1999). Moreover, immortalized cell lines established from these primary cultures continued to display reduced spreading, proliferation and colony forming efficiencies (Troy and Turksen, 1999). In a more recent study, knockdown of K14 in HaCaT cells was shown to cause similar effects, including reduced adhesion on collagen, reduced cell proliferation and delay in cell cycle progression (Alam et al., 2011). These defects were traced to decreased activation of Akt and increased Notch pathway activation (Alam et al., 2011). Interstingly, K14 knockdown HaCaT cells also expressed increased levels of terminal differentiation-associated proteins including involucrin, which we found to be upregulated also in three out of five *Plec*$^{Ogna/Ogna}$*/p53*$^{-/-}$ clonal cell lines. Intriguingly, plectin's IF-binding domain was shown to bind to K14, but not K5 (Geerts et al., 1999). However, primary *Plec*$^{Ogna/Ogna}$ *keratinocytes display a normal K5/K14 network under unstressed conditions (Walko et al., 2011), challenging the hypothesis that alterations in the cytoarchitecture of K5/K14 networks in EBS-Ogna keratinocytes could be responsible for the observed defects in cell adhesion and growth. However, a re-evaluation of keratin IF network cyotarchitecture and particularly filament dynamics and stability could help to detect yet unnoticed defects of the keratin IF network in EBS-Ogna*

keratinocytes and maybe reveal functions of P1a that go beyond keratin IF-HPC attachment. In keratinocytes, keratin IFs are not only anchored to HDs/HPCs via P1a and BPAG1e, but also to the nucleus via nesprin 3 (Wilhelmsen et al., 2005; Ketema and Sonnenberg, 2011), whereby the keratin IF network could act as a mechanosensory element ultimately leading to altered gene expression (Wang et al., 2009). In fact, several junctional proteins including BPAG2 and ITGα6 were found to be downregulated on the mRNA level in EBS cell lines carrying Dowling Meara-type mutations in K14 (Liovic et al., 2009). Of note, in EBS-Ogna keratinocytes, K5/K14 filaments retract from residual HPCs at the cell periphery and collapse onto the nucleus upon mechanical stress (Walko et al., 2011). Thus, the hypothetical mechanosensing axis from HPCs to the nucleus would be easily disrupted by the mechanical stress occurring during cell isolation and passaging. It remains to be established whether compromised anchorage of keratin IFs to HDs/HPCs and potential defects in keratin IF network dynamics in EBS-Ogna keratinocytes could indeed impact on mechanosensing and cause gene expression alterations leading to cell proliferation defects. The immortalized clonal keratinocyte cell lines would be a good tool to test this hypothesis. However, more detailed studies (including statistical analyse on the proliferation behaviour of primary wild-type and EBS-Ogna mouse keratinocytes, as well as of the corresponding immortalized clonal cell lines must be performed initially.

In conclusion, this diploma-thesis adds new aspects to the current model of HPC disassembly at the onset of keratinocyte terminal differentiation presented by Kostan et al. in 2009. Calpains were shown to be potential candidates for filling the gap between the calmodulin/phosphorylation-mediated dissociation of ITGβ4 and P1a and the consequent degradation of the two proteins during terminal differentiation of keratinocytes. In vivo studies of epidermal homeostasis in epidermis depleted of calpains would bring more light on the role of calpain isoforms during keratinocyte differentiation. Furthermore, this diploma thesis highlights the role of calpains in the pathology of EBS-Ogna proposing a new target for prospect future therapies. Additionally, this work provides evidence that hemidesmosomal plectin could be involved in cell survival and proliferation of keratinocytes

MATERIALS AND METHODS

Homogenisation of mouse keratinocytes

All steps were performed on ice.

Mouse keratinocytes from 2 x 10 cm culture dishes were washed two times with 5 ml of cold PBS, scraped into 5 ml of cold PBS and transferred to a fresh tube. The cells were pelleted using Haereus Megafuge 1.0R centrifuge with 335.40 g for 3 minutes. The supernatant was discarded and the pellet resuspended with 5 ml of swelling buffer with subsequent incubation for 2 minutes. The cell suspension was pelleted again and resuspended with 800 µl of sample preparation buffer (that was always prepared fresh).

The Dounce Ball Homogenisator was washed with sample preparation buffer prior to homogenisation. The cell suspension was homogenised with 8 strokes and using a ball with 8 µm clearance. The homogenate was centrifuged for 30 minutes with 167.70 g in an eppendorf 5415C centrifuge. The supernatant was aliquoted into fresh tubes, shock frozen with fluid nitrogen and subsequently stored at -80 °C. The supernatant was mixed 1:3 with 5 x SDS sample buffer for staining with Coomassie brilliant blue and immunoblotting analysis.

Swelling Buffer:

- 10mM Tris/HCl
- 2mM $MgCl_2$
- 10mM NaCl
- pH=7,5

Sample Preparation Buffer:.

- 20mM Tris/HCl
- 5mM EDTA
- 5mM EGTA
- 1mM DTT
- pH=7,5

Subcellular fractionation

The subcellular fractionation was performed with Fermentas ProteoJET Membrane Protein Extraction Kit (#K0321).
Mouse keratinocyte cell monolayers at a density of ~ 90% were washed with cold cell washing solution and subsequently incubated in cell permeabilisation solution for 10 minutes at 4°C gently rocking. The supernatant (cytosolic fraction) was transferred into fresh tubes and stored at -80°C. Membrane protein extraction buffer was then added to the permeabilised cells and they were then incubated gently rocking for 30 minutes at 4°C. Subsequently, the remaining fractions were scraped off from the dish, transferred to a fresh tube and centrifuged at 28341 g for 20 minutes at 4 °C. The supernatant (membrane fraction) was transferred into fresh tubes and aliquots were stored at -80°C. The pellet (cytoskeletal fraction) was resuspended in 5 x SDS sample buffer and incubated at 95 °C for 5 minutes with vigorous shaking. Immediately after heating, the suspension was passaged ten times through a 27G syringe, aliquoted and stored at -80 °C. Cytosolic and membrane fractions were mixed 1:10 with 5 x SDS sample buffer for staining with Coomassie brilliant blue and immunoblotting analysis.

Preparation of cell lysates

Keratinocytes were washed twice with 5 ml PBS. The cells were lysed in an appropriate volume of hot 5 x SDS sample buffer and passaged ten times through a 27G needle. The lysate was then incubated for 5 minutes at 95°C with gentle shaking. Finally, the total cell lysate was aliquoted and stored at -80°C.

5 x SDS sample buffer:

- 60 mM Tris-Cl pH6.8
- 2% (w/v) SDS
- 10% (v/v) glycerol
- 5% (v/v) β-mercaptoethanol
- 0.01% (w/v) bromophenol blue

SDS-polyacrylamide gel electrophoresis

The SDS-polyacrylamide gels were casted using the volumes listed in table 2 according to the desired acrylamid concentration. Separation gels were overlaid with isopropanol during polymerization for at least 30 minutes. The isopropanol was then removed, the stacking gel was poured onto the separation gel and the comb (10 or 15 slots) was inserted. After 30 minutes of polymerization samples premixed with 5 x SDS sample buffer were incubated for 5 minutes at 95°C and pipetted into the slots of the stacking gel. The gel was covered with electrophoresis buffer and gel-electrophoresis was performed using BioRAD Protean Mini II apparatures with 20 mA per gel for about 2 hours.

Table 2: Solutions for preparing SDS polyacrylamide gels. Quantities given are for 5 ml separation and 1 ml stacking gels respectively.

					stacking gel
	6%	8%	10%	12%	4%
ddH_2O	2.6 ml	2.3 ml	1.9 ml	1.6 ml	0.68 ml
30% acrylamid	1.0 ml	1.3 ml	1.7 ml	2.0 ml	170 µl
1.5 mM Tris/ HCl (pH 8.8)	1.3 ml	1.3 ml	1.3 ml	1.3 ml	130 µl
10% SDS	50 µl	50 µl	50 µl	50 µl	10 µl
10% ammonium persulfate	50 µl	50 µl	50 µl	50 µl	10µl
TEMED	10 µl	8 µl	6 µl	6 µl	2 µl

Electrophoresis buffer:

- 25 mM Tris
- 250 mM Glycine
- 0.1% SDS

Coomassie staining

The SDS polyacrylamid separation gel was briefly washed with ddH_2O and incubated in Coomassie staining solution for 30 minutes at RT. After incubation, the gel was de-stained using de-staining solution until an appropriate contrast was achieved. Subsequently, the gel was washed 5 minutes with ddH_2O for 5 minutes.

Coomassie staining solution:

- 0.5 g Coomassie blue R250
- 400 ml methanol
- 70 ml acetic acid
- ddH_2O at 1L

Coomassie de-staining solution:

- 10% (v/v) acetic acid
- 30% (v/v) methanol
- 60% (v/v) ddH_2O

Electrotransfer of proteins onto nitrocellulose membranes

The blot sandwich for electro transfer was prepared as depicted in Fig. 28:

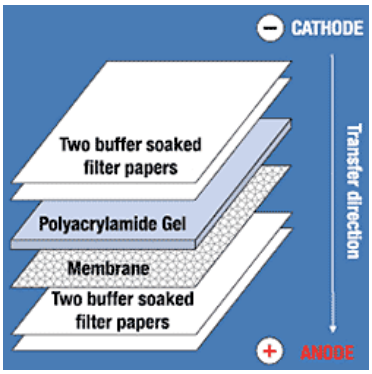

Figure 28: Preparation of blot sandwich for immunoblotting.
Source: http://www.fermentas.com/templates/files/tiny_mce/support_images/wblotting.gif

All components were pre-incubated in transfer buffer before the sandwich was assembled. Electrotransfer of proteins with a molecular mass higher than 300 kDa was performed in a wet-blotting tank filled with transfer buffer at 4°C o/n at 25 V. For proteins with a molecular mass lower than 300 kDa semidry electro transfer was performed using BioRAD Trans-Blot Turbo Transfer System. For turbo blotting, the filter papers prepared for the anode and the membrane were pre incubated in 2 x transfer buffer containing methanol for at least one hour. The filter paper prepared for the cathode was pre-wetted in 2 x transfer buffer for at least one hour. The blot sandwich was assembled and covered with 2 x transfer buffer. Transfer was performed by setting currents as listed in table 3 according to the molecular mass of the desired proteins to be transferred.

Table 3: Settings for semidry blotting using BioRAD Turbo Blotter

molecular mass	settings
20 kDa - 70 kDa	1.0 mA, 10 minutes
70 kDa - 200 kDa	1.5 mA, 15 minutes
200 kDa - 300 kDa	2.0 mA, 20 minutes

The protein transfer was verified by staining with 1 x Ponceau red solution for approx. 5 minutes and subsequent washing with ddH_2O. The size marker bands were marked with a soft pencil and the membrane was cropped. Finally, the membrane was de-stained with PBS-T.

Transfer buffer:

- 25 mM Tris
- 250 mM Glycine
- For semidry blotting: 20% (v/v) methanol

10 x Ponceau red solution:

- 0.1% (w/v) Ponceau S
- 5% (v/v) acetic acid
- ddH_2O to 100%

Immunoblotting

All steps were carried out on a shaker.

The nitrocellulose membrane was first incubated in blocking solution (5% (w/v) skimmed milk in PBS-T or 10% (v/v) horse serum in PBS-T) for one hour at RT to block unspecific binding of the primary antibody. After blocking, the membrane was briefly washed with PBS and subsequently the primary antibody diluted in 4% BSA/ PBS-T was added to the membrane, followed by incubation o/n at 4°C. On the next day, the membrane was washed 3 x 10 minutes with PBS-T and incubated with the secondary antibody, which was diluted in PBS-T, for two hours at RT. The secondary antibodies used were either coupled to HRPO (horseradish peroxidase) or AP (alkaline phosphatase) to allow for chemi-luminescence or AP reacting detection. Afterwards, the membrane was washed 3 x 10 minutes with PBS-T.

Chemiluminescent detection of HRPO-coupled secondary antibodies was performed using the Chemiluminescence substrate (SuperSignal West Pico of Thermo Scientific or BioRAD) and X-Ray films.
For detection of AP-coupled secondary antibodies the membrane was washed 1 x 10 minutes with AP buffer and subsequently incubated in AP substrate solution until optimal intensities of blotting bands had been achieved. The reaction was stopped with PBS-T.

AP buffer:

- 100 mM Tris
- 100 mM NaCl
- 5 mM $MgCl_2$
- pH 9.5

AP substrate solution:

- 66 µl BCIP stock solution
- 33 µl NBT stock solution
- 10 ml AP buffer

BCIP stock solution:

- 50 mg/ml BCIP in 100% (v/v) DMF

NBT stock solution:

- 50 mg/ml NBT in 70% (v/v) DMF

Cell culture:

All cell culture methods were carried out under aseptic conditions in a laminar flow hood. Unless otherwise mentioned all media and solutions used were preheated to 37 °C in a water bath.

Thawing of frozen cells

Cells stored in liquid nitrogen were rapidly thawed via gently shaking in a 37 °C water bath. The cell solutions were immediately added to 6 ml culture medium and centrifuged using a Haereus Megafuge 1.0R at 184 g for 3 minutes. The su-

pernatant was aspirated and the cell pellet re-suspended in 5 ml culture medium. The cell suspension was subsequently seeded into a 10 cm uncoated culture dish that was filled with 5 ml of culture medium. The cells were incubated at 37 °C and 5% CO_2.

Passaging of cells

The cells of a 10 cm culture dish were briefly washed with trypsin-EDTA and incubated with 2ml trypsin-EDTA for 8-12 minutes at 37 °C so that the cells clearly detached from the dish. The reaction was stopped by addition of 5 ml growth medium. The cells were resuspended by pipetting to obtain a single cell suspension and subsequently transferred into a fresh tube containing 5 ml growth medium. The cell suspension was centrifuged for 3 minutes at 184 g. The supernatant was aspirated and then the cell pellet was thoroughly resuspended in an appropriate volume of fresh growth medium and the cell suspension was seeded into fresh culture dishes. The cells were incubated at 37 °C and 5% CO_2.

Freezing of cells

After trypsination the cell pellet was resuspended carefully in 500 µl warm chelex treated FCS. 500 µl of the freezing medium was added drop wise and the mixture was then transferred to a cryo-tube that was placed in a cell freezing device (Nalgene). The cell suspension was frozen slowly at -80 °C and 48 hours later the cryo-tubes were transferred to liquid nitrogen.

Freezing medium:

- 20% DMSO
- 80% chelex treated FCS

Chelex treatment of FCS

200 ml of ddH_2O were added to 20 mg of chelex-100 resin. The pH was adjusted to 7.2 with 10 N HCl and the suspension was incubated o/n at 4°C. On the next day, the supernatant was discarded and fresh 200 ml ddH_2O were added. The pH was re-adjusted to 7.2 and the suspension was incubated for 1 hour at 4°C. Subsequently, the water was decantated and 50 ml of FCS (Sigma) were added. The mixture was incubated for one hour at 4°C while stirring slowly. The suspension

was incubated for another hour at 4°C without stirring to allow the chelex to settle. The mixture was sterile filtrated, aliquoted into fresh tubes and stored at -20 °C.

Isolation of primary keratinocytes from adult and newborn mice

Adult mice were anesthetized with 200 µl isofluran (Abbott) and killed by cervical dislocation. The tail was cut off and washed in PBS containing 2 x Penicillin/Streptomycin. In case of newborn mice the pubs were killed by decapitation. The trunk skin was isolated from new mice under aseptic conditions in a laminar flow hood.

Skin pieces isolated from adult mouse tails or trunk skin from newborn mice were incubated over night floating on 5 ml of Dispase solution (10 mg/ml in KGM/GA-1000). On the next day, the epidermis was peeled of the dermis and the epidermis pieces were minced with scalpels on a sterile glass object carrier and suspended in 3 ml trypsin-EDTA solution. Then the suspensions were incubated for 8 minutes in a 37°C water-bath and the tubes were shaken every minute. Epidermal cells were finally released from minced and trypsin-EDTA treated epidermis by pipetting 60 times up and down with a plastic pipette. Then the cell suspensions were filtered through a 40 µm cell strainer into a fresh tube filled with cold KGM supplemented with 8% (adult stem cells) or 2% (neonatal stem cells). The cell suspensions were then centrifuged for 7 minutes at 800 rpm, the medium discarded and the pellets dissolved in warm adhesion medium. Finally, the epidermal cells were plated onto Collagen 1 (neonatal keratinocytes) or Collagen 4 (adult keratinocytes) -coated dishes (filled with adhesion medium) and incubated o/n at 5% CO_2 and 37°C. After 24 hours the medium was changed to the appropriate growth medium.

<u>Adhesion medium:</u>

- KGM- Gold BulletKit (Lonza, 00192060)
- 8% for adult stem cells, 2% for neonatal stem cells of chelex treated FCS
- 1% ITS
- 100 µM $CaCl_2$

Growth medium:

- KGM- Gold BulletKit (Lonza, 00192060)
- 8% for adult stem cells, 2% for neonatal stem cells of chelex treated FCS
- 1% ITS
- 50 µM $CaCl_2$

Establishment of immortalized mouse keratinocyte cell lines

Wild-type and wt/Ogna primary mouse keratinocytes in a p53-null background were isolated from neonatal mice and cultured on 6 cm culture dishes (Lonza) under standard conditions until they reached senescence. Surviving colonies were trypsinised with 50 µl of trypsin-EDTA using clonal cylinders (Hilgenberg, 1980004). After successful detachment of the colonies, the reaction was stopped with 100 µl of growth medium and the suspension was transferred to a fresh eppendorf tube. The remaining cells were detached from the plate by washing twice with 1 x PBS and transferred to the tube. The suspension was centrifuged for 5 minutes with 186 g and the cell pellet was re-suspended in 100 µl of growth medium. The cell suspension was consequently seeded into a 24-well culture dish. The cells were cultured until they reached ~70% of confluency and subsequently trypsinised and seeded into 6-well culture dishes. Before reaching confluency, the cells were trypsinised again and seeded into 6 cm culture dishes and finally into 10 cm culture dishes. The cells were purified from contaminating fibroblasts and melanocytes by several rounds of selective trypsinisation for 4 minutes at 37 °C.

Growth medium:

- KGM BulletKit (Lonza, CC-3111)
- 2% chelexed FCS
- 1% ITS
- 50 µM $CaCl_2$

Calpain activity assay

$1x10^5$ cells were seeded into each well of a 6-well plate and the cells were cultured in 0.05 mM Ca^{2+} growth medium to a density of ~70%. The cells in two wells were then incubated with either 50 µM MDL-28170 or 10 µM ALLN for

one hour. In a next step 50 µM t-BOC-Leu-Met (CMAC) was applied to all wells and incubated for 20 minutes.

Immunostaining of cells for immunofluorescence microscopy

Cultured cells were methanol–fixed (2 min, −20°C) for immunostaining and then incubated with 4% (w/v) BSA/PBS o/n at 4°C to block unspecific bindings. On the next day, cells were incubated with the first antibody solution (antibody diluted in 4% BSA/PBS) for 1 hour and subsequently washed three times for 10 minutes with PBS. The cells were then incubated with the secondary antibody (diluted in PBS) for another hour and again washed with PBS as previously described. In a next step, the cells were incubated with Hoechst dilution (1:3000 in PBS) for 10 minutes and then washed with PBS for 10 minutes and washed twice with ddH2O for 5 minutes. Finally, the cells were mounted in Mowiol and covered with cover slips.

Fluorescence and phase contrast microscopy image acquisition

A Zeiss laser scanning microscope (LSM) 510 equipped with Plan-Apochromat 40x/1.3NA, Plan-Apochromat 63x/1.4NA and Plan-Apochromat 100x/1.4NA objective lenses was used confocal immunofluorescence microscopy. Digital images were processed using LSM 5 image browser and Adobe software package.
Phase contrast images of immortalized keratinocytess were obtained with an AxioObserver Z1 microscope coupled to AxioCam MRm (Carl Zeiss MicroImaging, Inc.) and equipped with phase contrast optics. Images were processed with Zeiss AxioVision 4.8.1 image analysis software (Carl Zeiss Microimaging, Inc.).

Quantification of HPC formation in cultured keratinocytes

Cultured keratinocytes were fixed with methanol and immunolabeled using anti-ITGα6 and anti-pan-plectin antibodies. Images were obtained using a LSM 510 microscope equipped with a Plan-Apochromat 63x/1.4NA objective lens. Cells displaying co distribution of ITGα6 and plectin in dense clusters at the basal cell surface were scored as HPC-positive. Cells that did not display this criterion were scored as HPC-negative.

Common buffers and solutions

10 x PBS:

- 81.8 g NaCl
- 2.01 g KCl
- 2.04 g KH_2PO_4
- 11.3 g K_2HPO_4
- pH 7.4; ddH_2O to 1000 ml

PBS-T:

- PBS containing 0.05% Tween-20

List of antibodies used for immunoblotting

Antigen/Epitope	Antibody	Vendor/Catalog# Reference/Name or Clone#	Dilution
Plectin (N-terminal domain of rat plectin, protein fragment encoded by exons 9-12)	rabbit antiserum	Andrä et al., 2003 / antiserum #9	1:3000
Plectin 1a (N-terminal domain of plectin isoform 1a, protein fragment encoded by exon 1a)	rabbit antiserum, purified	Rezniczek et al., 1998; Andrä et al., 2003	1:150
Plectin 1c (N-terminal domain of plectin isoform 1c, protein fragment encoded by exon 1c)	rabbit antiserum, purified	Andrä et al., 2003; Fuchs et al., 2009	1:400
Integrin β4 (N-terminal domain of human ITGb4)	rabbit antiserum, purified	Santa Cruz Biotechnology, Santa Cruz, CA / H-101	1:200
Keratin 5 (C-terminal end of mouse keratin 5)	rabbit antiserum, purified	Covance, Princeton, NJ / PR-B160-P	1:1000
E-Cadherin (C-terminal end of human E-Cadherin)	mouse mAb	BD Transduction Laboratories, Lexington, KY / clone LP 36	1:2000
Calpain-1 large Subunit (synthetic peptide corre-	rabbit antiserum,	Cell Signaling Technology / #2556	1:700

sponding to human sequence of calpain-1)	purified		
Calpain-2 large Subunit (synthetic peptide corresponding to human sequence of calpain-1)	rabbit, antiserum, purified	Cell Signalling Technology/ #2539	1:700
Desmoplakin 1+2 (bovine desmoplakin 1+2)	mouse mAb	Progen, Heidelberg, Germany DP-2.15, DP-2.17, DP-2.20	1:100
Keratin 10 (human squamous keratinizing epithelium)	mouse mAb	Millipore/ MAB3230	1:100
Vimentin	goat antiserum	P. Traub	1:30.000
Involucrin (peptide of mouse involucrin)	rabbit antiserum, purified	Covance, Princeton, NJ, USA PRB-140C	1:1000
Keratin 18 (human stratum corneum keratin preparation)	mouse mAb	Progen, Heidelberg, Germany Ks 18.04	1:250
p27 Kip1 (peptide of rabbit p27 Kip1)	rabbit antiserum, purified	Cell Signalling Technology, Boston, USA #2552	1:1000
α-Tubulin (Strongylocentrus purpuratus sperm axoneme filaments	mouse mAb	Sigma-Aldrich, Austria B-5-1-2	1:500

List of antibodies used for immunofluorescence microscopy

Antigen	**Antibody**	**Vendor/Catalog# Reference/Name or Clone#**	**Dilution (tissue/cells)**
Plectin (purified plectin from rat glioma C6 cells)	rabbit antiserum	Wiche and Baker, 1982 / serum #46	1:400 (c)
Integrin α6	rat mAb	BD Biosciences / clone GoH3	1:100 (c)

List of secondary antibodies

Immuno-fluorescence microscopy	Species	Dilution	Vendor
RRX anti-rabbit IgG	goat	1:200	Jackson Lab.
Alexa 488 anti-goat	donkey	1:800	Molecular Probes
Immunoblotting			
AP anti-rabbit IgG	goat	1:5.000	Jackson Lab.
AP anti-mouse IgG	goat	1:5.000	Jackson Lab.
HRPO anti-mouse IgG	goat	1:10.000	Jackson Lab.
HRPO anti-rabbit IgG	goat	1:20.000	Vector Labs
HRPO anti-goat IgG	donkey	1:20.000	Jackson Lab.

IC50 values (nM) for the calpain inhibitors used in this study

Table 4: IC50 values (nM) of calpain inhibitors used in this study from different proteases

Inhibitor	Calpain-1	Calpain-2	CTS-B	CTS-K	CTS-L	CTS-S	Proteasome
ALLN	190[1]	220[1]	150[1]			0.5[1]	6000[1]
E64	570[2]			1.4[3]	2.5[3]	4.1[3]	
MDL-28170	23[4]	23[4]	100[4]		57[4]		>1000[4]

[1] (Sasaki et al., 1990)
[2] (Trinchese et al., 2008)
[3] (Susa et al., 2004)
[4] (Briguet et al., 2008)

List of chemicals

Reagent	Vendor	Article number
30% Acrylamid mix (29:1)	Gerbu	1108
Acetic acid	Merck	100063
Ammoniumpersulfat	Serva	13375
AEBSF	Fluka	76307
ALLN	Calbiochem	208719
BCIP (X-Phos)	Gerbu	71290
Beta-Mercaptoethanol	Sigma	M625
Bovine Serum Albumin	Gerbu	1063
Bromphenol-blue solution	Sigma-Aldrich	B7021

$CaCl_2$, dihydrat	Fluka	21097
Chelex-100 mesh, sodium	BioRAD	142 2842
CMAC	invitrogen	A6520
Collagen I solution	Sigma	C-8919
Coomassie blue R250	Gerbu	1097
Dispase Typ I	Gibco	17105-041
Dithiothreiol (DTT)	Gerbu	1008
Dimethylformamid (DMF)	Loba	402480
Dimethylsulfoxid (DMSO)	Fluka	41640
EDTA	Gerbu	1034
EGTA	Sigma-Aldrich	E-4378
E64d	Sigma	E-3132
Ethanol	Merck	100983 RdH 32221
fetal calve serum (FCS)	Sigma-Aldrich	F-7524
Glycerol	Gerbu	2006
Glycine	Gerbu	1023
Hoechst-dye (#33258)	Hoechst	382061
Horse serum	Gibco	26050-088
Hydrochlorid acid (HCl)	Merck	100317
Insulin transferase solution (IST)	Gibco	41400-045
Isofluron (Isoflo)	Abbott	34480VA
Isopropanol	Merck	109634
K2HPO4	Fluka	56750
KCl	Loba	58648
KH2PO4	Fluka	60230
KGM BulletKit	Lonza	CC-3111
KGM-Gold BulletKit	Lonza	00192060
MDL-28270	Enzo	BML-PI130
Methanol	Merck	10600-RdH 32213
MgCl2	Fluka	63072
Na2HPO4	Fluka	71645
NaH2PO4	Fluka	71506
NaCl	Gerbu	1112
NaN3	Fluka	71290
NBT	Sigma-Aldrich	N-6876
Phosphate buffered saline (PBS)	Gibco	21300-074
Phenylmethanesulfonylflouride (PMSF)	Sigma-Aldrich	P-7626
Ponceau S	Sigma-Aldrich	P-3504
Skim milk powder	Gerbu	1602
Sodiumbicarbonate	Gibco	043-5080
Sodiumdodecylsulfate	Sigma-Aldrich	L-4509
Sodium hydroxide solution (NaOH)	Gerbu	2020
TEMED	Serva	35925
Tris-X	Gerbu	1018

Trypsin	Gibco	15090-046
Tween-20	Gerbu	2001

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Abstract

Plectin is a large (~500 kDa) and versatile member of a family of proteins called plakins or cytolinkers. It is able to interact with a multitude of cytoskeletal proteins including components of all major cytoskeletal filament systems of the cytoskeleton, and is spliced into various isoforms. Plectin isoform 1a (P1a), which is predominantly expressed in skin, is an essential component of hemidesmosomes (HDs) where it binds to integrin β4 (ITGβ4). HDs are transmembrane adhesion complexes, which anchor basal keratinocytes of the epidermis to the underlying basal lamina, thus conferring mechanical stability to the skin. A single autosomal dominant missense mutation in the plectin gene causes the skin blistering disease Epidermolysis Bullosa Simplex-Ogna (EBS-Ogna), which manifests with dramatically diminished P1a protein levels and reduced numbers of HDs. Calpains, cysteine proteases expressed in several tissues including the epidermis, have been demonstrated to degrade ITGβ4. Proteolytic degradation of HD components could be an important prerequisite for terminal differentiation of keratinocytes and could also be involved in the pathomechanism underlying EBS-Ogna. For these reasons, I assessed the role of calpains as possible candidates for P1a proteolysis at the onset of terminal differentiation and in the pathogenesis of EBS-Ogna.

Performing an enzymatic activity assay, I could demonstrate that calpains are active in immortalized mouse keratinocytes. I also found that in immortalized mouse keratinocytes a fraction of calpains is localized at the plasma membrane and associated with the cytoskeleton. Furthermore, hyperactivation of calpains in immortalized mouse keratinocytes led to P1a and ITGβ4 degradation, which could be blocked by treatment with a cell permeable peptide inhibitor of calpains. Results with the analysis of primary mouse keratinocytes undergoing differentiation suggested that a fraction of calpains must be active during terminal differentiation since treatment of the cells with a pharmacological inhibitor of cysteine proteases increased the protein levels of the inactive full length form of calpains. Furthermore, using a mouse model mimicking EBS-Ogna I found that calpains are involved in the proteolysis of P1a in primary keratinocytes derived from mutant animals. Upon inhibition of calpains in primary EBS-Ogna keratinocytes, elevated P1a protein levels could be detected along with increased numbers of HD-like

protein complexes (HPCs). Finally, I established clonal keratinocyte cell lines carrying the heterozygous EBS-Ogna mutation ($Plec^{wt/Ogna}$). Compared to wild-type clones, $Plec^{wt/Ogna}$ keratinocytes showed signs of proliferation defects pointing towards a hitherto undetected role of HDs as regulators of cell proliferation.

Zusammenfassung

Plectin ist ein ungewöhnlich großes (500 kDa) und vielseitiges cytoskelettäres Vernetzungsprotein. Es interagiert mit einer Vielzahl von Zytoskelettkomponenten, unter anderem Aktin Filamenten, Mikrotubuli und Intermediärfilamenten. Plectin kommt in unterschiedlichen Isoformen vor. Die in der Epidermis expremierte Isoform 1a (P1a) bildet gemeinsam mit Integrin β4 (ITGβ4) Hemidesmosomen (HD) aus. HD sind trans-membranöse Adhesionskomplexe, welche die basalen Keratinozyten der Epidermis an der darunterliegenden Basallamina verankern und so der Haut Stabilität verleihen. Eine autosomal-dominante Mutation einer einzelnen Base im Plectin Gen verursacht die Hautkrankheit Epidermolysis Bullosa Simplex-Ogna (EBS-Ogna). In der Epidermis von EBS-Ogna Modellmäusen sind die Proteinniveaus von P1a, sowie die entsprechende Anzahl von HDen stark reduziert. Calpaine sind weit verbreitete Cystein Proteasen welche unter anderm ITGβ4 proteolytisch abbauen können. Der proteolytische Abbau von HD-Proteinen könnte einen essenziellen Schritt im Zuge der terminalen Differenzierung von Keratinozyten darstellen. Weiters besteht die Möglichkeit, dass die gezielte Proteolyse von HD-Proteinen zum Pathomechanimsus von EBS-Ogna beiträgt. Im Zuge dieser Diplomarbeit wurde daher die Rolle von Calpainen in der Pathogenese von EBS-Ogna, sowie der terminalen Differenzierung von Keratinozyten untersucht.

Mithilfe einer enzymatischen in vitro Messmethode konnte ich die Aktivität von Calpainen in immortalisierten Mauskeratinozyten nachweisen. Weiters konnte ich mittels subzellulärer Fraktionierung zeigen, dass Calpaine sowohl an der Plasmamembran lokalisiert als auch mit dem Cytoskellett assoziiert sind. Aktivierung von Calpainen in immortalisierten Keratinozyten zeigte, dass Calpaine P1a und ITGβ4 abbauen können, und dass sich dieser Abbau durch Inaktivierung von Calpainen mittels spezifischer Peptid-Inhibitoren verhindern ließ. In terminal diffe-

renzierenden primären Mauskeratinozyten erhöhte die Inhibierung von Calpainen die Proportion an inaktiven Calpainen und gab somit Hinweise darauf, dass Calpaine auch im Zuge der terminalen Differenzierung aktiv sind. Mithilfe einer EBS-Ogna Mauslinie konnte ich des Weiteren die Beteiligung von Calpainen im Zuge der Pathogenese von EBS-Ogna zeigen. In primären Keratinozyten von EBS-Ogna Mäusen führte die Inhibierung von Calpainen zu einer Erhöhung der P1a Proteinspiegel und Anzahl an HD-Proteinen angereicherten Komplexen. Zusätzlich wurde eine klonale Zelllinie von Keratinozyten etabliert, welche die EBS-Ogna Mutation heterozygot exprimieren ($Plec^{wt/Ogna}$). Im Zuge der Etablierung zeigten die $Plec^{wt/Ogna}$ Klone ein langsameres Wachstum verglichen mit den Zellen des Wildtyps. Diese Beobachtung könnte eventuell einen Hinweis auf eine bisher nicht beschriebene Funktion von HDen (und HPCs) als Zellproliferationregulatoren liefern.

Curriculum Vitae

Personal Data

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