Flotillins as novel regulators of desmosome dynamics

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Frauke Völlner

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Johann Wolfgang Goethe - Universität als Dissertation angenommen.

Dekanin:

Prof. Dr. Meike Piepenbring

Gutachter:

Prof. Dr. Anna Starzinski-Powitz (Johann Wolfgang Goethe-Universität, Frankfurt a. M.)

Prof. Dr. Ritva Tikkanen (Justus Liebig-Universität, Gießen)

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Für meine Kinder

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Abbreviations

AA	Amino acid
AJ	Adherens junction
Ala	Alanin
ANOVA	Analysis of variance
APS	Ammonium persulfate
ARM	Armadillo
BACE1	Beta-site APP cleaving enzyme
BSA	Bovine serum albumin
Cad	Cadherin
CAM	Cell adhesion molecule
Cas9	CRISPR-related
Cat	Catenin
CIE	Clathrin independent endocytosis
CME	Clathrin mediated endocytosis
CoIP	Co-Immunoprecipitation
CPD	Cytoplasmic domain
CRISPR	Clustered regularly interspaced short palindromic repeats
Cys	Cystein
DABCO	1,4-diazabicyclo[2.2.2]octane
DNA	Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DBDA	Dispase based dissociation assay
DMEM	Dulbecco's Modified Eagle Medium
Dsc	Desmocollin
Dsg	Desmoglein
DSM	Desmosome
DTD	Desmoglein terminal domain
DTT	Dithiothreitol
EA	Extracellular anchor region
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growt factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ESCRT	Endosomal sorting complex required for transport
F1-KO	Flotillin-1 knockdown
F2-KO	Flotillin-2 knockdown
FCS	Fetal calf serum
Flot-1	Flotillin-1
Flot-2	Flotillin-2
FRS2	Fibroblast growth factor substrate 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGA	Golgi-associated, gamma-ear containing
Gly	Glycine

GPI	Glycophosphatidylinositol
GST	Glutathione S transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HRS	Hepatocyte growth factor regulated tyrosine kinase substrate
HS	Horse serum
IA	Intracellular anchor region
ICS	Intracellular cadherin like sequence
IF	Immunofluorescence
Ig	Immunoglobulin
iPS	Induced pluripotent stem cells
IP	Immunoprecipitation
IPL	Intracellular proline-rich linker
IPTG	Isopropyl-ß-D-thiogalactopyranosid
KD	Knockdown
kDa	Kilo Dalton
KO	Knockout
MAPK/MAP kinase	Mitogen-activated protein kinase
MBP	Maltose-binding protein
MiTMAB	Tetradecyltrimethylammonium bromide
MW	Molecular weight
NEAA	Non essential amino acids
NOG	N-Octyl-s-D-glucopyranoside
NP-40	Nonidet P-40
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHB	Prohibitin homology
PI	Protease inhibitor
PKP	Plakophilin
PV IgG	Pemphigus vulgaris immunoglobulin/ auto-antibodies
RNA	Ribonucleic acid
RT	Room temperature
RTK	Receptor tyrosine kinase
RUD	Repeat unit domain
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
shRNA/siRNA	Small hairpin RNA/ small interfering RNA
SPFH	Stomatin/prohibitin/flotillin/HflK/C
TBS-T	Tris buffered saline tween
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
WB	Western blot
WT	Wild type

Summary

Multicellular organisms require that cells adhere to each other. This cell-cell adhesion is indispensable for the formation and the integrity of epithelial structures, tissues and organs. Mammals have developed four different cell-cell adhesion structures, the adhering junctions, which ensure the tight contact between cells but are also important platforms for communication and exchange in tissues. Two of these adhering junctions are cadherin based, the belt-like adherens junctions and the spot-like desmosomes. Both structures have in common that they are composed of single membrane spanning proteins, the cadherins, which accomplish adhesion in a calcium-dependent manner. The intracellular parts of classical as well as desmosomal cadherins bind to different adaptor proteins of the armadillo-protein family and to other proteins which build a protein plaque underneath the membrane and link the cadherins to the actin or intermediate filament cytoskeleton.

Desmosomes are of special importance for tissues that have to withstand mechanical stress. Although they are essential to stabilize tissues, they have to be highly flexible and dynamic structures, as processes like wound healing or tissue remodeling require that adhesive interactions can be modulated. The molecular dynamics within desmosomes are not jet understood in detail, but it is assumed that two different membrane associated pools of desmosomal cadherins exist in cells. Cadherins that are incorporated in mature desmosomes are part of the junctional pool, whereas cadherins that are not associated with firm desmosomes and the intermediate filament cytoskeleton belong to the non-junctional pool. Lateral movements between the two pools result in a dynamic equilibrium and allow for example the replacement of old cadherins. Little is known about the breakdown of desmosomal cadherins. Several studies found that desmosome assembly and endocytosis are cholesterol dependent processes and suggested that membrane microdomains play a role in the regulation of desmosome dynamics. Moreover, membrane rafts may be involved in the pathomechanism of the desmosome associated disease pemphigus, were autoantibodies bind to the cadherin desmoglein-3 and trigger its internalization, which results in loss of adhesion in skin cells.

Membrane rafts are cholesterol dependent nanoscale structures of cellular membranes that are able to regulate the distribution of proteins within the plasma membrane and thus form platforms for cell signaling and membrane trafficking. Flotillins are proteins that are associated with membrane rafts and are reported to be involved in processes like endocytosis, endosomal sorting and a multitude of different signaling events. We could recently show that the membrane raft associated proteins flotillin-1 and flotillin-2 directly bind to the armadillo protein y-catenin which can be part of both the adherens junction and the desmosome. The aim of this study was to elucidate a possible role of flotillins in the regulation of desmosomes.

HaCaT keratinocytes were chosen as the main cell system for this study, and the association of desmosomal components with flotillins was first analyzed in detail. It was found that flotillins are clearly associated with desmosomal proteins. They colocalize with desmoglein-3 at cell borders and precipitate with the other desmogleins. Further binding assays revealed that both flotillins bind to all desmogleins and the long isoforms of the

second class of desmosomal cadherins, the desmocollins. The interaction is direct, and it was mapped to the ICS sequence within the cadherins. This close association rendered the question whether flotillins are functionally implicated in desmosome regulation. To address this issue, stable flotillin knockdown HaCaT cells were analyzed in detail. The cellular staining pattern of desmoglein-3, desmoglein-1 and two plaque proteins was clearly altered in the absence of flotillins. The membrane staining of all tested desmosomal proteins was derailed and disordered. Furthermore, loss of flotillins had an impact on the adhesive capacity of HaCaT keratinocytes. The cell-cell adhesion was weakened in the absence of flotillins, which was manifested by an increased fragmentation of knockdown cells in a cell dissociation assay.

In order to find out the mechanism by which flotillins influence the membrane morphology and the adhesiveness in keratinocytes, the association of desmosomal proteins with membrane microdomains was examined. A predominant part of desmoglein-3 is associated with membrane rafts in HaCaT keratinocytes, whereas only a minor part of desmoglein-1 is found there. However, the raft-association of none of the examined proteins was altered in the absence of flotillins. Furthermore, flotillin depletion did not change the distribution of desmogleins with the two different cadherin membrane pools. Less desmoglein-3 was found in the junctional pool of the flotillin depleted cells as compared to the control cells, but this was due to an overall diminished desmoglein-3 protein level in these cells.

Flotillins are involved in endocytic processes but their exact role therein is under debate. The endocytic uptake of desmosomal cadherins requires intact membrane rafts, but the precise mechanism is still unknown. A possible involvement of flotillins in the endocytosis of desmoglein-3 was addressed next. It is known that the internalization of desmoglein-2 is dependent on the GTPase dynamin, arguing for an involvement of dynamin in the endocytosis of desmoglein-3 as well. When dynamin and thus desmoglein-3 endocytosis was inhibited using chemical compounds, the mislocalization of desmoglein-3 that was observed in flotillin knockdown cells was ameliorated. This suggest that inhibition of desmoglein-3 endocytosis enhances the amount and/or availability of desmoglein-3 at the plasma membrane, which then normalizes the morphological alterations caused by a knockdown of flotillins. Furthermore, the morphological alterations in the flotillin knockdown HaCaT cells were found to be similar to the localization of desmoglein-3 that was observed upon treatment of keratinocytes with PV IgG. These structures have been described before as linear arrays and are assumed to be sites of endocytic uptake. This strengthens the idea that enhanced desmoglein-3 internalization takes place in the absence of flotillins, which then results in a weakened adhesion.

Taken together, this study revealed flotillins as novel players in desmosome mediated cell-cell adhesion processes. By binding to desmosomal cadherins and desmosomal plaque proteins, flotillins stabilize desmosomes at the plasma membrane and are required for a proper cell-cell adhesion.

1 Introduction

1.1 Cell-cell adhesion

Cell-cell adhesion is essential for the formation and integrity of all epithelial structures like the skin or mammary gland. Different types of adhesion structures do not only ensure the tight contact between neighboring cells, but are also crucial for communication and regeneration processes like wound healing. There are four different cell-cell adhesion structures found in mammalian cells. The tight junctions build an impermeable seal between the apical and basolateral membrane in epithelial cells and are crucial for the establishment of the epithelial barrier function. The gap junctions are channel forming connections between cells that are important for the fast exchange of ions or small molecules between some cells, like myocardial cells. Furthermore, there are two anchoring junctions, the adherens junctions and the desmosomes, which are thought to link cells to each other in cooperation with the cytoskeleton. The anchoring junctions and their molecular composition will be addressed here in more detail.

1.1.1 The anchoring junctions

Within the group of cell adhesion molecules (CAMs) the cadherin family belongs to the calcium-sensitive adhesion proteins. In epithelial cell layers, cadherins are distributed along the whole lateral membrane of cells. Either they appear as single molecules or disordered oligomers, which represent an extra-junctional pool of cadherins, or they are found as concentrated, well-ordered structures, the cell junctions. Cadherins are the main constituents of the anchoring junctions, the belt-like adherens junctions (AJs) and the spot-like desmosomes (DSMs) (Delva et al., 2009; Harris and Tepass, 2010).

In general, adherens junctions and desmosomes share a common molecular structure and are composed of the same protein classes, but they are constituted by distinct proteins. For example, the so-called classical cadherins like E-cadherin or N-cadherin are part of the adherens junctions, whereas desmosomes have their own desmosomal cadherins, the desmogleins and desmocollins. The only protein that has so far been found to be part of both structures is the armadillo-protein γ-catenin or plakoglobin. However, the structure of desmosomes and adherens junctions is very similar and can be simplified as follows: Cadherins are large single-span transmembrane proteins that bind with their extracellular parts to cadherins of the adjacent cells in a calcium dependent manner. In the cytosol, cadherins are associated with a multifunctional and dynamic set of adaptor molecules like the armadillo family proteins and others which build a protein plaque underneath the membrane and link the cadherins to the actin or intermediate filament cytoskeleton. Association to cytoskeletal structures is a prerequisite for proper cell adhesion.

The pools of junctional and extra-junctional cadherin-catenin complexes in a cell are in a dynamic equilibrium. New cell-cell contacts are formed by lateral cis-interactions of cadherins at the plasma membrane. This leads to the formation of dimers or higher-ordered oligomers in one cell. Thereafter, trans interactions with cadherin complexes from adjacent

cells are formed, followed by more cis interactions, and so on. This results in a clustering of cadherin complexes and the formation of new junctions (Steinberg and McNutt, 1999).

Cadherins are continuously synthesized and supplied to the plasma membrane and internalized via a clathrin-dependent pathway (Green et al., 2010). However, also other routes of internalization are possible, as E-cadherin has been reported to be internalized by macropinocytosis (Bryant et al., 2007). Disordered internalization of cadherin complexes is associated with diseases like cancer and the autoimmune disease pemphigus vulgaris (Delva et al., 2008; Mosesson et al., 2008).

1.2 The desmosome

Desmosomes are of particular importance for tissues that have to resist extensive and permanent mechanical stress like the bladder, the heart and the skin. In simple epithelium and the upper layer of stratified epithelial structures, they are distributed along the lateral membrane, whereas in stratified epithelium, they connect all cells and cell layers. Specialized structures, the hemidesmosomes, connect the basal layer of epithelial cells to the basal membrane. Desmosomes are associated with intermediate filaments in cells and serve as anchoring points of a filament meshwork that draws through the whole epithelium.

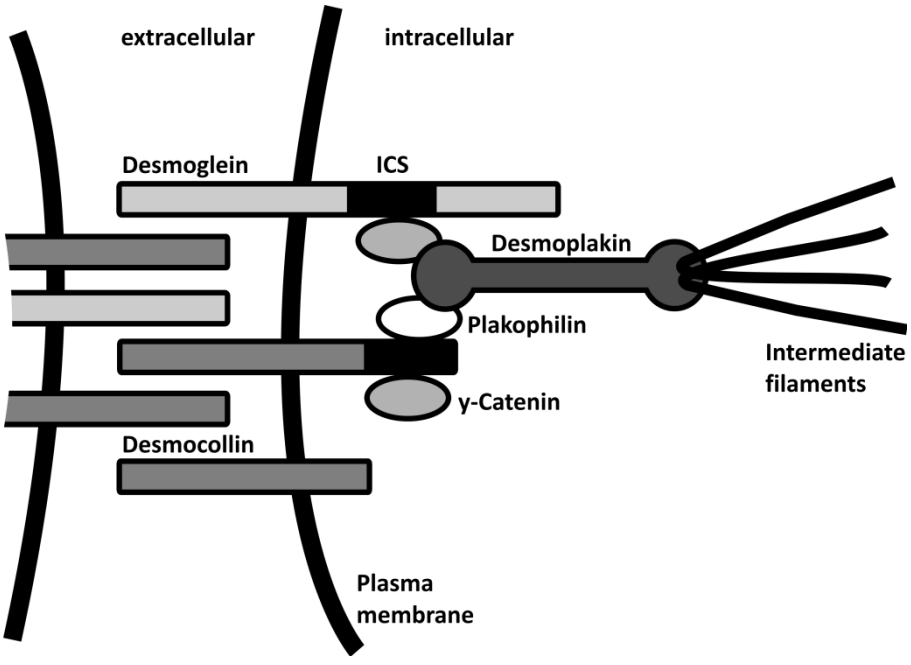

Figure 1.1: Model of the molecular structure of the desmosome.
The desmosomal cadherins, desmogleins and desmocollins are single span transmembrane proteins that bind to cadherins of adjacent cells in the extracellular space. Intracellularly, they are attached to proteins of the armadillo family (γ-catenin and plakophilins) which bind to the intracellular cadherin-like sequence (ICS) in the cadherins. The armadillo proteins are associated with the giant protein desmoplakin that facilitates attachment to the intermediate filament cytoskeleton of the cell.

1.2.1 Structure

The molecular structure of the desmosome can be subdivided into three parts (Fig 1.1): Desmosomal cadherins (I) interact with cadherins from adjacent cells. Intracellularly, they are connected to plaque proteins of the armadillo protein family (II) which interact with proteins of the plakin family (III). Plakin family members facilitate the interaction to the intermediate filaments. The desmosomal cadherins are the desmogleins and desmocollins. In mammals, four different desmogleins (Dsg) and three desmocollins (Dsc) exist. Their extracellular parts consist of four cadherin repeats with calcium binding sites and an extracellular anchor region (EA) close to the plasma membrane. In the cytosol, they share an intracellular anchor region (IA) and an intracellular cadherin-like sequence (ICS) which serves as a binding site for plaque proteins and is also present in classical cadherins (Delva et al., 2009; Mathur et al., 1994; Troyanovsky et al., 1994). Due to alternative splicing, each desmocollin exists as a long isoform (a) which contains the ICS sequence and a truncated splice variant (b) that lacks the ICS (Troyanovsky et al., 1993). Both isoforms are expressed in equal quantities, although the function of the b isoforms is still unknown (Goonesinghe et al., 2012).

The desmogleins have additional intracellular parts that are absent in the desmocollins. An intracellular proline-rich linker (IPL) is followed by a variable number of repeat unit domains (RUD) and a desmoglein terminal domain (DTD) (Fig 1.2). The function of the desmoglein unique sequences is not known at the moment (Delva et al., 2009). All desmosomal cadherins are expressed in a differentiation and tissue-dependent manner. In the skin, the cadherin expression changes with the cell layers in the stratified epithelium (Getsios et al., 2009).

The desmosomal plaque proteins comprise plakoglobin, also known as γ-catenin, and the plakophilins. These proteins belong, together with proteins like ß-catenin, p120-catenin or δ-catenin, to the armadillo protein family. Members of this protein family share a variable number of repeating units of a 42 amino acid sequence, the so called arm repeat (Kowalczyk and Green, 2013). γ-Catenin is so far the only protein that is associated with both the adherens junction and the desmosome, although its affinity for desmosomes is greater than for adherens junctions (Chitaev et al., 1996). It binds to the ICS sequence in cadherins and to desmoplakin (Chitaev et al., 1998). The three different human plakophilins (Pkp 1-3) are also able to bind desmoplakin and the desmosomal cadherins. It was proposed that plakophilins are especially important for the lateral interactions in the desmosomal plaque (Kowalczyk et al., 1999). However, not only the linkage to the cytoskeletal adaptors is facilitated by the armadillo proteins, but also signaling pathways are influenced. Catenin family members are known regulators of gene expression. Both ß-catenin and γ-catenin are able to translocate to the nucleus and function as transcription factors (Swope et al., 2013).

Plakin family members link cytoskeletal networks to the plasma membrane. The two splice variants desmoplakin I and II are able to bind intermediate filaments directly and are the key linkers between intermediate filaments and desmosomal plaque components in humans (Green et al., 1992). Desmoplakin is of essential importance for the function of

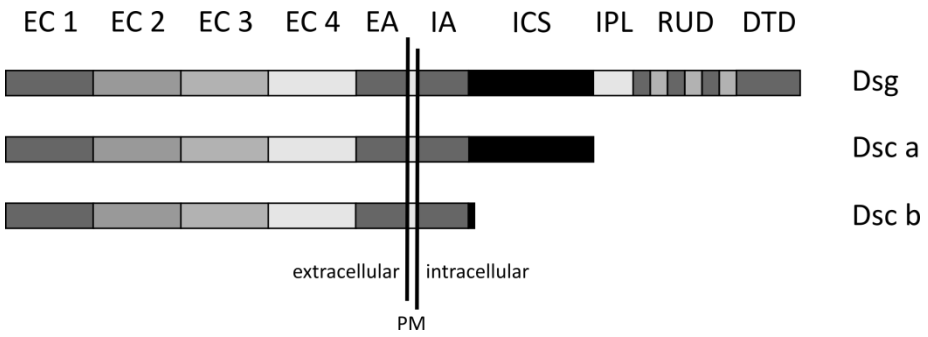

Figure 1.2: Domain structure of the desmosomal cadherins.
Desmogleins (Dsg) and desmocollins share extracellular domains and parts of their intracellular structure. The extracellular parts are composed of four extracellular cadherin homology repeats (EC), followed by an extracellular anchor domain (EA) and a transmembrane domain. In the cytosol, all desmosomal cadherins share an intracellular anchor domain (IA). The intracellular cadherin-like sequence (ICS) is only present in the desmogleins and in the long isoforms "a" of the desmocollins. The desmogleins have additional desmoglein unique regions, an intracellular proline-rich linker (IPL) is followed by a varying number of repeat unit domains (RUD) and a desmoglein terminal domain (DTD). Number of RUDs in the desmogleins: Dsg-1: 5 repeats; Dsg-2: 6 repeats; Dsg-3: 2 repeats; Dsg-4: 3 repeats. Abbreviations: PM, plasma membrane

desmosomes, as evidenced by desmoplakin knockout mice that die early in embryogenesis due to the lack of proper desmosomal adhesion (Gallicano et al., 1998).

1.2.2 Homeostasis and dynamics

Although desmosomes are important for the stabilization of tissues, they are highly flexible structures. Processes like wound healing, tissue remodeling or the basal to apical proliferation of cells in stratified tissues require that adhesive interactions can be modulated.

The formation of desmosomes requires the presence of mature adherens junctions. In keratinocytes depleted of classical cadherins, the assembly of desmosomes is hampered (Michels et al., 2009). Like classical cadherins, newly synthesized desmosomal cadherins follow a typical secretory route. Precursor propeptides are synthesized into the ER lumen, transported to the Golgi where they are glycosylated, followed by a transport to the plasma membrane (Green et al., 2010; Pasdar and Nelson, 1989; Shore and Nelson, 1991). In addition, an association of γ-catenin to the desmoglein tails seems to be important for the correct transport as well as for the stability of the cadherins at the plasma membrane (Andl and Stanley, 2001; Pasdar et al., 1995). Once at the plasma membrane, the desmogleins are probably stabilized by dimerization of their intracellular unique regions (Chen et al., 2012). However, desmosome assembly seems to be a complex process that requires the coordinated delivery of plaque proteins and cytoskeleton components to the plasma membrane and its details are not entirely understood yet (Nekrasova and Green, 2013).

Based on FRAP microscopy data, it was proposed that two pools of plasma membrane-associated desmosomal cadherins (junctional and nonjunctional) exist. Both pools are in an equilibrium with a rapid mutual exchange of contents (Windoffer et al., 2002). This idea was further supported by observations in migrating cells, where the clustering of desmosomal cadherins into desmosomes arises from proteins already present at the cell surface rather than from a cytosolic vesicle pool (Roberts et al., 2011). Due to its

association with the intermediate filament network, the junctional pool of desmosomal cadherins is insoluble in buffers containing Triton X-100. The nonjunctional desmosomal pool is considered to lack the association to the intermediate filaments and is therefore soluble in such buffers, which allows the separation of the two protein pools by simple sequential detergent extraction (Stahley et al., 2014). It was proposed that desmosomal cadherins in the nonjunctional pool may be rapidly endocytosed and recycled or even degraded, although this has not been experimantally shown. In contrast, it was stated that cadherins from the junctional pool can be internalized as complete desmosomal halves (Mattey and Garrod, 1986; Nekrasova and Green, 2013). For E-cadherin, it was shown that growth factor stimulation resulted in its phosphorylation and subsequent ubiquitination, followed by a clathrin-dependent endocytosis and lysosomal degradation (Fujita et al., 2002). It remains open whether desmosomal cadherins follow a similar degradation pathway.

Studies investigating the breakdown of desmosomes most often take advantage of the autoimmune disease pemphigus, where autoantibodies against desmosomal components result in the loss of desmosomal adhesion in keratinocytes (see below). Less is known about the nonpathogenic regulation of desmosomal cadherin endocytosis. However, the endocytosis and stability of desmosomal cadherins was addressed in some studies. A connection between membrane rafts and desmosomal stability was observed. Therefore, this issue is described in the lipid raft chapter below (see 1.3.1).

1.2.3 Pemphigus

Pemphigus is a severe autoimmune bullous disease of the skin and mucosal areas that is painful and potentially life-threatening. It is a rare disease with less than 2000 new cases per year in Germany (Deutsches Ärzteblatt, 2011). Two major forms of pemphigus are distinguished with reference to the self-antigens that are affected. In pemphigus foliaceus, autoantibodies against desmoglein-1 lead to the dissociation of keratinocytes in the upper layers of the skin. In the more severe form pemphigus vulgaris, the autoantibodies are directed against desmoglein-3, whereby more basal epidermal layers as well as mucosal areas are affected (Waschke, 2008). Besides these two main self-antigens, several other desmosomal as well as nondesmosomal self-antigens have been described to contribute to the course of the disease (Kurzen and Brenner, 2006; Pan et al., 2011).

The blistering that occurs in pemphigus is always the consequence of the dissociation of epithelial cells, a process that is called acantholysis. Acantholysis is triggered by the autoantibodies (PV IgG) that bind to the extracellular parts of desmosomal cadherins (Amagai et al., 1991). However, the exact mechanisms that lead to the disruption of desmosomes are still under investigation and several different hypotheses are frequently discussed among researchers. The main difference in the discussed scenarios is whether the binding of the autoantibodies to desmoglein-3 results in the hindrance of cadherin-cadherin interaction in the extracellular space (steric hindrance) or whether the binding to the cadherin activates signaling cascades that indirectly lead to the dissociation of cells (Caldelari et al., 2001; Futei et al., 2000; Heupel et al., 2008). Also a combination of these events is conceivable (Sharma et al., 2007). Moreover, it is unclear which signaling

pathways are activated by the PV IgGs and which cellular events are triggered by the signaling. It was published that signaling via acetylcholine receptors, Rho GTPases, Src-kinases or tyrosine kinases may be influenced by PV IgG (Chernyavsky et al., 2008; Delva et al., 2008; Tsang et al., 2012; Waschke et al., 2006) and that apoptosis or depletion of cell surface desmoglein-3 are induced (Grando, 2012; Pan et al., 2011).

Calkins and colleagues found that desmoglein-3 is internalized in response to PV IgG and colocalizes with endosomal and lysosomal markers, suggesting that it is targeted for degradation (Calkins et al., 2006). Later, the same group was able to show that the endocytosis of desmoglein-3 in response to PV IgG follows a temporally predictable sequence of events. In a first phase, nonjunctional desmoglein-3 is internalized, followed by a change in desmoglein-3 distribution at the plasma membrane and a rearrangement of junctional desmoglein-3 into so called linear arrays. These linear arrays are structures perpendicular to the plasma membrane and are considered to be sites of endocytic uptake. Finally, the virtually complete depletion of cell surface desmoglein-3 results in the loss of cell-cell adhesion (Jennings et al., 2011). The endocytosis of desmoglein-3 is presumably regulated by the p38 MAP kinase which is activated in response to PV IgG and whose inhibition blocks the internalization of desmoglein-3 (Berkowitz et al., 2005; Jolly et al., 2010). The mechanism of desmoglein-3 endocytosis was investigated further. Upon PV IgG treatment desmoglein-3 is endocytosed by a clathrin and dynamin-independent mechanism. The internalization of desmoglein turned out to be dependent on cholesterol, hence an involvement of the lipid raft protein caveolin-1 was investigated. However, the endocytic uptake of desmoglein-3 emerged to be independent of caveolin-1 as well (Delva et al., 2008). Nevertheless, this points to a role for lipid rafts in the regulation of desmoglein endocytosis in the pathomechanism of pemphigus vulgaris.

1.3 Lipid rafts in cell adhesion

Membrane or lipid rafts (also membrane microdomains) are nanoscale structures of cellular membranes that are enriched in cholesterol and glycosphingolipids (Fig 1.3). One characteristic feature of rafts is their ability to facilitate compartmentalization of lipid membranes. They are supposed to be flexible and mobile structures that are able to move freely within the lipid bilayer and, upon certain stimuli, are able to fuse to larger platforms. As a wide range of membrane associated proteins is either permanently or transiently associated with lipid rafts, the membrane-organizing capacity of lipid rafts enables cells to cluster certain proteins at defined positions within the plasma membrane. These protein clusters serve as platforms for diverse cellular functions in a spatiotemporal manner. They are implicated in a multitude of functions like cellular trafficking, endosomal sorting, signal transduction or virus budding (Lingwood and Simons, 2010; Staubach and Hanisch, 2011).

Studies from the late 1990s linked lipid rafts for the first time to immunological processes. In contrast to the strong and stable adhesion that is found in tissues, immune cells require adhesive interactions that are rather transient and weaker for their functions (e.g. the rolling of blood cells along endothelial cells to sides of infection). It was found that lipid

rafts help to cluster proteins involved in the formation of the so-called immunological synapse, the transient contact zone between a T cell and an antigen-presenting cell that contains adhesion and signaling proteins (Dykstra et al., 2003; Moran and Miceli, 1998; Viola, 1999). In addition, lipid rafts influence clustering of adhesion molecules upon activation of the CD4 receptor of T cells (Nguyen et al., 2005). Furthermore, cell adhesion between B cells and target cells is diminished upon depletion of membrane cholesterol which destroys lipid rafts (Zhang et al., 2009).

Beside their function in immune cells, lipid rafts are also implicated in strong cell-cell adhesion processes. Mukai and colleagues could show that intact lipid rafts are required for a proper cell fusion of myogenic cells and that the adhesion proteins M-cadherin, p120-catenin and ß-catenin accumulate at cell fusion sites in a lipid raft dependent manner. The fusion of these cells may also be dependent on the dynamic properties of lipid rafts, as it was found that the fusion site is cadherin and raft-free directly prior to the process of membrane fusion (Mukai et al., 2009). In a later study, the same group found that src kinase activity is required to recruit the p120-catenin-M-cadherin complex to lipid rafts (Mukai and Hashimoto, 2013). Also N-cadherin was found to colocalize with lipid rafts at cell contact sites in myoblasts, and disruption of rafts resulted in a weakened cell-cell adhesion and affected N-cadherin accumulation at cell-cell contacts in these cells (Causeret et al., 2005).

Cell-cell adhesion is indispensable for the structure, function, polarization and integrity of epithelial layers. Like in nonepithelial cells, intact lipid rafts seem to be involved in cell-cell adhesion in epithelial cells. In endothelial cells, nonjunctional VE-cadherin clusters and is localized in cholesterol rich membrane microdomains. Furthermore, an ample reduction in cholesterol levels results in a loss of VE-cadherin function (Baumgartner et al., 2014). In mammary epithelial cancer cells, N-cadherin was found to co-localize with lipid rafts at cell-cell junctions (Boscher et al., 2012). In addition, there is again evidence for an involvement of src kinase activity, as a restricted inhibition of src kinase in lipid rafts interferes with a proper cell-cell adhesion in the mammary epithelial cancer cell line MCF7 (Hitosugi et al., 2007).

1.3.1 Lipid rafts and desmosome dynamics

Several studies found desmosomal components associated with lipid rafts and postulated that desmosome assembly or endocytosis are cholesterol dependent processes (Brennan et al., 2011; Chen et al., 2012; Delva et al., 2008; Stahley et al., 2014). However, it is not entirely clear which raft associated proteins are implicated in the regulation of desmosome dynamics. Caveolin-1 was found to interact with desmoglein-1 and -2 (Brennan et al., 2011), but endocytosis of desmoglein-2 and -3 seems to be a caveolin-1 independent process (Chen et al., 2012; Delva et al., 2008). The importance of dynamin for desmoglein endocytosis is unclear as well, as it was seen that inhibition of dynamin is able to restore desmoglein-2 level at the cell surface (Chen et al., 2012), whereas others found the endocytosis of desmoglein-3 (in response to PV IgG) to be independent of dynamin (Delva et al., 2008). It is very likely that the mechanism of normal desmoglein endocytosis is

different from the pathomechanism of PV IgG induced desmoglein endocytosis and/or that the different desmogleins are internalized by different mechanisms.

1.4 Flotillins

Flotillin proteins are expressed in almost every mammalian cell type. They show a divergent intracellular localization pattern but are assumed to be permanently associated with membranes. Already their discovery in Triton X-100 -insoluble membrane fractions led to the assumption that flotillins are membrane associated proteins, although no typical transmembrane domains were found (Schulte et al., 1997). Moreover, they were thought to be located in membrane microdomains (Bickel, 1997). Future studies found that flotillins are cytosolic proteins that mainly reside in noncaveolar lipid rafts, a special type of membrane microdomains that are enriched in cholesterol and glycosphingolipids (Fernow et al., 2007; Frick et al., 2007; Glebov et al., 2006). Meanwhile, flotillins, especially flotillin-1, serve as well established marker proteins for lipid rafts.

1.4.1 Structure

The two homologues, flotillin-1 and flotillin-2 share 44 % sequence identity on protein level and are predicted to have a similar topological structure (Schulte et al., 1997). In their N-terminus, both proteins contain a stomatin/prohibitin/flotillin/HflK/C (SPFH) domain, also known as the prohibitin homology (PHB) domain. Towards the C-terminus, flotillins share a conserved unique flotillin domain (Fig 1.3 B).

The exact function of the SPFH domain is not yet evident, but proteins sharing this structure are known to be associated with lipid rafts and to be able to form oligomers, both features also being true for flotillins. SPFH domains contain several hydrophobic parts which are supposed to form loops into the inner leaflet of the membrane, as well as sites for acylation (Browman et al., 2007). Hence, also flotillins carry fatty acid modifications in their N-terminal parts, although they differ in the modified site and the kind of fatty acid that is attached. Flotillin-1 carries a single palmitoylation site at Cys34 that has been shown to be necessary for its membrane association in kidney cells (Morrow et al., 2002). Later it was found in adipocytes that a mutation of this site did not affect membrane localization but that two short hydrophobic amino acid stretches in the N-Terminus are important for membrane association, indicating that cell type specific differences concerning flotillin-1 membrane association may exist (Liu et al., 2005). Flotillin-2 differs from flotillin-1 in the mechanism of its membrane attachment. Studies by our group show that the protein is myristoylated at Gly2 and carries palmitoylation sites at cysteins Cys4, 19 and 20. The myristoylation is presumably a prerequisite for the palmitoylation, as a mutant Gly2Ala protein is not acylated at all. Furthermore, the nonacylated mutant is located in the cytoplasm, which indicates that fatty acid modifications are necessary for the membrane association of flotillin-2 (Neumann-Giesen et al., 2004, 2007).

Less is known about the C-terminal flotillin domain. It contains three predicted coiled-coil stretches and seems to be important for the oligomerisation of flotillins (Solis et al., 2007). Flotillins are capable of forming homo as well as hetero-oligomers, and this clustering

appears to be an essential feature for many functions of the proteins. For example, hetero-oligomerization is required for the endocytosis of flotillins in response to growth factor stimulation (Babuke et al., 2009). Furthermore, oligomerization is important for membrane and also raft-association of flotillins per se (Solis et al., 2007).

Flotillin-1 and flotillin-2 are expressed from two different genetic loci. Despite their almost ubiquitous expression, it was shown that activation of the MAP kinase pathway results in increased expression of both flotillins (Banning et al., 2012). The expression of flotillin-1 depends very much on flotillin-2 levels, whereas the other way around, the dependence is less strong (Solis et al., 2007). A flotillin-2 knockout mouse model was recently generated to study the effects of flotillin ablation in the living animal. Reduced protein expression of flotillin-1 is detectable in the flotillin-2 knockout mouse, whereas flotillin-1 mRNA levels are even elevated, due to increased MAP kinase signaling (Banning et al., 2014a).

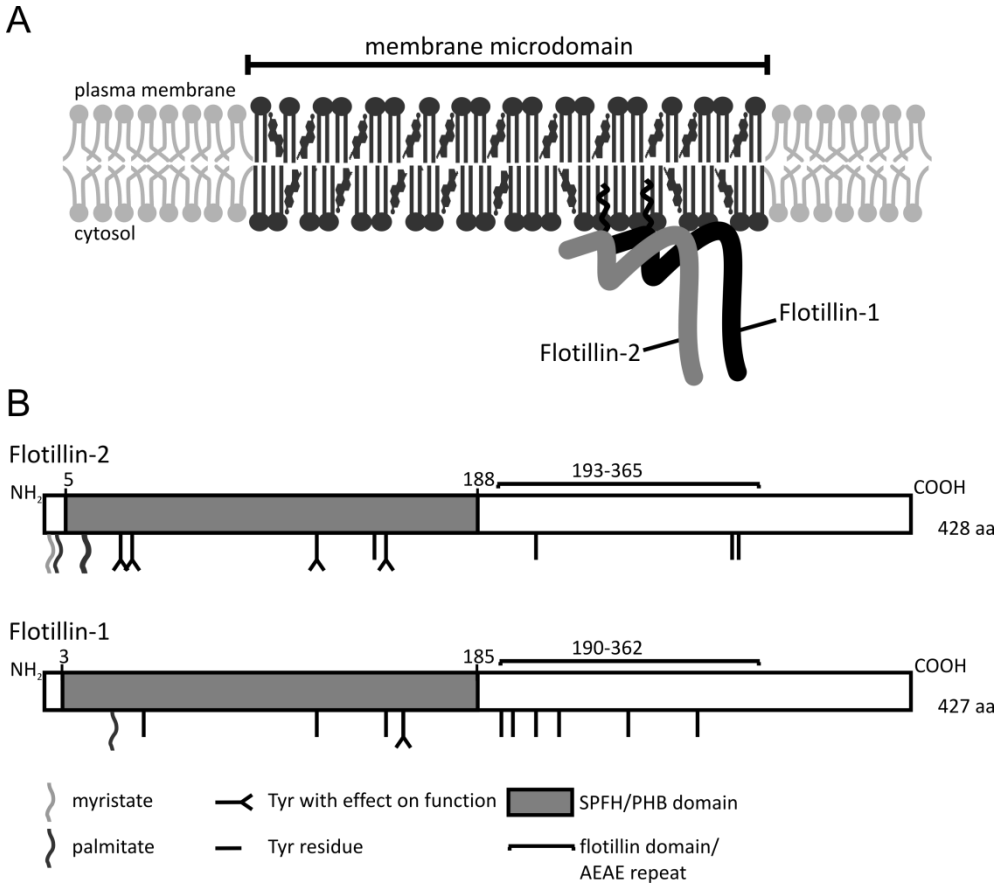

Figure 1.3: Flotillins are membrane raft associated proteins.
A) Membrane microdomain in the plasma membrane. Membrane microdomains are mobile structures in lipid membranes that are enriched in cholesterol and glycosphingolipids. In the cytosol flotillin-1 and flotillin-2 are associated with membrane microdomains by fatty acid modifications in their N-terminus. B) Domain structure of flotillin-1 and flotillin-2 proteins. Flotillins share 44% sequence identity on protein level. Both proteins contain an N-terminal SPFH/ PHB domain and a C-terminal flotillin domain. Flotillin-1 carries a single palmitoylation site, whereas flotillin-2 is myristoylated and carries repeated palmitoylation sites. Abbreviations: Tyr, tyrosine. Modified from M. Meister, PhD Thesis.

Flotillins are very well conserved in mammals, for example rat and human flotillin-2 share 97 % sequence identity on protein level. Also among other species, vertebrates as well as invertebrates, flotillins are found and are quite well conserved (Banning et al., 2014a). Even in some plants, bacteria and fungi flotillin-related proteins are found (Otto and Nichols, 2011).

1.4.2 Localization

The subcellular localization of flotillins is dependent on the cell type, culturing conditions, nutrition, and differentiation status of the cells. Flotillins are either localized at the plasma membrane or in diverse intracellular compartments. So far, they have been reported to reside e.g. in early and late endosomes, lysosomes, exosomes and phagosomes (Babuke et al., 2009; Dermine, 2001; Glebov et al., 2006; Staubach et al., 2009). Their localization can change rapidly, as they translocate from the plasma membrane to the endosomal compartment within minutes after growth factor stimulation (Neumann-Giesen et al., 2007). It is a tendency that flotillins are predominantly found at the plasma membrane in differentiated/confluent cells, whereas in undifferentiated/subconfluent cells, flotillins are mainly localized in vesicular structures in the cytoplasm (Ha, 2003; Kurrle et al., 2013; Langhorst et al., 2008).

1.4.3 Function

Flotillins are involved in endocytic processes. It was even proposed that they would define a new clathrin-independent endocytosis (CIE) pathway that is independent of dynamin (Frick et al., 2007; Glebov et al., 2006). On the other hand, there is evidence that flotillins are important for clathrin-mediated endocytosis (CME), as they cluster different cargo molecules at the plasma membrane prior to endocytic uptake (Amaddii et al., 2012; Schneider et al., 2008; Sorkina et al., 2013). Our group could recently show that flotillin uptake itself upon growth factor stimulation is a dynamin-dependent process (Meister et al., 2014). Hence, the exact molecular role of flotillins in endocytosis is still under debate (Meister and Tikkanen, 2014).

Flotillins are also involved in transport and sorting processes within the cell. They have been reported to play a role in exosome release from the plasma membrane (Phuyal et al., 2014) and to participate in endosomal sorting (John et al., 2014; Saslowsky et al., 2010). It was shown by our group that flotillin-1 directly binds to the β-secretase BACE1 and that this interaction is required for the recycling of BACE1 from endosomes back to the plasma membrane (John et al., 2014).

As mentioned above, flotillins are responsive to growth factor stimulation and can influence MAP kinase signaling. Upon EGF stimulation, they are phosphorylated by Src family kinases at different tyrosine residues, which is important for their endocytosis (Neumann-Giesen et al., 2007; Riento et al., 2009). However, they are dispensable for the endocytosis of the EGFR. Nevertheless, it was shown that flotillin-1 is an important regulator of EGFR signaling by serving as scaffolding protein and enhancer of the MAPK signaling cascade (Amaddii et al., 2012). Flotillins presumably influence signaling via other RTKs, as flotillin-1 is able to bind to FRS2, an important adaptor protein of many

RTKs (Tomasovic et al., 2012). In addition, flotillins are supposed to participate in several other signaling events like insulin signaling, T cell receptor signaling or signaling via GPI-anchored proteins (Banning et al., 2014b; Otto and Nichols, 2011).

1.5 Flotillins in cell adhesion

When Schulte and colleagues discovered flotillins in 1997 they found that flotillin-2 showed sequence identity with a known protein, the epidermal surface antigen protein (ESA) that was first described in 1994. This protein was suggested to play a role in cell adhesion because it was detected by an antibody that caused cell detachment and stained human epidermis in a pemphigus vulgaris-like pattern (Schroeder et al., 1991, 1994). Although it was found later that the protein does not react with the antibody (Hazarika et al., 1999), these reports in the 1990s initially linked flotillins initially with cell adhesion processes.

1.5.1 Adhesion proteins are associated with flotillin microdomains

Since then, many biochemical studies found adhesion molecules partially associated with flotillin microdomains in various cell lines. For example, in human kidney cells a part of the endogenous E-cadherin and ß-catenin is found in flotillin-2 membrane fractions (Roitbak et al., 2005). Further, in the human colon carcinoma cell line HT-29, E-cadherin and p120-catenin associate with lipid rafts, defined by flotillin-1, in a differentiation dependent manner (Chartier et al., 2011). Recently, we could show that in the mammary epithelial cell line MCF10A, a minor part of endogenous E-cadherin as well as γ-catenin is associated with flotillin rafts (Kurrle et al., 2013). Also desmosomal cadherins were found in flotillin-1 microdomains (Resnik et al., 2011), which indicates that association with adhesion molecules is a general feature of flotillin microdomains.

In several epithelial cell lines as well as in myoblasts, flotillins were found to colocalize with E-cadherin and N-cadherin and catenin family members at the plasma membrane. This colocalization increases when cells grow confluent and adherens junctions mature. Flotillin depletion affects adherens junction morphology or organization at the plasma membrane. In epithelial cells, flotillin-2 depletion results in an abnormal E-cadherin staining at the plasma membrane, insofar as the signal is distributed over a wide and diffuse area, which might be explained by an overlap of cell membranes (Chartier et al., 2011; Kurrle et al., 2013; Solis et al., 2012). In MCF-7 breast cancer cells, E-cadherin and p120catenin staining was reduced upon flotillin-1 depletion, whereas in MCF10A breast epithelial cells, no effect on E-cadherin or γ-catenin was observed (Guillaume et al., 2013; Kurrle et al., 2013).

1.5.2 Flotillins interact with adhesion proteins

Flotillins exist in a complex with several classical cadherins and catenin-family members in diverse cell types. For example, they co-precipitate with the cadherins E-cadherin and N-cadherin, as well as with the catenins ß-catenin, α-catenin, p120 catenin and γ-catenin (Bodrikov et al., 2011; Guillaume et al., 2013; Kurrle et al., 2013). The composition of these complexes is very likely dependent on the differentiation status of cells and/ or the

maturation status of cell junctions, since only flotillin-2 is found in a complex with E-cadherin and γ-catenin in cells grown confluent for 5 days. Flotillin-1 is part of that complex only under a prolonged culture of confluent cells (Kurrle et al., 2013).

An association of flotillin-2 with γ-catenin was found in various cell lines, whereas the interaction with cadherins seems to be more cell type dependent. A knockdown of flotillin-2 results in an altered staining of γ-catenin at the plasma membrane in MCF10A cells, comparable to that of E-cadherin. This points towards an important connection of flotillins and γ-catenin. Indeed, work from our group revealed that both flotillins bind γ-catenin directly in an in-vitro system. The interaction site in γ-catenin was mapped to be the ARM domains 6-8 with a major contribution of ARM domain number 7 (Kurrle et al., 2013). Among the interaction partners of flotillins that are involved in cell adhesion, the interaction between γ-catenin and flotillins is the only direct one that is reported so far. As γ-catenin is a major component of desmosomes, it may serve as an adaptor that links flotillins to desmosomes.

1.5.3 Functional aspects

Flotillin depletion seems not only to affect the morphology of adherens junctions, but also interferes with cell-cell adhesion. A knockdown of flotillin-2 in A432 cells resulted in an increased fragmentation of cell sheets in a dispase based dissociation assay, a bona fide assay to measure the adhesive strength in a cell layer (Solis et al., 2012). In an aggregation assay that measures the ability of cells to re-aggregate out of a single cell suspension, the re-aggregation capacity of MCF7 cells and myoblasts was dramatically reduced upon flotillin-1 knockdown (Guillaume et al., 2013). The same study observed defects in epithelial integrity in MCF-7 cells depleted of flotillin-1, as those cells showed a reduction in monolayer thickness and tightness (measured by transepithelial resistance). The defects in cell-cell adhesion and epithelial integrity may be due to problems in the assembly of adherens junctions that have been observed in A431 cells where flotillin-2 depletion resulted in an uncoordinated and slow basal to apical flow of E-cadherin along the membrane (Solis et al., 2012).

The impact of flotillins on the endocytosis of desmosomal cadherins was addressed only in one publication so far. Chen et al. showed that the endocytosis of desmoglein-2 does not require flotillin-1 (Chen et al., 2012). However, the functional aspects of flotillins in cell-cell adhesion processes are not clear at the moment.

1.6 Aims of the present study

Several studies have linked flotillins to adhesion processes. Most of these studies focused on cell-cell adhesion that is mediated by adherens junctions and examined the role of flotillins in the regulation of classical cadherins (Bodin et al., 2014). On the other hand, there is strong evidence for an involvement of membrane rafts in the regulation of normal desmosomal function and homeostasis, as well as in the pathomechanism of the autoimmune disease pemphigus that targets desmosomes. However, a connection of flotillins and desmosomes was so far never examined in detail. This study was carried out to investigate whether and how flotillins are implicated in desmosomal adhesion in epithelial cells.

2 Materials

2.1 Chemicals and consumables

Common chemicals, reagents and enzymes are listed in table 2.1. All restriction enzymes were purchased from New England Biolabs (Frankfurt a. M., Germany). Chemicals that are not listed in table 2.1 were purchased from either AppliChem (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Taufkirchen, Germany) or Roth (Karlsruhe, Germany). Consumables like one way plastic ware, reaction tubes, etc. were purchased from BD Biosciences (Heidelberg, Germany), Greiner Bio-One (Frickenhausen, Germany), Sarstedt (Nümbrecht, Germany) or TPP (Trasadingen, Switzerland).

Table 2.1 Chemicals, reagents and enzymes

Name	Company
2-Mercapthoethanol	Roth, Karlsruhe, Germany
4',6-diamidino-2-phenylindole (DAPI)	Merck, Darmstadt, Germany
Ampicillin	Roth, Karlsruhe, Germany
Aprotinin	Roth, Karlsruhe, Germany
Bio-Rad protein assay reagent	Bio-Rad, Munich, Germany
Bovine serum albumin	GE Healthcare, Munich, Germany
Bovine serum albumin (BSA)	PAA Laboratories, Pasching, Austria
Bovine serum albumin (Protease free)	PAA Laboratories, Pasching, Austria
Bromphenol blue sodium salt	Roth, Karlsruhe, Germany
Chloramphenicol	Roth, Karlsruhe, Germany
Cholera Toxin	Sigma-Aldrich, Taufkirchen, Germany
Coomassie brilliant blue G-250	AppliChem, Darmstadt, Germany
DABCO (1,4-diazabicyclo[2.2.2]octane)	Fluka, Neu-Ulm, Germany
Dexamethasone	Sigma-Aldrich, Taufkirchen, Germany
Digitonin	Roth, Karlsruhe, Germany
Dispase II	Sigma-Aldrich, Taufkirchen, Germany
Dithiothreitol (DTT)	AppliChem, Darmstadt, Germany
DMEM (high glucose)	Life Technologies, Darmstadt, Germany
DMEM/F12	Life Technologies, Darmstadt, Germany
Dynabeads (Protein A and G)	Life Technologies, Darmstadt, Germany
Dynasore	Sigma-Aldrich, Taufkirchen, Germany
ECL Western blotting detection reagents	GE Healthcare, Munich, Germany
Fetal calf serum (FCS)	Life Technologies, Darmstadt, Germany
Gelmount mounting medium	Biomeda, Foster City, CA, USA
Glutathione sepharose beads	GE Healthcare, Munich, Germany

Horse Serum (HS)	PAA Laboratories, Pasching, Austria
Insulin 40 I.E. (Insuman Rapid, human)	Sanovi-Aventis, Frankfurt, Germany
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	Roth, Karlsruhe, Germany
Kanamycin	Roth, Karlsruhe, Germany
LB-Broth (Luria/ Miller)	Roth, Karlsruhe, Germany
Leupeptin	Roth, Karlsruhe, Germany
Lipofectamine2000	Life Technologies, Darmstadt, Germany
Luminol (3-Aminophthalhydrazide)	AppliChem, Darmstadt, Germany
Lysozyme	Roth, Karlsruhe, Germany
MiTMAB	Abcam, Cambridge, UK
NEAA (Non-essential amino acids)	Life Technologies, Darmstadt, Germany
Octyl-ß-D-glucopyranoside (NOG)	Sigma-Aldrich, Taufkirchen, Germany
OptiMEM	Life Technologies, Darmstadt, Germany
OptiPrep	Alere Technologies AS, Oslo, Norway
Pansorbin cells	Merck, Darmstadt, Germany
Penicillin/Streptomycin	GE Healthcare, Munich, Germany
Pepstatin A	Roth, Karlsruhe, Germany
Phenylmethylsulfonylfluoride (PMSF)	Roth, Karlsruhe, Germany
Phusion High-Fidelity DNA Polymerase	New England Biolabs, Frankfurt a. M., Germany
Ponceau S	AppliChem, Darmstadt, Germany
Precision Plus Protein Standard	Bio-Rad, Munich, Germany
Proteinase Inhibitor Cocktail (PI)	Sigma-Aldrich, Taufkirchen, Germany
Puromycin	Life Technologies, Darmstadt, Germany
Sodium pyruvate	PAA Laboratories, Pasching, Austria
Super Script Reverse Transkriptase	Thermo Fisher Scientific, Waltham, MA, USA
TEMED	Roth, Karlsruhe, Germany
Thrombin	Roth, Karlsruhe, Germany
Trypsin	Life Technologies, Darmstadt, Germany
Hydrogen peroxide 30% p.a.	Roth, Karlsruhe, Germany

2.2 Buffers

Self-made buffers, solutions and their composition are listed in table 2.2.

Table 2.2 Composition of homemade buffers and solutions

Buffer	**Composition**
Lysis buffer	50 mM Tris pH 7.4; 150 mM NaCl; 2 mM EDTA; 1% NP-40
Co-IP buffer	10 mM Tris; 150 mM NaCl; 5 mM EDTA 0,5% Triton; pH 8
GST lysis buffer	50 mM HEPES pH 7.5; 150 mM NaCl; 1 mM EDTA; 5% v/v glycerol; 0.1% NP-40

Blocking solution for WB	5% non-fat milk powder in TBS-T
Coomassie blue staining solution	0.1% Coomassie brilliant blue; 42.5% ethanol; 5% methanol; 10% acetic acid
Coomassie destaining solution	20% methanol; 7.5% acetic acid
ECL solution	1250 µM 3-aminophtalhydrazide (Luminol); 200 µM p-coumaric acid; 100 mM Tris-HCl pH 8.5; fresh: 1:1000 10% H_2O_2
LB media	25 g LB-Broth in 1 l water
PBS	150 mM NaCl; 20 mM NaH_2PO_4; adjust to pH 7,4 with NaOH
PFA solution	80 mM Pipes, pH 6,8; 2 mM $MgCl_2$; 4% paraformaldehyde; 5 mM EGTA, pH 8
Ponceau staining solution	0,1% ponceau; 5% acedic acid
SDS electrophoresis buffer	192 mM glycin, 25 mM Tris, 0.1% SDS
SDS sample buffer 4x	250 mM Tris-HCl pH 6,8; 8% SDS; 40% glycerin; 0,4% Bromphenolblue; 100 mM DTT; 20% 2-mercaptoethanol
SOC medium	0,8% glucose in LB medium
Stripping solution acidic for WB	0,1 M glycin pH 2,5 (acetic acid)
Stripping solution basic for WB	0,1 M NaOH
TAE 1 x (0,5 x for DNA gels)	40 mM Tris; 20 mM acetic acid; 1 mM EDTA (pH 8.0)
TE	10 mM Tris pH 8,0; 0,1 mM EDTA
TNE buffer 1 (Harder et al., 1998)	25 mM Tris-HCl, pH 7,4; 150 mM NaCl; 5 mM EDTA; 1 mM DTT, 2% Triton X-100; 10% sucrose
TNE buffer 2 (Mc Guinn and Mahoney., 2014)	25 mM Tris-HCl, pH 7,5; 150 mM NaCl; 5 mM EDTA; 1,5% Triton X-100; 1mM PMSF
Transferbuffer for WB	192 mM glycin, 25 mM Tris, 10% v/v methanol
TBS-T	10 mM Tris; 150 mM NaCl; 0.05% Tween-20; pH 7.4; adjust to pH 7,4 with HCl
DNA sample buffer	75% (v/v) glycerol; 2 mg/ml Bromphenolblue; 4 mg/ml Xylencyanol; 50mM EDTA
GST elution buffer	50 mM Tris-HCl pH 8; 150 mM NaCl; 0,1% Triton X-100; 1 mM DTT, 40 mM reduced glutathione
Direct pulldown buffer	50 mM Tris-HCl pH 7,5; 150 mM NaCl; 0,01% Triton X-100; 1 mM DTT; 1 mM EDTA; 1 % BSA
Triton buffer for sequential detergent extraction	1% Triton X-100; 10 mM Tris-HCl pH 7,5; 140 mM NaCl; 5 mM EDTA; 2 mM EGTA; 1mM PMSF; 1 µg/ml Leupeptin; 1 µg/ml Pepstatin A
SDS-urea buffer for sequential detergent extraction	1% SDS; 8 M urea; 10 mM Tris-HCl pH 7,5; 140 mM NaCl; 5 mM EDTA; 2 mM EGTA

2.3 Antibodies

Antibodies were used for either Western blotting (WB), Immunofluorescence microscopy (IF) or Immunorecipitation (IP). Primary antibodies are listed in table 2.3. Secondary antibodies with coupled enzymes or fluorophores are listed in table 2.4. Other fluorophores or enzyme-coupled reagents are listed in table 2.5.

Table 2.3 Primary antibodies

Antibody	Host	WB	IF	IP	company
Actin cl. AC40	mouse	1:1000			Sigma
c-myc (A-14)	rabbit			2 µg	Santa Cruz
γ-Catenin	mouse	1:2500	1:250		BD
Desmoglein 1	mouse	1:1000			BD
Desmoglein 1	rabbit		1:50		Santa Cruz
Desmoglein 2	mouse	1:1000			Santa Cruz
Desmoglein 3	mouse	1:500	1:100	2 µg	AbD Serotec
Desmoplakin	rabbit	1:500	1:50		Santa Cruz
E-Cadherin cl.36	mouse	1:2000	1:200		BD
E-Cadherin	rabbit	1:2000			Tebu Bio
Flotillin-2 (F2C)	rabbit	1:150	1:1000	2 µg	Sigma
Flotillin-2	rabbit	1:1000			Cell Signalling
Flotillin-2/ESA	mouse	1:1000	1:50		BD
Flotillin-2	rabbit		1:100		Abcam
Flotillin-1 (FLOT)	mouse	1:1000	1:50		BD
Flotillin-1	rabbit	1:2000	1:150	2 µg	Sigma
GAPDH	mouse	1:10000			Abcam
GST	mouse	1:8000			Novagen
HRS	rabbit	1:1000			Santa Cruz
MAT 1	mouse			2 µg	Santa Cruz
PCNA	mouse	1:1000			Santa Cruz

Abbreviations: GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GST, Glutathione S-transferase; HRS, Hepatocyte growth factor-regulated tyrosine kinase substrate; PCNA, Proliferating cell nuclear antigen

Table 2.4 Secondary antibodies

Antibody/ conjugate	WB	IF	Company
Goat anti mouse/ HRP	1:10000		Dako
Goat anti rabbit/ HRP	1:10000		Dako
Goat anti mouse/ Cy3		1:300	Jackson ImmunoResearch
Goat anti rabbit/ AlexaFluor488		1:300	Jackson ImmunoResearch

Abbreviations: HRP, Horseradish peroxidase

Table 2.5 Fluorescent dyes and enzyme coupled reagents

Dye/ enzyme	DNA Gels	IF	Company
Ethidium bromide	1:10000		**Dako**
DAPI		1:10000	**Dako**

Abbreviations: DAPI, 4',6-Diamidin-2-phenylindol; HRP, Horseradish peroxidase

2.4 Synthetic oligonucleotides

DNA primers were used for PCR amplification of several human genes (coding sequence). Primers were designed based on respective NCBI Reference sequences. All primers were purchased from Sigma-Aldrich, Taufkirchen, Germany. All primers are listed in Table 2.6. Nucleotide sequences are indicated from 5´to 3´.

Table 2.6 DNA primer

Name	Sequence 5´to 3´	NCBI Ref Seq.
hDsg1_cyt_BamH1_fw	CTATAGGATCCTGTGATTGTGGAGGTGCTCCTC	NM_001942.2
hDsg1_STOP_EcoR1_rev	CTATAGAATTCCTACTTGCTATATTGCACGGTAC	NM_001942.2
hDsg2_cyt_BamH1_fw	CTATAGGATCCATGTGCCATTGCGGAAAGGGC	NM_001943.3
hDsg2_STOP_Sal1_rev	CTATAGTCGACTTAGGAGTAAGAATGCTGTACAG	NM_001943.3
hDsg3_cyt_EcoR1_fw	CTATAGAATTCTTGACCTGTGACTGTGGGGCAG	NM_001944.2
hDsg3_STOP_Sal1_rev	CTATAGTCGACTCATATTAGACGGGAGCAAGGATC	NM_001944.2
hDsc1a_cyt_BamH1_fw	CTATAGGATCCAAGAGAACAGTCAAGAAATGTTTTC	NM_024421.2
hDsc1a_STOP_EcoR1_rev	CTATAGAATTCTTATTTCTTGATGCATGTCTTTGC	NM_024421.2
hDsc2a_cyt_BamH1_fw	CTATAGGATCCGCTTCTGGGACGTCTAAAC	NM_024422.3
hDsc2a_STOP_EcoR1_rev	CTATAGAATTCTCATCTCTTCATGCATGCTTC	NM_024422.3
hDsc2b_STOP_EcoR1_rev	CTATAGAATTCTTAATTTTTAATCAGAGTGTGTC	NM_004949.3
hDsc3a_cyt_BamH1_fw	CTATAGGATCCAAAGGGAAACGTTTTCCTGAAG	NM_001941.3
hDsc3a_STOP_EcoR1_rev	CTATAGAATTCTTATCTCTTTGTGCATGCTTC	NM_001941.3

2.5 Plasmids

Plasmids and DNA constructs are listed in table 2.7.

Table 2.7 Plasmids

Name	Vector	Insert/ Tag	Reference/ NCBI Ref Seq
GST pET41a	**pET41a**	**GST**	**Novagen**
Reggie 1 GST	pET41a	R Flot 2/ GST	AG Tikkanen
Reggie 2 GST	pET41a	R Flot 1 /GST	AG Tikkanen
GST pGEX4T-1	**pGEX4T-1**	**GST**	**GE Healthcare**
Flotillin 1 GST	pGEX4T-1	H Flot 1/ GST	AG Tikkanen
Flotillin 2 GST	pGEX4T-1	H Flot 2/ GST	AG Tikkanen

Dsg 1 CPD GST	pGEX4T-1	H Dsg1 (1923-3363)/ GST	FV; NM_001942.2
Dsg 2 CPD GST	pGEX4T-1	H Dsg2 (2088-3545)/ GST	FV;NM_001943.3; (G2506A); n.v. (Posch et al., 2008)
Dsg 3 CPD GST	pGEX4T-1	H Dsg3 (2024-3108)/ GST	FV; NM_001944.2; (A2843G); n.v. (Amagai et al., 1991)
Dsc 1a CPD GST	pGEX4T-1	H Dsc1a (2411-2947)/ GST	FV; NM_024421.2
Dsc 1b CPD GST	pGEX4T-1	H Dsc1b(2411-2993)/ GST	FV; NM_004948.3
Dsc 2a CPD GST	pGEX4T-1	H Dsc2a (2606-3160)/ GST	FV; NM_024422.3
Dsc 2b CPD GST	pGEX4T-1	H Dsc2b (2606-2998)/ GST	FV; NM_004949.3
Dsc 3a CPD GST	pGEX4T-1	H Dsc3a (2310-2846)/ GST	FV; NM_001941.3
Dsc 3b CPD GST	pGEX4T-1	H Dsc3b (2310-2675)/ GST	FV; NM_024423.2
MBP pMAL-c2X	**pMAL-c2X**	**MBP**	**New England Biolabs**
Dsg 1 CPD MBP	pMAL-c2X	H Dsg1 (1923-3363)/ MBP	FV; NM_001942.2
Dsg 2 CPD MBP	pMAL-c2X	H Dsg2 (2088-3545)/ MBP	FV;NM_001943.3; (G2506A); n.v. (Posch et al., 2008)
Dsg 3 CPD MBP	pMAL-c2X	H Dsg3 (2024-3108)/ MBP	FV; NM_001944.2; (A2843G); n.v. (Amagai et al., 1991)
Dsc 1a CPD MBP	pMAL-c2X	H Dsc1a (2411-2947)/ MBP	FV; NM_024421.2
Dsc 1b CPD MBP	pMAL-c2X	H Dsc1b(2411-2993)/ MBP	FV; NM_004948.3
Dsc 2a CPD MBP	pMAL-c2X	H Dsc2a (2606-3160)/ MBP	FV; NM_024422.3
Dsc 2b CPD MBP	pMAL-c2X	H Dsc2b (2606-2998)/ MBP	FV; NM_004949.3
Dsc 3a CPD MBP	pMAL-c2X	H Dsc3a (2310-2846)/ MBP	FV; NM_001941.3
Dsc 3b CPD MBP	pMAL-c2X	H Dsc3b (2310-2675)/ MBP	FV; NM_024423.2

Abbreviations: CPD, cytoplasmic domain; FV, Frauke Völlner AG Tikkanen; GST, Glutathione S-transferase; H, human; MBP, Maltose-binding protein; n.v. natural variant; R, rat.

2.6 Bacterial strains

Bacterial strains are listed in table 2.8. All bacterial strains were cultured in LB medium or on LB agar plates at 37°C.

Table 2.8 Bacterial strains

Name	Organism	Application	Reference
XL-1 Blue	*E. coli*	Plasmid amplification	Stratagene, La Jolla, CA, USA
Rosetta(DE3)pLysS	*E. coli*	Protein expression	Novagen, Darmstadt, Germany

2.7 Cell lines

Human cell lines and their culturing conditions are listed in table 2.9. The composition of the different growth media is shown in table 2.10.

Table 2.9 Human cell lines

Name	Description	Culture
HaCaT	Immortal keratinocytes (Boukamp et al., 1988)	DMEM ++/++; 8% CO2; 37°C
MCF 7	Breast cancer cells (Soule et al., 1973)	DMEM +/+; 8% CO2; 37°C
MCF 10A	Mammary epithelial cells (Soule et al., 1990)	DMEM F12; 5% CO2; 37°C

Table 2.10 Cell culture media compositions

Media	supplements
DMEM ++/++	10% FCS, 1% Pen/ Strep, 1% NEAA, 1% Sodium pyruvate
DMEM +/+	10% FCS, 1% Pen/ Strep
DMEM F12	5% horse serum, 1% Pen/ Strep, 10 mg/ml insulin, 20 ng/ml human recombinant EGF, 1 mM dexamethasone, 100 ng/ml cholera toxin

Abbreviations: FCS, Fetal calf serum; Pen/ Strep, Penicillin/ streptomycin; NEAA, Non-essential amino acids

All cell lines were used as wild type cells as well as knockdown variants in which the expression of flotillin-1 or flotillin-2 is stably knocked down with the help of a lentiviral shRNA system (described in Kurrle et al., 2013). Control cells are transfected with a non-sense shRNA. 2 µg/ml Puromycin was added to the medium to maintain the selection pressure for a permanent knockdown.

2.8 Mouse strains

Flotillin-1 and flotillin-2 knockout mice were generated and housed as described in (Banning et al., 2014a). Adult wild type and knockout littermates were used in the experiments.

2.9 Pemphigus vulgaris antibodies

Pemphigus vulgaris Immune-apherisate (purified IgG) and control-IgG (from a healthy person) were purified by Yvonne Exner, AG Hertl, University of Marburg. For the experiments, a mixture of four Pemphigus vulgaris patient IgG was used.

2.10 Technical devices

Technical devices are listed in table 2.11.

Table 2.11 Technical devices

Device	Model / Company
Analytival balance	SI-64 / Denver Instrument, Göttingen, Germany
Balance	PB 602-S / Mettler-Toledo, Columbus, Ohio, USA
Benchtop centrifuges	Mikro 200R / Hettich, Tuttlingen, Germany
Centrifuge	J2-21 / Beckman Coulter, Brea, CA, USA
Ultra centrifuge	Optima LE-80K / Beckman Coulter, Brea, CA, USA

Agarose gel electrophoresis camber	Typ Midi-Large / Neo Lab, Heidelberg, Germany
Acrylamide Gel electrophoresis camber	Mighty-Small II / GE Healthcare, Chalfont St Giles, GB
Transfer tank for western blot	Criterion blotter / Bio-Rad, Munich, Germany
Bacteria shaker	KS-15 / Edmund Bühler GmbH, Hechingen, Germany
Incubator for bacteria	Incucell / MMM, München, Germany
Incubator for cell cultures	CO2 Incubator / Sanyo, Moriguchi, Japan
Clean bench	HeraSafe KS / Thermo Fisher Scientific, Waltham, MA, USA
PCR termocycler	TPersonal / Biometra, Göttingen, Germany
Photometer	Bio Photometer Plus / Eppendorf, Hamburg, Germany
Microplate reader	Iinfinite M200 / Tecan, Männedorf, Switzerland
Sonicator	Sonopuls / Bandelin, Berlin, Germany
Light microscope	AE 31 / Motic, Xiamen, China
Confocal laser scanning microscope	LSM710 Confocal LSM, Carl Zeiss, Jena, Germany
Water purification system	Millipore / Merck, Darmstadt, Germany
Tissue lyzer	MM 200 / Retsch, Haan, Germany

2.11 Software and open resources

Microsoft office 2007

Prism 5.02, GraphPad Software Inc.

ZEN 2009, Carl Zeiss MicroImaging

Quantity one 4.6.8, Bio-Rad

Image J 1.46r, Wayne Rasband, USA

Motic Image Plus 2.0, Motic China Group CO., LTD

Zotero 4.0.23, George Mason University, Fairfax, VA, USA

NCBI: http://www.ncbi.nlm.nih.gov/

EXPASY: http://www.expasy.org/

HGNC: http://www.genenames.org/

Free life science tools: http://www.fr33.net/

NEBcutter: http://nc2.neb.com/NEBcutter2/

Human Protein Reference Database: http://www.hprd.org/

Dictionary: http://dict.leo.org/ende/index_de.html

3 Methods

3.1 Molecular biological methods

3.1.1 RNA isolation and cDNA synthesis

RNA was isolated from human cell lines with the PureLink RNA Mini Kit from Ambion (Life Technologies, Darmstadt, Germany) according to the manufacturer guidelines for purifying RNA from animal and plant cells. The RNA was recovered in 50 µl of DEPC water and used for subsequent cDNA synthesis.

An amount of 1 µg RNA was used for cDNA synthesis with the reverse transcriptase SuperScript III according to the manufacturer guidelines.

3.1.2 Cloning: Polymerase chain reaction, restriction and ligation

The Phusion High-Fidelity DNA Polymerase was used to amplify all required DNA fragments from HaCaT or MCF7 cDNA. The PCR protocol as well as the thermocycling conditions were adopted from NEB. The primer annealing temperatures were calculated with the help of the NEB web resources. The conditions are depicted in table 3.1 and 3.2.

Table 3.1 PCR protocol

Component	50 µl Reaction
Nuclease-free water	to 50 µl
5X Phusion GC Buffer	10 µl
10 mM dNTPs	200 µM
10 µM Forward Primer	0.5 µM
10 µM Reverse Primer	0.5 µM
Template DNA	variable
DMSO	3%
Phusion Polymerase	0.5 µl

Table 3.2 PCR thermocycling conditions

Step	Tem	Time
1 Initial Denaturation	98°C	2 min
2 Denaturation	98°C	10 sec
3 Primer Annealing	variable	10 sec
4 Amplification	72°C	1,5 min
5 Final Extension	72°C	10 min

Steps 2 – 4: 30 cycles

The PCR products were separated on 1% -1,5% Agarose gels, and purified with the help of the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chalfont St Giles, GB). PCR products as well as the plasmids were digested with restriction enzymes from NEB according to the manufacturer guidelines. Shrimp Alkaline Phosphatase from NEB was used to de-phosphorylate the linearized plasmids. 20 µl ligation reactions were carried out at room temperature for 2 hours with the T4 DNA ligase from NEB according to the manufacturer guidelines.

3.1.3 Transformation of bacteria and selection of clones

Competent *E.coli.* bacteria (XL1-blue) were transformed with 10 µl of a ligation reaction on ice for 30 minutes, followed by a heat shock at 42°C for 45 seconds. The bacteria were

allowed to grow in pre-warmed SOC media for 1 hour at 37°C before they were plated on LB agar plates with the appropriate antibiotics and incubated at 37°C over night.

Single bacteria colonies were picked and transferred into 5 ml of LB media with the appropriate antibiotics and allowed to grow at 37°C in a bacteria shaker.

3.1.4 Isolation of plasmid DNA

Depending on the culture volume, plasmid DNA was isolated from bacteria either with the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Taufkirchen, Germany) for mini-cultures up to 5 ml, or with the NucleoBond Xtra Midi / Maxi Kit from Macherey-Nagel (Düren, Germany) for culture volumes up to 100 ml.

3.2 Biochemical methods

3.2.1 Expression and purification of recombinant proteins

The *E.coli* strain Rosetta DE3 was used for the expression of all GST- and MBP-fusion proteins. Transformed bacteria cultures were grown at 37°C in LB media until an OD_{600} of 0,4 – 0,6 was reached. Thereafter, the expression of the recombinant proteins was induced with IPTG. All desmoglein fusion proteins were induced with 0,5 - 1 mM IPTG for 4 – 5 hours at 37°C. The expression of the flotillin-GST proteins was induced with 0,15 mM IPTG for 20 hours at 19°C. Cells were pelleted by centrifugation and washed once with cold PBS. Lysis was performed at 4°C with GST lysis buffer supplemented with 100 µg/ml lysozyme and two sonications for 30 seconds with 95% amplitude. Lysates were cleared by centrifugation and the GST- or MBP-fusion proteins were immobilized on either glutathione-sepharose or amylose resin at 4°C on a rotating wheel over night. After washing, the immobilized proteins were stored as aliquots in PBS supplemented with 10% glycerol at -20°C.

3.2.2 Indirect GST pulldown

HaCaT cells were lysed for 20 minutes on ice in Co-IP buffer, supplemented with protease inhibitor cocktail. The lysates were cleared by centrifugation, incubated with 5 mg of GST-tagged proteins or GST and immobilized on glutathione sepharose over night at 4°C on a rotating wheel. The sepharose was washed four times, resuspended in sample loading buffer and boiled for 5 min at 94°C. Bound proteins were separated by SDS PAGE and detected by Western blot.

3.2.3 Elution of GST proteins and direct GST pulldown

GST and flotillin-GST proteins were eluted from glutathione sepharose in GST elution buffer containing protease inhibitor cocktail for 2 h at 4°C. Eluted proteins were captured by centrifugation and immediately used for direct pulldown experiments. For this, the eluted GST-proteins were incubated with MBP or Dsg-3-MBP proteins immobilized on amylose resin, in direct pulldown buffer on ice for 2 - 3 hours. Thereafter, the resin was washed 3 times in direct pulldown buffer, resuspended in sample loading buffer and

cooked for 5 minutes at 94°C. Proteins were separated by SDS-PAGE and detected by Western blot.

3.2.4 Preparation of protein lysates from human cells

Cells were washed twice with cold PBS and directly scraped into lysis buffer supplemented with protease inhibitor cocktail. The cell lysate was cleared by centrifugation and the protein amount was measured with the Bio-Rad Protein Assay reagent. Equal protein concentrations were analyzed by SDS-PAGE and Western blot.

3.2.5 Co-Immunoprecipitation

Cells were washed twice with cold PBS and lysed on ice for 30 minutes in CoIP buffer supplemented with protease inhibitor cocktail. Lysed cells were directly scraped and cleared by centrifugation. Antibodies were pre-coupled to 50 µl of magnetic protein A or protein G beads in the presence of 0,5% BSA for one hour at RT on a rotating wheel. The antibody-coupled magnetic beads were combined with equal amounts of protein lysate (750 µg – 1200 µg, depending on experiment) and rotated at 4°C over night. The next day, the beads were washed four times with cold Co-IP buffer with the help of a magnetic tube holder on ice. Afterwards the beads were boiled in SDS sample buffer for 5 minutes to release the precipitated proteins. Proteins were separated by SDS-PAGE and Western blot.

3.2.6 Preparation of protein lysates from mouse skin

Mouse skin samples were taken from the lateral left side during the dissection of adult wildtype and flotillin-2 knockout mice. Per sample, approximately 100 mg of skin was taken and homogenized in 500 µl of lysis buffer (see cell lysis) supplemented with protease inhibitor cocktail using a tissue lyzer (three times for 2 minutes at 25 Hz). The samples were lysed on ice for 45 minutes and homogenized again with the tissue lyzer twice for 2 minutes. Lysates were cleared by centrifugation. The protein concentration was measured with the Bio-Rad Protein Assay reagent. Equal protein amounts were analyzed by SDS-PAGE and Western blot.

3.2.7 Isolation of lipid rafts

Isolation of lipid rafts was carried out as described in Harder et al. (Harder et al., 1998). In detail: HaCaT cells were seeded in 15 cm dishes and allowed to grow confluent. Solutions and equipment were pre-chilled, and the whole experiment was strictly carried out on ice. Cells were washed in PBS, and afterwards scraped into PBS. After centrifugation at 2000 rpm for 5 minutes the supernatant was discarded and the pellet was resuspended in 600 µl of TNE buffer 1. Samples were resuspended with a 200 µl pipette and afterwards incubated on ice for 30 minutes. The whole sample was transferred into a 13-ml ultracentrifuge tube and mixed with 1,2 ml of OptiPrep (60% sucrose) to bring the sucrose concentration to 40%. Samples were carefully overlaid with 1,8 ml of five different sucrose concentrations mixed of OptiPrep and TNE buffer 1 (35%, 30%, 25%, 20% and 0%). Gradients were centrifuged at 35.000 rpm for 16 h in a SW41Ti rotor at 4°C without using the brake. Nine fractions (1,2 ml each) were collected from the top to the bottom. The fractions were mixed with SDS (2% end concentration) and 150 µl of the mixture were boiled with 50 µl

SDS sample buffer. Equal amounts were loaded on 10% PAA gels and analyzed by SDS-PAGE and Western blot.

To verify the results, lipid rafts were isolated according to a second protocol that was developed for HaCaT keratinocytes (McGuinn and Mahoney, 2014). In detail: HaCaT cells were seeded in a density of 2 x 10^6 in 10 cm dishes and allowed to grow confluent. Prior to experimentation, the cells were serum starved over night. Solutions and equipment were pre-chilled, and the whole experiment was strictly carried out on ice. Cells were washed three times in PBS, and afterwards directly scraped into 1 ml of TNE buffer 2. Cells were aspired through a 23-gauge needle for 20 times and vortexed. 900 µl of the lysate were transferred into a 13 ml ultracentrifuge tube and mixed with 2,7 ml of OptiPrep (60% sucrose) to bring the sucrose concentration to 45%. Samples were carefully overlaid with 3,6 ml of 30% and 5% sucrose solution (Optiprep in TNE buffer 2). Gradients were centrifuged at 36.000 rpm for 18 h in a SW41Ti rotor at 4°C without using the brake. Twelve 900 µl fractions were collected from the top to the bottom. The fractions were mixed with SDS (2% end concentration) and 150 µl of the mixture were boiled with 50 µl SDS sample buffer. Equal amounts were loaded on 10% PAA gels and analyzed by SDS-PAGE and Western blot.

3.2.8 Sequential detergent extraction

Sequential detergent extraction was done according to Stahley et al. (Stahley et al., 2014). HaCaT cells were grown until confluent, washed with cold PBS and incubated with 200 µl Triton buffer for 10 minutes on ice. Cells were scraped, vortexed for 30 seconds and centrifuged at 14.000 x g for 30 minutes. The supernatant containing the Triton-soluble proteins was saved. The Triton-insoluble proteins (pellet) were subsequently extracted with 400 µl of the SDS-Urea buffer. 15 µl of each sample were analyzed by SDS-PAGE and Western blot. The Triton X-100 insoluble pool has double the volume compared to the Triton X-100 soluble pool. This was taken into account in the quantitative analysis.

3.2.9 SDS PAGE and Western blot

SDS-PAGE was carried out using 10% polyacrylamide gels or commercial 4-12% gradient gels (NuPAGE Novex 4-12% Bis-Tris Protein Gels, Thermo Fisher Scientific, Waltham, MA USA). The separated proteins were transferred to a nitrocellulose membrane with a tank blot system at 400 mA for 1 - 2,5 h. If necessary, transfer quality to the membrane was monitored with Ponceau staining solution. After washing with TBS-T, the membrane was incubated in 5% milk powder in TBS-T to block unspecific protein binding. Primary antibodies were incubated over night at 4°C. After washing, the appropriate HRP-coupled secondary antibodies were applied for 1 h at RT. Detection was performed in the presence of H_2O_2 using either a handmade ECL solution or ECL Western blotting detection reagents (GE Healthcare, Munich, Germany). If necessary, the membranes were stripped for 5 minutes with an acidic stripping solution, followed by a short incubation with a basic stripping solution and again blocked with milk and incubated with antibodies as described before.

3.3 Cell biological methods

3.3.1 Cultivation of human cell lines

General culturing conditions for the human cell lines are listed in tables 2.9 and 2.10. Cells were maintained in T25 plastic flasks. Cells were passaged at ~100% confluency. Medium was removed, cells were washed once with PBS and incubated with 0,5% EDTA in PBS at 37°C for 5 minutes or for up to 12 minutes (HaCaT) to chelate calcium ions that are needed for cell-cell adhesion. Cells were then incubated with 1 x Trypsin in PBS for 5 minutes at 37°C. Culture medium was added to inactivate the trypsin and the cells were suspended several times with a Pasteur pipette to produce a single cell suspension. A suitable part of the cell suspension was transferred back into a flask for prolonged culture.

Cells were frozen by resuspending a cell pellet in FCS supplemented with 10% DMSO. Frozen cells were stored in cryo-tubes in a -160°C freezer.

Cells were thawn from a -160°C freezer by heating the cryo-tubes in 37°C warm water until the cell suspension began to melt. The cells were directly transferred to pre-warmed culturing medium and allowed to settle at 37°C. The freezing medium containing DMSO was removed from the cells as fast as possible after the cells attached to the surface and replaced by normal culturing medium. In the case of knockdown cells, the selection medium was applied the next day.

3.3.2 Dispase-based dissociation assay

2 x 10^5 HaCaT cells were seeded in 12-well plates and grown until confluent. Two hours before the assay, the cells were treated with exfoliative toxin A (ETA, 0,5 µg/ml). After washing twice with PBS, 1,5 U/ ml Dispase II were added and incubated at 37°C for 30 to 40 minutes or until monolayers detached as a sheet from the plastic surface. After washing the monolayers carefully in PBS (supplemented with 0,5 mM $MgCl_2$) mechanical stress was applied to the monolayers by pipetting up and down for 5 times with a 1 ml pipette. The resulting fragments were fixed with paraformaldehyde and stained with crystalviolett. Photos were taken and the fragments were counted automatically with the help of the ImageJ software.

3.3.3 Aggregation assay

Cells were washed twice with PBS, followed by incubation with 0,5% EDTA in PBS for 10 minutes at 37°C. Cells were carefully detached using a 2,5 mg/ml trypsin in PBS solution. After a short centrifugation step, the cell pellet was resuspended in HaCaT medium containing 20 mM HEPES pH 7.2. 1,5 x 10^5 cells were seeded into a 6-well and allowed to re-aggregate under constant horizontal rotation of 80 rpm at 37°C. To determine the calcium-dependency of the cell aggregates, 4 mM EGTA was optionally added to the medium. After different time points, the cell aggregates were documented using an inverted phase-contrast microscope (objective 4x, Motic, Wetzlar, Germany). Per 6-well, two randomly chosen visual fields were imaged and the number of particles in an area of 1500 µm x 1500 µm was counted. Cell aggregation index was calculated using the formula $A=(N_0-N_{4h})/N_0$, with N_0 representing the total particles at the beginning and N_{4h} the total

particles after 4 hours, as described in Jesse et al. (Jesse et al., 2009). The assays were performed in duplicates.

3.3.4 Immunofluorescence

Cells were seeded on glass cover slips in 12-well plates and grown for at least 3 days. Medium was aspired and the cells were directly fixed in cold 20% methanol for 5 to 8 minutes at -20°C. Cells were washed three times with PBS and either directly prepared for staining or stored for up to three weeks in PBS at 4°C. Fixed cells were blocked with 1% BSA in PBS for 15 minutes. Primary antibodies were diluted in 1% BSA in PBS and applied for 1 h at RT. Cover slips were washed three times and fluorophore coupled secondary antibodies were applied for 1 h at RT. DAPI was incubated together with the secondary antibodies to stain double-stranded DNA. After staining, the cells were washed three times in PBS and once in water before the cover slips were mounted on glass slides with Gelmount mounting medium supplemented with DABCO (50 mg/ml).

Mouse skin samples were frozen in liquid nitrogen in Tissue Tek Compound (Sakura, Tokyo, Japan) and stored at -80°C. 10 µM cryo-sections were produced, immobilized on glass slides and fixed with cold methanol at -20°C for 10 minutes. Samples were blocked with 10% mouse serum in PBS for 10 min. The primary antibodies were diluted in 1% mouse serum in PBS and incubated over night at 4°C. Incubation with the secondary antibodies as well as DAPI staining of the nuclei was performed at RT for 1 h.

All samples were analyzed with a Zeiss LSM710 Confocal Laser Scanning Microscope.

3.3.5 PV IgG treatment of HaCaT cells

HaCaT cells were seeded on cover slips and grown for a minimum of three days. Prior to experimentation, the cells were serum-starved for 16 h before treatment with 150 µg/ml Pemphigus vulgaris IgG mix or control-IgG (healthy person) for 20 hours.

3.3.6 Inhibitor treatment of HaCaT cells

Dynasore and MiTMAB are inhibitors of the GTPase dynamin and block the endocytosis of many membrane proteins. HaCaT cells were either treated with Dynasore or MiTMAB to block the endocytic uptake of desmoglein-3. In detail: Cells were serum-starved overnight and then treated with 80 µM Dynasore for 2 h at 37°C or with DMSO as a control. Alternatively, the cells were incubated with 30 µM MiTMAB for 30 minutes and then processed further, as described in 3.3.4.

3.4 Statistical analysis and manipulation of images

All experiments were performed at least three times, unless otherwise stated. For the quantification of Western blot signals, the films were scanned and the band density was calculated and subtracted by the background using the Quantity one software. To calculate the relative protein amount, the signals were normalized to GAPDH and set in relation to one sample. For the sequential detergent extractions and the raft isolations, the signals in the different pools/ fractions were combined and taken as 100%.

Data were analyzed with the GraphPad Prism software and are shown as the mean ± S.D (standard deviation). Statistical comparisons were made using one-way analysis of variance (ANOVA) with Bonferroni`s multiple comparison test. Values of $p < 0.05$ were considered significant (*), whereas values of $p < 0.01$ and $p < 0.001$ were defined very significant (**) and highly significant (***), respectively.

The images shown have in some cases as a whole been subjected to contrast or brightness adjustments. No other manipulations have been performed unless otherwise stated.

4 Results

4.1 Flotillins are linked to Desmosomes and influence their Morphology and Adhesion

4.1.1 Flotillins colocalize with desmoglein-3

Our group could recently show, that flotillins colocalize with several proteins of the adherens junction at the cell borders of diverse epithelial cell lines, as well as in human epidermis (Kurrle et al., 2013). In addition we were able to show that flotillins also colocalize with desmosomal structures. Desmoglein-3 and flotillin-2 colocalization at the plasma membrane in MCF10A cells (Figure 4.1 A, Kurrle et al., 2013) as well as in human skin sections (Völlner et al., 2016).

HaCaT keratinocytes were chosen as the main cell system for this study and the localization of desmoglein-3 and flotillins in this cell line was addressed. A prominent part of flotillins is found in intracellular structures in these cells (Fig 4.1). Only a minor part resides at the plasma membrane. Nevertheless, colocalization of flotillin-2 and desmoglein-3 is found at the cell borders of HaCaT cells (Fig 4.1 B). Due to technical reasons, a co-staining of flotillin-1 and desmoglein-3 was not feasible. However, flotillin-1 and -2 colocalize to a very large extent in HaCaT cells (Figure 4.1.C). Therefore, it can be assumed that flotillin-1 also colocalizes with desmoglein-3.

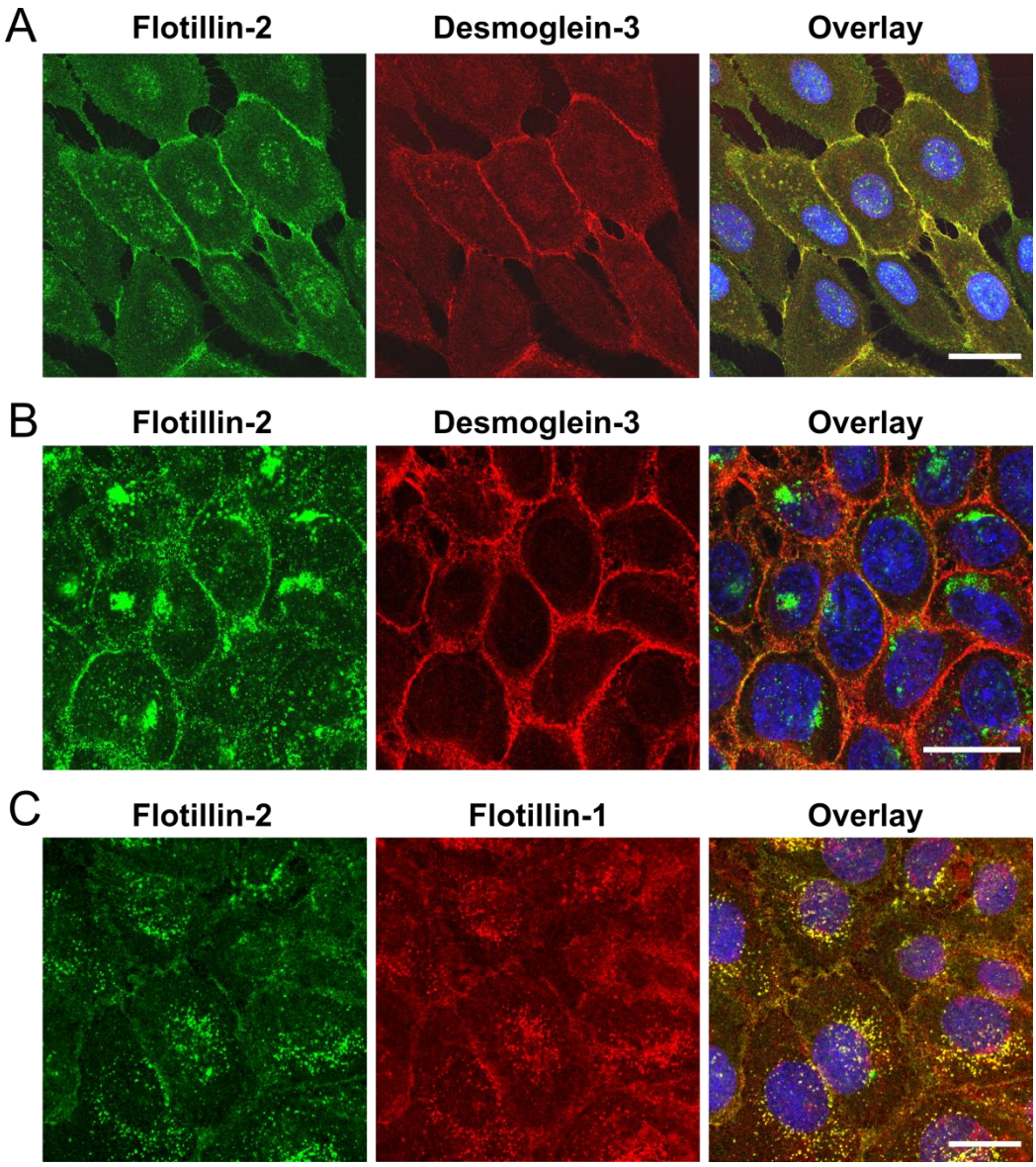

Figure 4.1: Flotillins colocalize with desmoglein-3 at the cell borders of epithelial cells.
Epithelial cells were grown on glass cover slips for three days, fixed and immunostained with the indicated antibodies. A) In MCF10A mammary epithelial cells flotillin-2 and desmoglein-3 colocalize to a large extent at the plasma membrane. B) In HaCaT cells, a prominent part of flotillin-2 is found in vesicular structures. Only a minor part is located at the plasma membrane where it colocalizes with desmoglein-3 which is mostly located at cell borders in these cells. C) Flotillin-2 and flotillin-1 localization is almost identical in HaCaT cells, with a predominant part of flotillin-1 found in intracellular structures and only a minor part at cell borders. Colocalization is shown in yellow. Scale bars: 20 µm. Fig 4.1 A and B were modified from Völlner et al., 2016.

4.1.2 Flotillins are found in a complex with desmosomal components

Colocalization is a prerequisite but does not provide any solid evidence for the interaction of two proteins. To elucidate if flotillins are only located in the proximity of desmosomal structures or found in a complex with them, co-immunoprecipitation experiments were performed in HaCaT keratinocytes. Desmogleins 1-3 co-precipitated from HaCaT cell lysates with endogenous flotillin-1 and -2 (Fig 4.2 A), whereby all proteins bound flotillin-

2 to a lesser extent. The interaction of flotillins with desmoglein-3 was also verified in reverse. Both flotillins co-precipitated with endogenous desmoglein-3 from HaCaT cell lysates (Fig 4.2 B). As expected on the basis of our previous data (Kurrle et al., 2013), y-catenin was also found in a complex with desmoglein-3 in these cells.

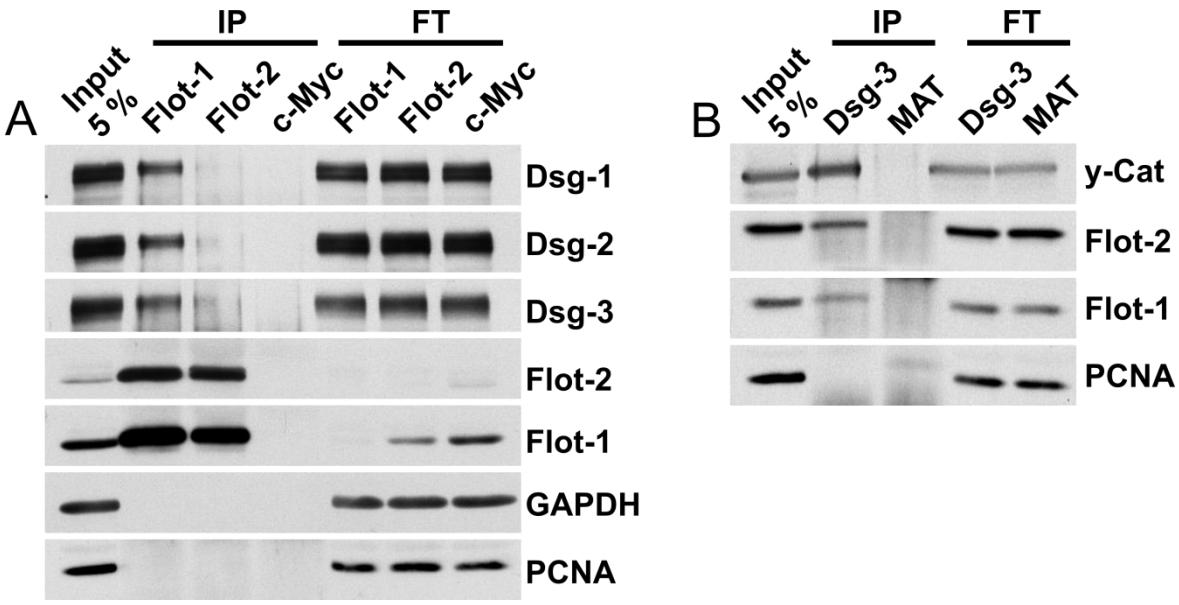

Figure 4.2: Flotillins and desmosomal cadherins are found in a complex in HaCaT keratinocytes.
A) Co-immunoprecipitation of desmogleins 1-3 from HaCaT cell lysates with antibodies against endogenous flotillin-1 and flotillin-2. Cells were grown confluent for four days and 1 mg of total protein was used per precipitation. B) Co-immunoprecipitation of flotillin-1, flotillin-2 and y-catenin from HaCaT cell lysates with an antibody against endogenous desmoglein-3. Cells were grown confluent for 9 days and 1 mg of total protein was used per precipitation. Fig 4.2 A was modified from Völlner et al., 2016.

4.1.3 Flotillins bind directly to the cytoplasmic domain of desmosomal cadherins

To further investigate the interaction of flotillins with desmogleins and to test whether flotillins are associated with the second class of desmosomal cadherins, the desmocollins, pulldown assays with purified proteins were performed. For this, GST-tagged constructs of the cytoplasmic domains of desmogleins 1-3 and of the isoforms (long and short) of the desmocollins were cloned. The purified proteins were used for GST-pulldown experiments with HaCaT cell lysates (Fig 4.3). The cytoplasmic domains of desmoglein 1-3 pulled down flotillin-1 as well as flotillin-2 and, as expected, also interacted with y-catenin (Fig 4.3 A).

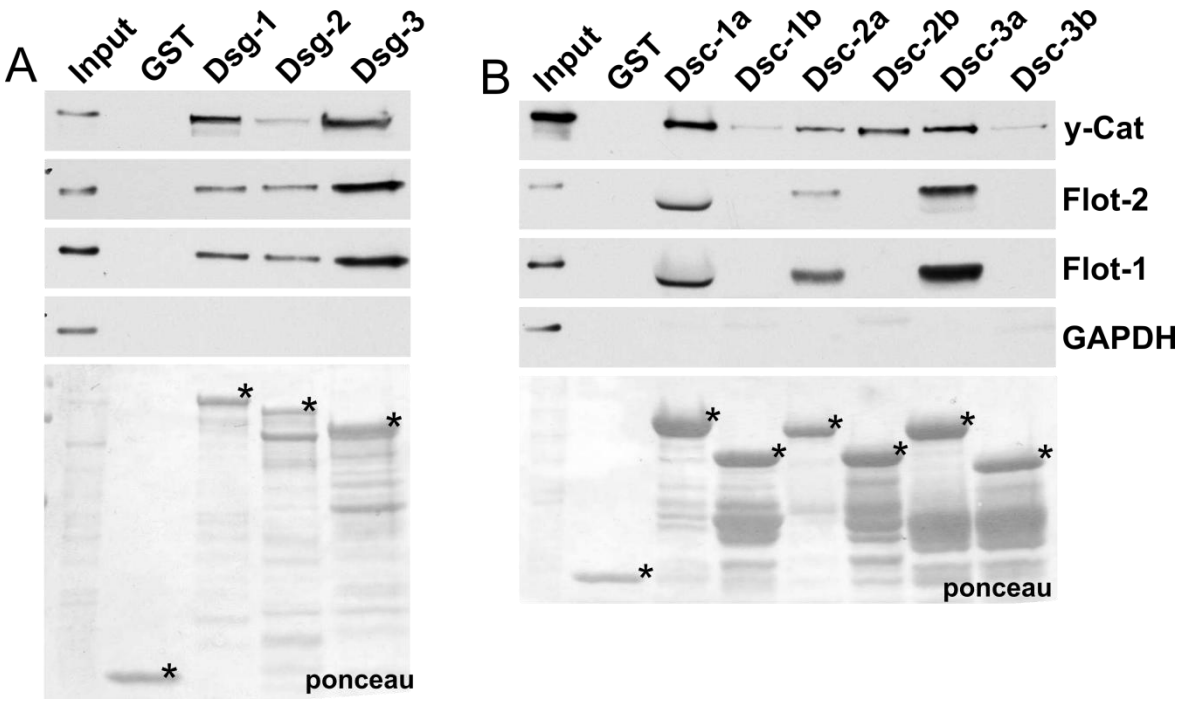

Figure 4.3: Flotillins interact with the cytoplasmic domain of desmosomal cadherins.
A) GST-pulldown from HaCaT cell lysate using GST-tagged purified proteins comprising the cytoplasmic domains of desmoglein 1-3. Flotillin-1 and -2 as well as y-catenin are pulled down with all tested desmogleins. B) GST-pulldown from HaCaT cell lysate using GST-tagged purified proteins comprising the long and short isoforms of the cytoplasmic tails of desmocollin 1-3. Flotillins are only pulled down by the long desmocollin isoforms (a) that contain the ICS sequence. According to the literature, y-catenin is pulled down mainly by the long isoforms. GST was used as a negative control in both experiments. For each pulldown, 1 mg of protein lysate and 5 µg of purified protein were used. Fig 4.3 A was modified from Völlner et al., 2016.

Due to alternative splicing, each desmocollin exists as two isoforms. The incorporation of a premature stop-codon results in the expression of the shorter isoform b that shares the extracellular part, the trans-membrane sequence and a short intracellular part with the longer isoform a. GST-pulldown assays with the cytoplasmic part of the desmocollin isoforms revealed that only the long isoforms were able to pull down flotillin-1 and flotillin-2 from HaCaT cell lysates (Fig 4.3 B). The longer isoforms exhibit an intracellular cadherin-like sequence (ICS) which is absent in the shorter desmocollin isoforms but present in all desmogleins (Fig 4.4). This sequence is known to serve as a binding motif for proteins like y-catenin. As expected, y-catenin was pulled down in the GST assay with desmocollin-1a and -3a but not with the short isoforms 1b and 3b. Intriguingly both isoforms of desmocollin-2 interact with y-catenin in the present assay, which is contradictory to the literature.

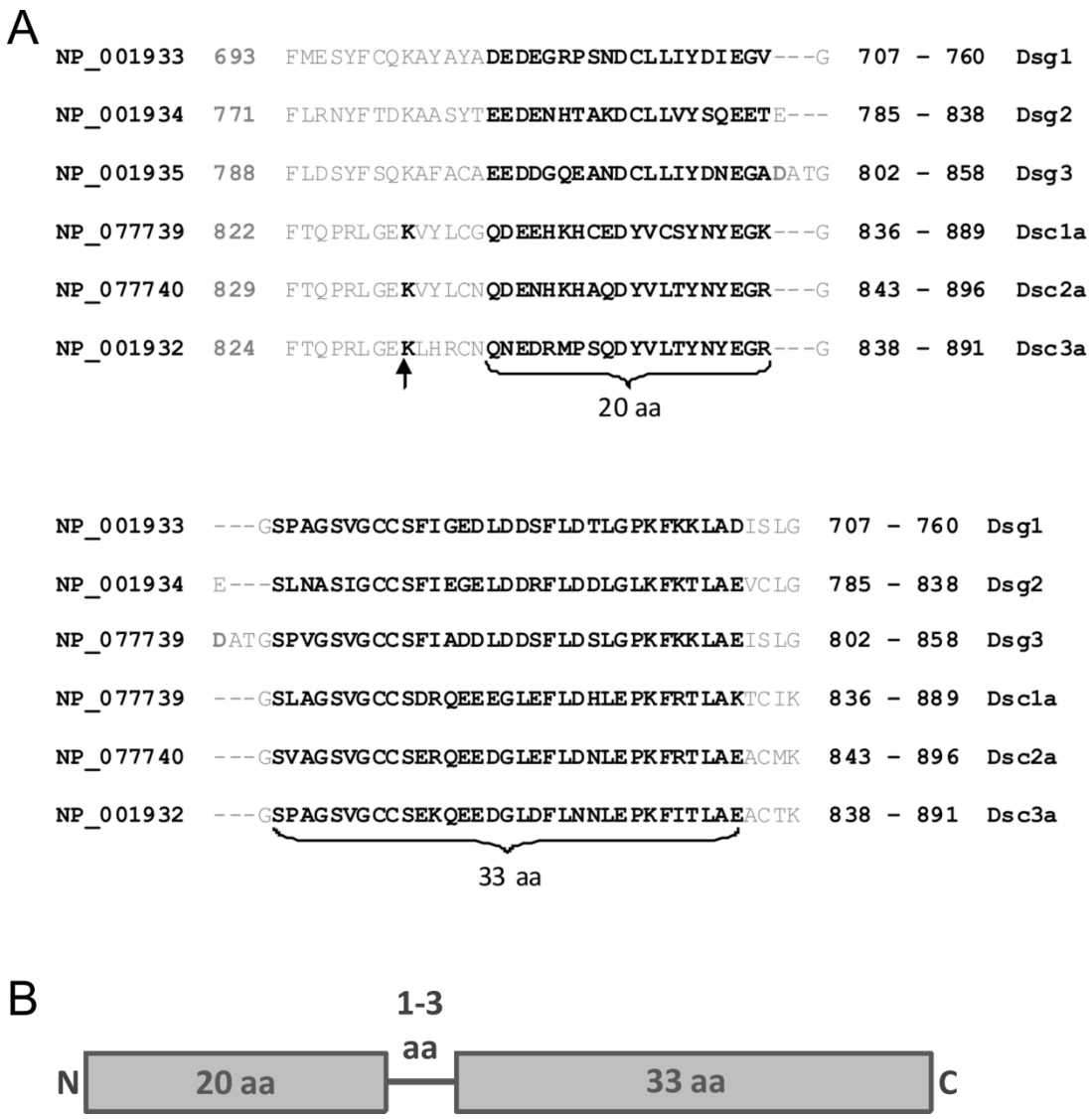

Figure 4.4: Alignment of the intracellular Cadherin-like (ICS) sequences of the desmosomal cadherins.
A) The protein sequences of desmoglein 1-3 and desmocollin 1a-3a were aligned with the help of the COBALT Multiple Alignment Tool from NCBI. Shown is a conserved sequence in the C-terminus which represents the ICS sequence found in all desmogleins and the long desmocollin isoforms. The bold letters indicate the lowest common sequence that is indicated as ICS in NCBI. The restricting sequences are: For the start Dsg-2 and for the end Dsg-1. Arrow: End point of the identical sequence of the long and short Dsc isoforms. B) Schematic prediction of the ICS sequence in desmosomal cadherins that should bind y-catenin and flotillins.

A former study revealed that y-catenin is able to bind flotillins directly via its ARM-domains 6 – 12 (Kurrle et al., 2013). y-Catenin also binds directly to the ICS sequence of desmosomal cadherins. The GST-pulldown experiments shown in figure 4.3 are indirect binding assays that reveal whether or not two proteins are found in a complex. The interaction may be a direct one or facilitated by another protein that binds both partners. To address the question whether flotillins bind directly to desmosomal cadherins or only indirectly via their interaction with y-catenin, direct pulldown assays were performed. For this, MBP-tagged fusion proteins of the desmosomal cadherins were purified and incubated with purified flotillin-GST proteins. Flotillin-1 directly binds to desmoglein-3 and

desmocollin-1a. Desmocollin-1b interacts with flotillin-1 but to a much lesser extent, as compared to the a isoform. (Fig 4.5 A). Surprisingly, the two flotillins show a different binding pattern, as flotillin-2 was only pulled down with desmoglein-3 but not with the desmocollin tails (Fig 4.5 B). Nevertheless, figure 4.3 B shows that flotillin-2 interacts with all long desmocollin isoforms indirectly. Flotillin-1 and y-catenin are able to bind flotillin-2 as well as the long desmocollin isoforms directly and may therefore enable this indirect interaction.

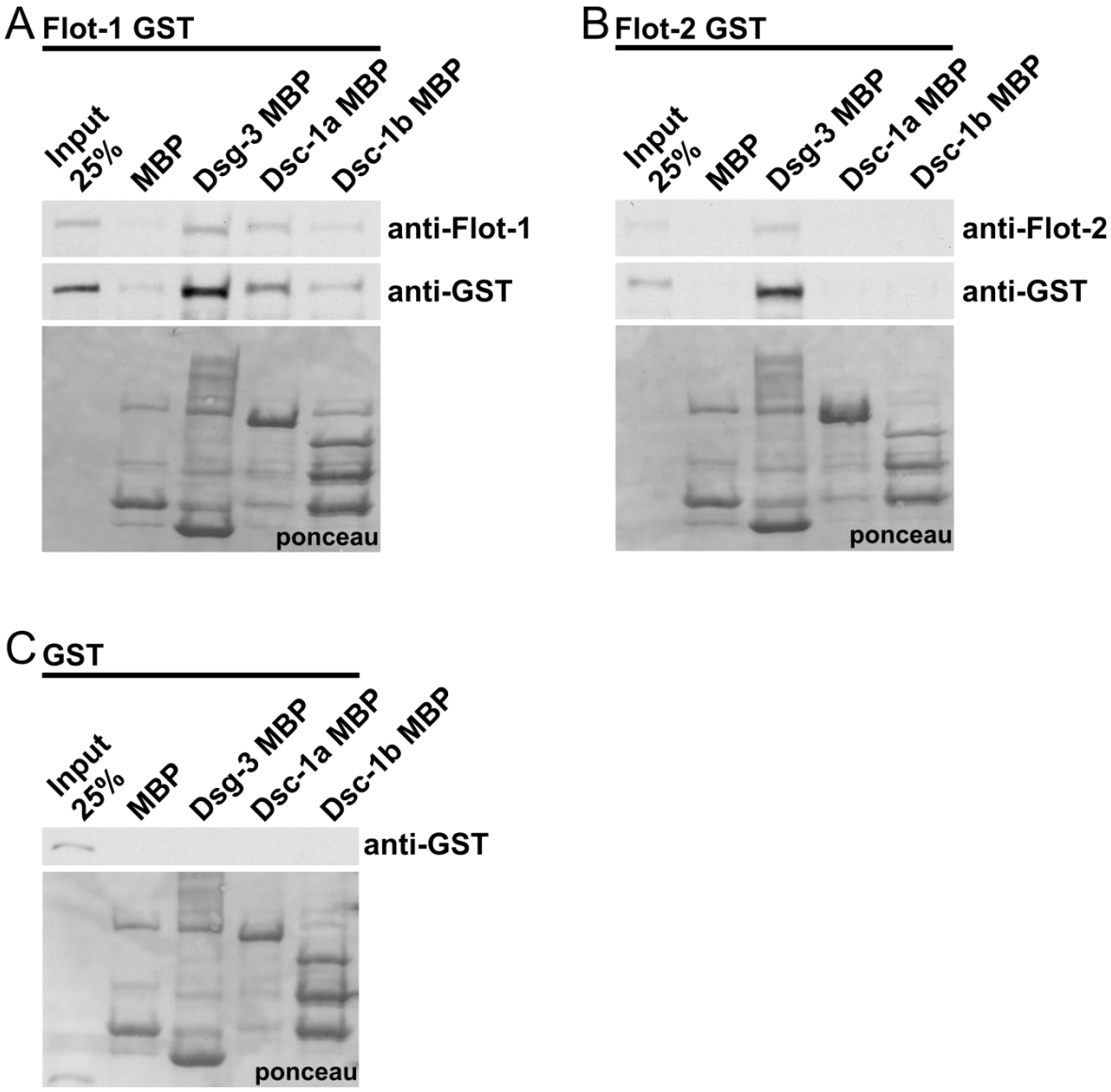

Figure 4.5: Flotillins directly interact with the cytoplasmic domain of desmoglein-3 and desmocollin-1. Direct pulldown experiments using purified proteins. For each pulldown 5 µg of Dsg-3-MBP, Dsc-1a-MBP or MBP as a negative control were used and incubated with flotillin-1-GST (A), flotillin-2-GST (B) or GST (C). Interaction of proteins was verified by Western blot using the indicated antibodies. Fig 4.5 A was modified from Völlner et al., 2016.

Figure 4.5 shows that flotillins are able to bind directly to desmosomal cadherins. Flotillin-1 binds directly to desmocollin-1a but only very weakly to desmocollin-1b, which allows conclusions about the binding site. As mentioned above desmogleins share the ICS sequence with the long desmocollin isoforms. The short desmocollin isoforms lack this sequence (Fig 1.2 and Fig 4.4). Therefore it is assumed that flotillins bind directly to the ICS sequence (Fig 4.4 B) of desmosomal cadherins.

4.1.4 Depletion of flotillins alters the morphology of desmosomal components

Flotillins are closely associated with desmosomal cadherins. Hence, the impact of flotillin depletion on the morphology of desmosomes was studied next. HaCaT cells with a stable lentivirus-mediated knockdown of flotillin-1 (F1-shRNA) or flotillin-2 (F2-shRNA) and control cells (sh-control) were available in the lab (Kühne et al., 2015). These cells show a good knockdown of the particular protein (Fig 4.10 A). The interdependency of flotillin expression observed in other cell lines (like HeLa) is not distinctive in these cells, as flotillin-2 protein level is not and flotillin-1 protein level is only slightly reduced in the other knockdown, respectively. Desmosome formation requires that cells grow over a certain time in proximity to each other. Therefore, the cells were cultured for at least three days and until the confluence reached about 70 – 90 %, as over-confluent HaCaT cells are not suitable for confocal laser scanning microscopy.

In sh-control HaCaT cells, the localization of flotillin-2 is comparable to wildtype HaCaT cells (Fig 4.1), with a large part of flotillin-2 found in intracellular structures and only a minor part at the plasma membrane (Fig 4.6). At sites of close cell-cell contact, desmoglein-3 resides in a sharp and defined zone at the plasma membrane. This sharp appearance is disturbed in F1-shRNA as well as F2-shRNA HaCaT cells. In both flotillin knockdown cell lines, the membrane staining of desmoglein-3 is diffuse and spread over a much wider area around the cell borders, as compared to the control cells (Fig 4.6). Frequently, linear extensions vertical to the plasma membrane seem to stretch out into the cytosol. These structures bear resemblance to so-called linear arrays, which are assumed to be sites of endocytic uptake (Jennings et al., 2011).

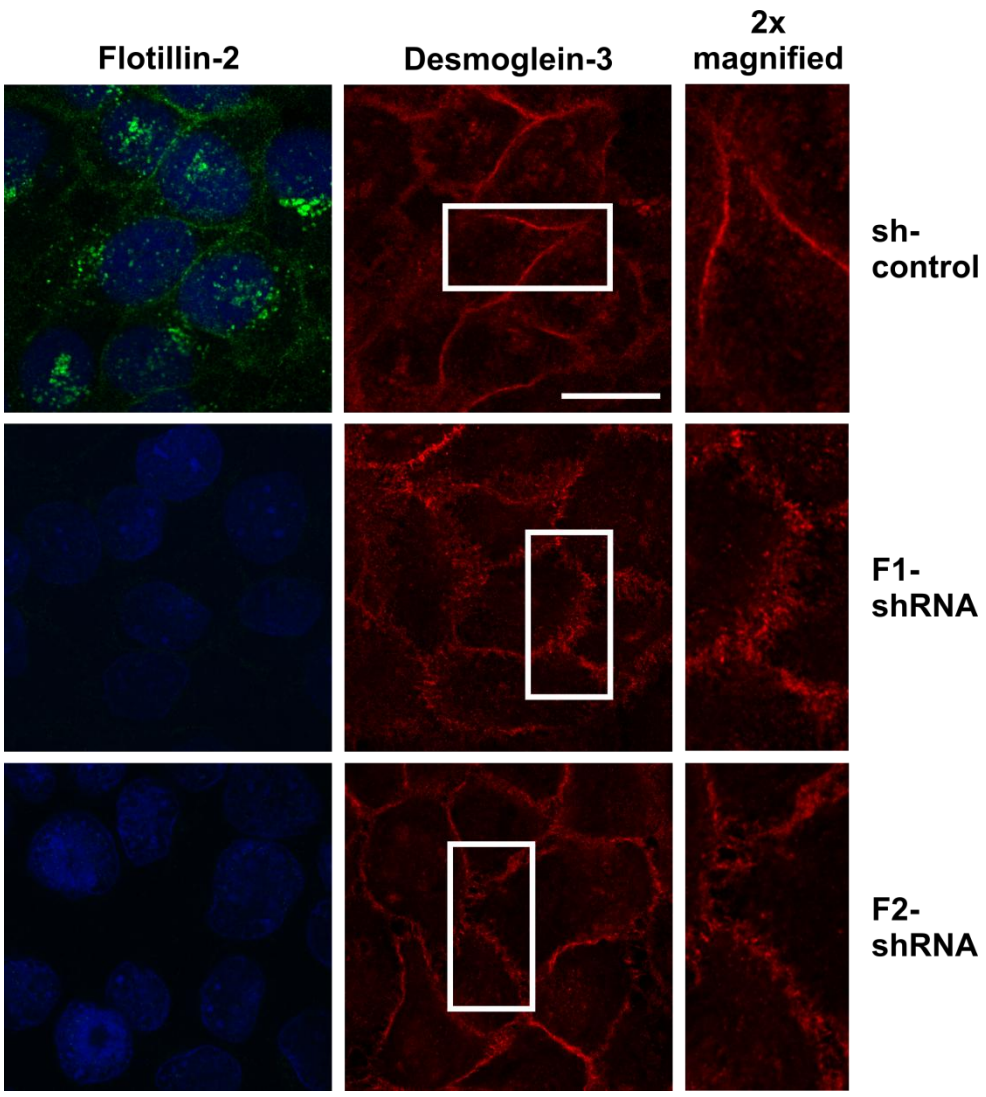

Figure 4.6: Knockdown of flotillins affects the plasma membrane localization of endogenous desmoglein-3.
HaCaT cells depleted of flotillin-1 (F1-shRNA), flotillin-2 (F2-shRNA) or control cells (sh-control) were grown on glass cover slips for three days. After fixation, cells were stained with antibodies against desmoglein-3 (red) and flotillin-2 (green). Nuclei were stained with DAPI (blue). Please note that the intensity of desmoglein-3 staining has been adjusted to give about equal intensities. Scale bar: 20 µm. Fig 4.6 was modified from Völlner et al., 2016.

Other desmosomal components were stained to verify if the structure of desmosomes is affected per se in flotillin knockdown cells or exclusively restricted to alterations in the appearance of desmoglein-3. In all tested cases, the staining pattern of desmosomal proteins was affected in the flotillin-depleted HaCaT keratinocytes (Figs. 4.7 – 4.9). Desmoglein-1 resides at the plasma membrane in a punctate pattern in control cells. However, in flotillin-1 depleted cells, the membrane staining is more or less lost, and the cell borders are no longer outlined. This effect is also observed in flotillin-2 knockdown cells, albeit to a much lesser extent. The desmoglein-1 localization at cell borders is still apparent in these cells, but the staining is blurred compared to the control cells (Fig 4.7).

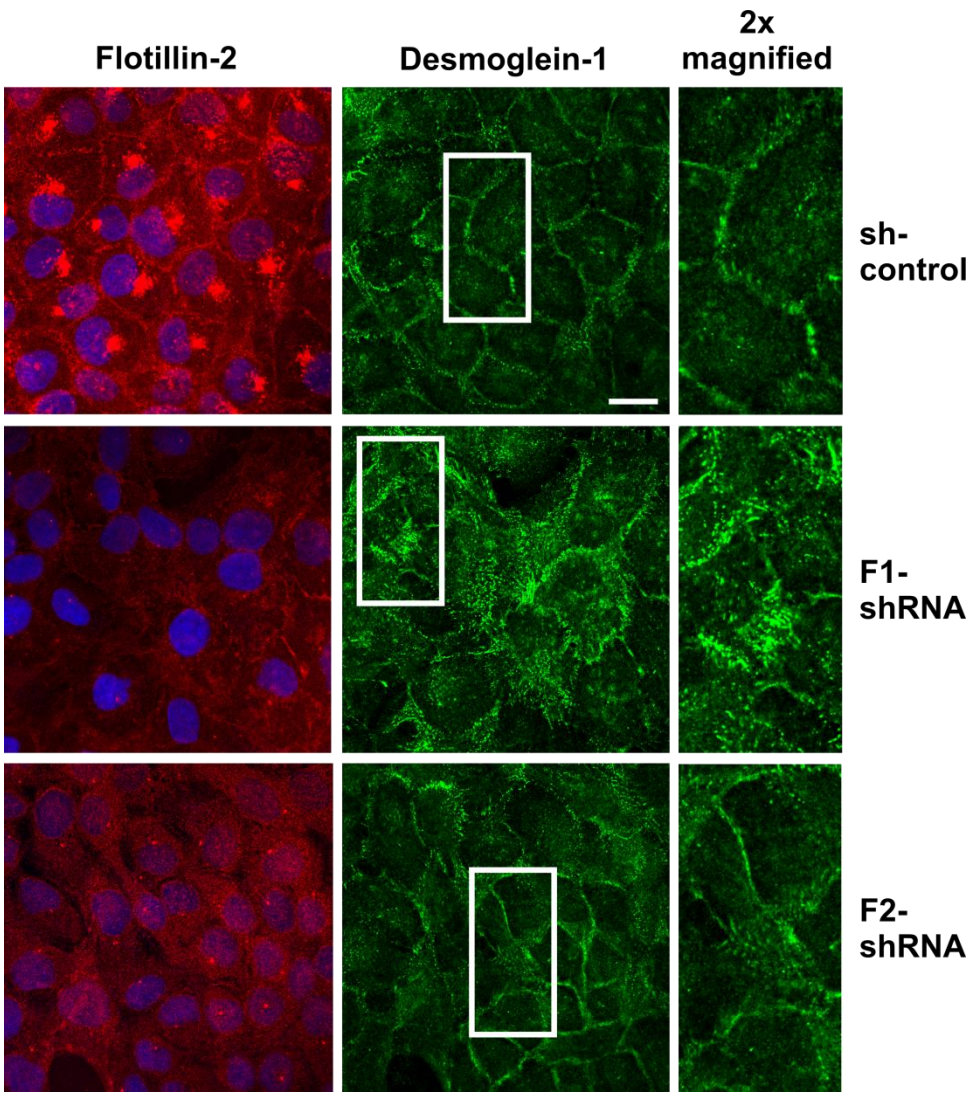

Figure 4.7: Knockdown of flotillins affects the plasma membrane localization of endogenous desmoglein-1.
HaCaT cells depleted of flotillin-1 (F1-shRNA), flotillin-2 (F2-shRNA) or control cells (sh-control) were grown for three days on glass coverslips, fixed and stained with desmoglein-1 (green) and flotillin-2 (red) antibodies. Nuclei were stained with DAPI (blue). Scale bar: 20 µm. Fig 4.7 was modified from Völlner et al., 2016.

The antibody that was used for the staining of desmoplakin is polyclonal and detects both desmoplakin I and desmoplakin II. In control cells, desmoplakin is found all over the cell body with a punctate concentration at the plasma membrane as well as in nuclear speckles. The staining pattern in the two flotillin knockdown cell lines is altered, but differs between each knockdown. In flotillin-1 knockdown HaCaT cells, the plasma membrane staining is almost lost, but the cytosolic staining is not affected. The nuclear speckles are moderately enriched. In contrast, in flotillin-2 knockdown cells, the nuclear speckles as well as the cytosolic staining are gone, and the desmoplakin staining shows a very punctate membrane pattern resembling that of desmoglein-3 (Fig 4.8).

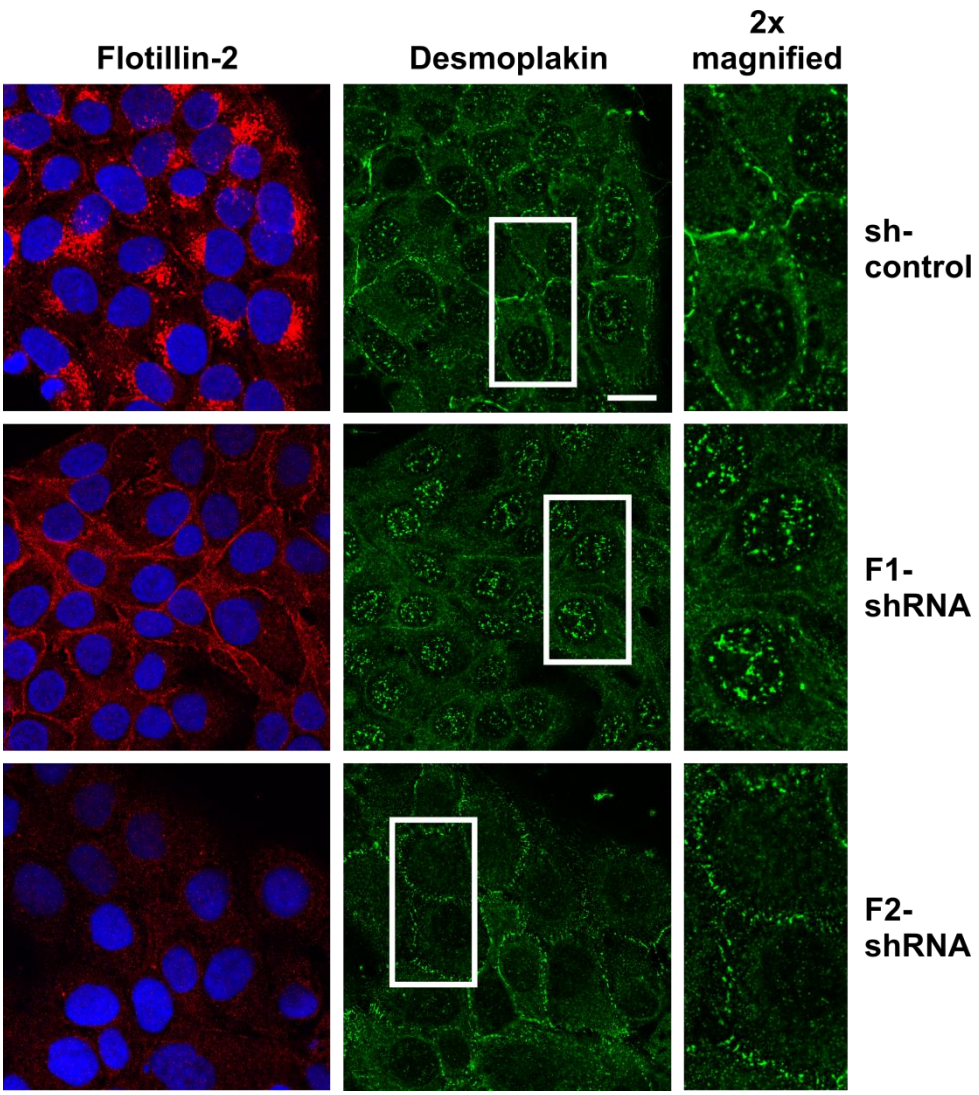

Figure 4.8: Knockdown of flotillins affects the plasma membrane localization of endogenous desmoplakin.
HaCaT cells depleted of flotillin-1 (F1-shRNA), flotillin-2 (F2-shRNA) or control cells (sh-control) were grown for three days on glass coverslips, fixed and stained with desmoplakin (green) and flotillin-2 (red) antibodies. Nuclei were stained with DAPI (blue). Scale bar: 20 µm. Fig 4.8 was modified from Völlner et al., 2016.

y-Catenin is mainly localized at the plasma membrane in HaCaT control but also in the flotillin knockdown cells (Fig 4.9). The cell borders are clearly defined by the y-catenin antibody in each cell line. In the flotillin knockdown cells, the membrane staining is disturbed in a way that the structure looks tattered and turbulent. Comparable to desmoglein-3 in these cells, y-catenin seems to exist in vertical extensions at the plasma membrane, or in linear arrays.

Figures 4.6 – 4.9 show that all tested desmosomal protein display alterations in their morphology and/or localization in flotillin-depleted HaCaT keratinocytes, although these alterations slightly differ among these proteins. Therefore, can be concluded that the structure of desmosomes is affected by the knockdown of one of the two flotillins in keratinocytes.

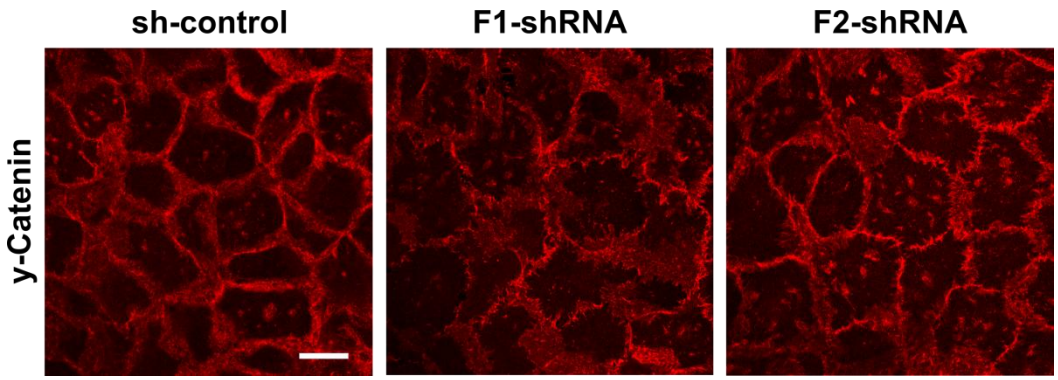

Figure 4.9: Knockdown of flotillins affects the plasma membrane localization of endogenous γ-catenin. HaCaT cells depleted of flotillin-1 (F1-shRNA), flotillin-2 (F2-shRNA) or control cells (sh-control) were grown for three days on glass coverslips, fixed and stained with a γ-catenin (red) antibody. Scale bar: 20 µm. Fig 4.9 was modified from Völlner et al., 2016.

4.1.5 Decreased desmoglein-3 protein level upon flotillin depletion

The fluorescence intensity of the desmoglein-3 staining in flotillin-depleted HaCaT cells appeared reduced under the microscope. To test if the expression levels of desmosomal components are affected upon knockdown of flotillins in HaCaT cells, protein lysates of confluent cells were analyzed by Western blot (Fig 4.10 A). The protein expression of desmoglein-1, -3, E-cadherin and γ-catenin appeared reduced in the flotillin knockdown cells, but varied among several experiments. Densitometric quantification of five independent experiments revealed that only the protein expression of desmoglein-3 was significantly reduced upon flotillin knockdown (Fig 4.10 B). E-cadherin protein levels were only reduced in the flotillin-2 knockdown cells but not in the flotillin-1 knockdown (Fig 4.10 E). Desmoglein-1 (Fig 4.10 C) and γ-catenin (Fig 4.10 D) exhibited large variations in protein expression upon flotillin knockdown. Both proteins showed a tendency to be reduced in the flotillin-2 knockdown and increased in the flotillin-1 knockdown cells, but the data failed to become significant.

Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and GAPDH were analyzed as reference proteins, to exclude that the variations in protein expression observed in the stable flotillin-knockdown HaCaT cells are the result of a general problem in protein synthesis in these cells. However, Hrs and GAPDH protein expression show minor variations, but are not significantly altered in the flotillin-knockdown cells (Fig 4.10 F).

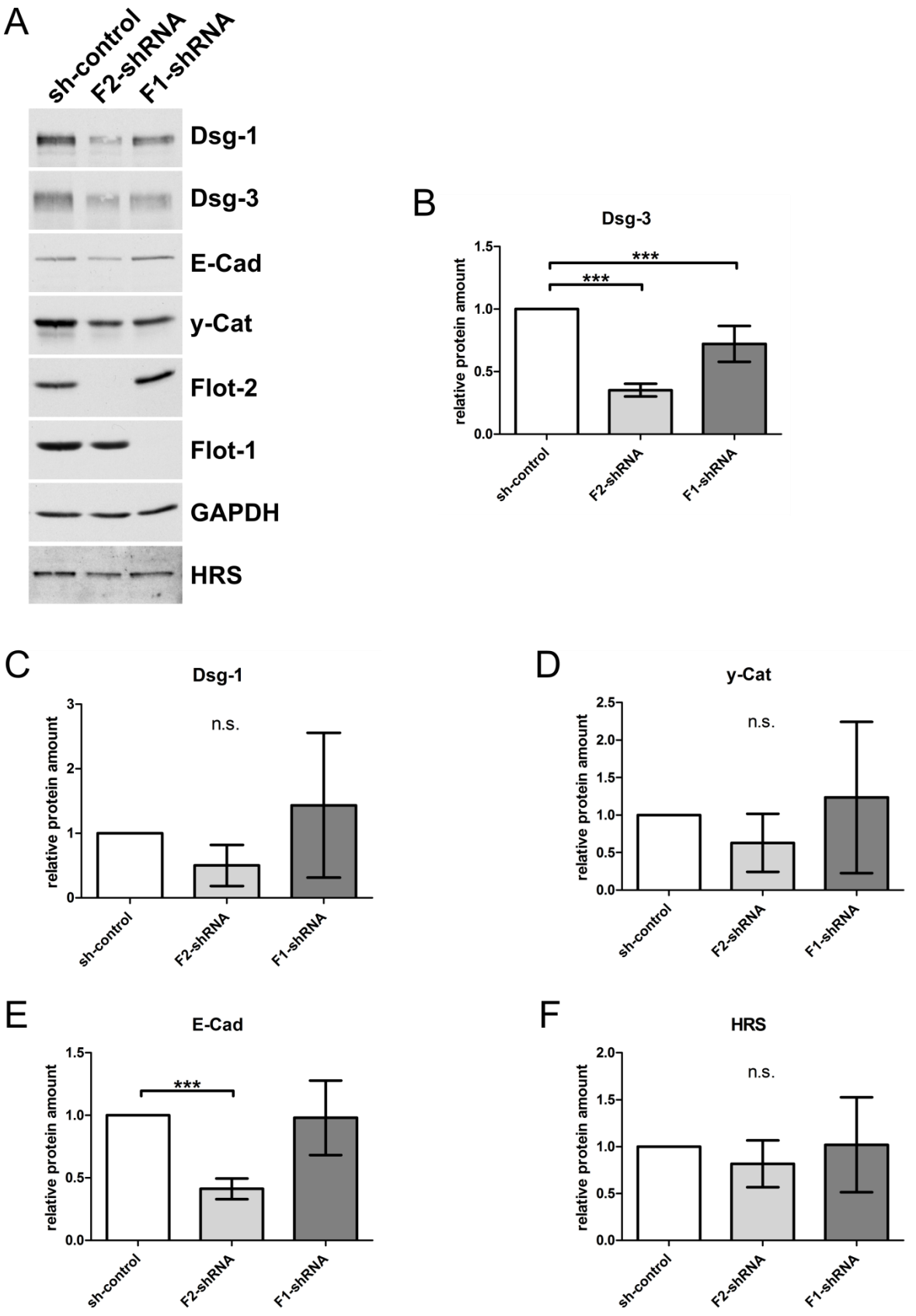

Figure 4.10: Flotillin depletion results in diminished desmoglein-3 and E-cadherin protein levels.
A) Flotillins were stably knocked down in HaCaT cells by lentivirus-mediated RNA interference. Cells were grown until confluent and equal protein amounts were analyzed by Western blot. B-F) Western blot bands of proteins were quantified by scanning densitometry and normalized against GAPDH. B) Desmoglein-3

protein levels are reduced in HaCaT cells depleted of flotillin-1 or -2. C-D; F) Protein levels of desmoglein-1, y-catenin and HRS are not significantly affected in flotillin knockdown HaCaTs E). E-cadherin levels are reduced upon flotillin-2 knockdown. B-E) Bars represent the mean ± SD of five independent experiments. F) Bars represent the mean ± SD of four independent experiments B-F) One-way ANOVA with Bonferroni`s multiple comparison test. ***, $p < 0.001$. Fig 4.10 was modified from Völlner et al., 2016.

4.1.6 Flotillin depletion interferes with epithelial adhesion

Flotillin depletion in HaCaT keratinocytes alters the morphology of desmosomes and results in diminished desmoglein-3 protein levels. Thus, the impact of flotillin depletion on cell-cell adhesion was addressed. A *bona fide* assay to measure epithelial strength and therefore cell-cell adhesion is a Dispase-based dissociation assay (DBDA). In this assay, the enzyme dispase II is used to liberate intact, confluent cell monolayers as a sheet from the surface of the culture dishes. The floating monolayer is then exposed to mechanical forces by e.g. pipetting up and down several times. Applying these sheer forces on the cell monolayer results in the appearance of more or less fragments, depending on the cell-cell adhesion strength. This assay was used to test a potential effect of flotillin depletion on the adhesion strength in HaCaT keratinocytes.

The adhesion strength in HaCaT keratinocytes depleted of flotillin-1 or -2 was dramatically reduced (Fig 4.11). When mechanical stress was applied on the monolayers of flotillin-depleted cells, they dissociated into numerous fragments, whereas the control cells were released as virtually intact monolayer (Fig 4.11 A upper panel). The *staphylococcus aureus* enzyme exfoliative toxin A (ETA) specifically cleaves desmoglein-1 and is used in DBD assays to monitor exclusively the impact of desmoglein-3 on adhesion strength. However, when the above experiment was carried out with a pre-incubation of ETA on the cultured cells, no alterations in the fragmentation behavior were observed (compare Fig 4.11 A upper and lower panel). The assay was carried out six times, fragments were counted and the relative fragment number compared to the control was monitored (Fig 4.11 C and D). Quantification revealed that indeed no further fragmentation was observed after the elimination of desmoglein-1 by ETA. This would point to the possibility that desmoglein-1 is less important than desmoglein-3 for the adhesion in these cells. However, a verification of the activity of the used ETA was not successful (not shown), and therefore, a final conclusion remains elusive.

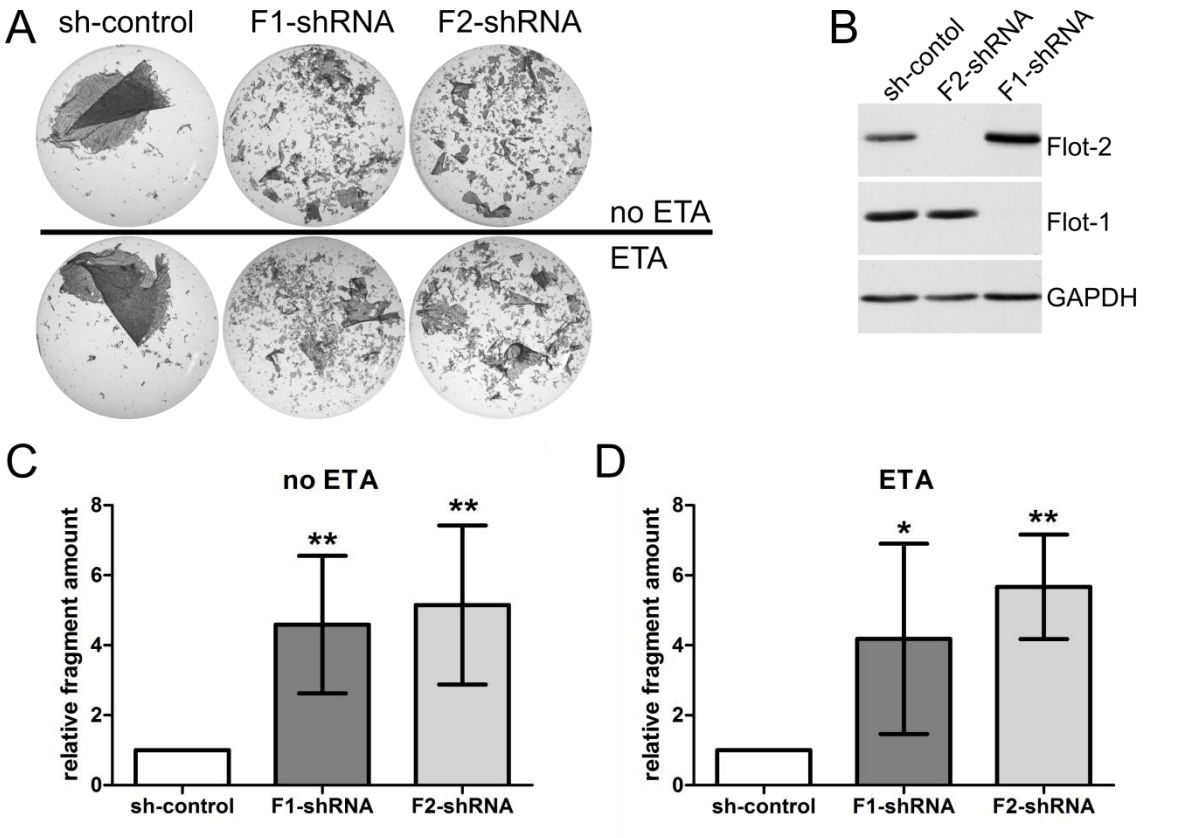

Figure 4.11: Flotillin knockdown weakens the epithelial strength in HaCaT keratinocytes.
A) Dispase-based dissociation assay of HaCaT cells depleted of flotillin-1 (F1-shRNA) and flotillin-2 (F2-shRNA). Cells were either treated or not with ETA for 2 hours. Dispase II treatment for 30 minutes released cell monolayers. Shear forces were applied, and the resulting fragments were fixed and stained. B) HaCaT cells used for the assay shown in A have reduced flotillin-1 and -2 protein level. Lysates of HaCaT cells depleted of flotillin-1 or -2 were separated by SDS PAGE and analyzed for flotillin expression. (C-D) Relative fragment number of six independent experiments. Bars represent the mean ± SD. One-way ANOVA with Bonferroni`s multiple comparison test. *, $p < 0,05$, **, $p < 0,01$. (C) Exclusive effect of flotillin knockdown on fragment number. Cells were not treated with ETA before experimentation. (D) Flotillin-depleted cells were treated with 0,5 µg/ml ETA for 2 hours before experimentation. Fig 4.11 A and C were modified from Völllner et al., 2016.

Another common assay to measure the adhesiveness of cells is a re-aggregation assay. With this assay the ability of single cells to form aggregates with each other out of a single cell suspension is measured over time. After four hours of shaking at 37°C, flotillin-depleted cells form bigger cell aggregates than the control cells (Fig 4.12 A-C). The aggregation index was calculated by counting the newly formed aggregates in a defined area, and it is a direct measure of the re-aggregation capacity of the cells. The aggregation index of the flotillin-depleted HaCaT cells is five times higher compared to the control cells (Fig 4.12 D). To confirm that the observed effects are due to the action of cadherins, EGTA was added to chelate the calcium ions that are required for cadherin-based adhesion. When EGTA was added to the single cell suspension, the aggregation index was significantly reduced and comparable to the aggregation capacity in the control cells (Fig 4.12 E). This shows that the re-aggregation of the cells is dependent on cadherins. The

stronger re-aggregation capacity of the flotillin-depleted HaCaT keratinocytes was somewhat surprising, as it seems to interfere with the diminished epithelial strength that was observed in the DBDA (Fig 4.11). However, the DBDA measures the strength of cell-cell adhesion in an existing cell sheet and is a bona fide assay for desmosomal strength. In contrast, the aggregation assay gives information about the capacity of single cells to form new cell contacts, a relative fast process, which is supposed to be dependent on classical cadherins and thus on adherens junctions. Summing up, flotillin depletion in HaCaT keratinocytes diminishes the desmosomal strength, whereas the re-aggregation capacity is enhanced.

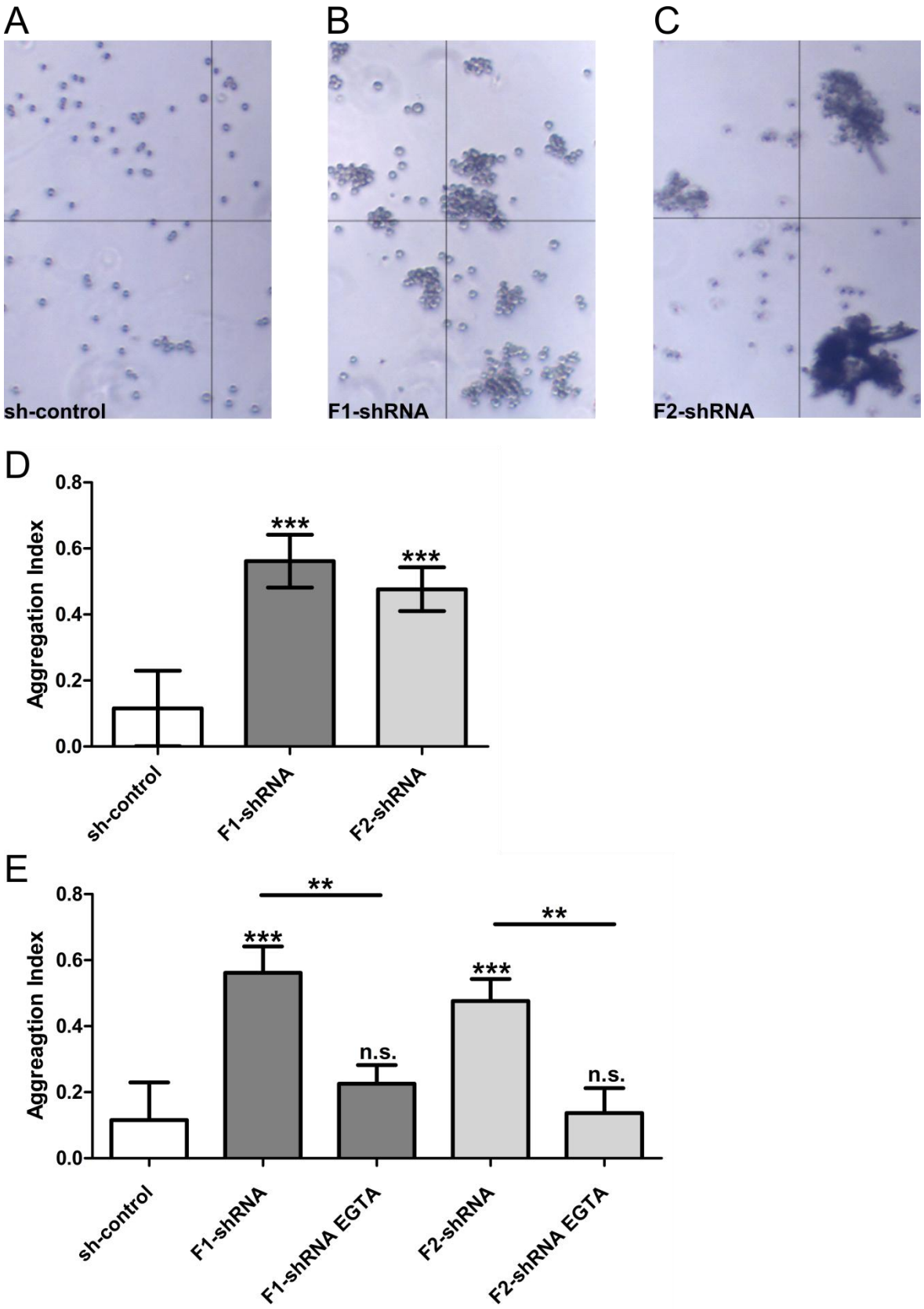

Figure 4.12: The re-aggregation capacity of HaCaT cells is enhanced upon flotillin knockdown.
A) Aggregation of control and flotillin-depleted HaCaT cells after 4 hours of horizontal shaking at 37 °C. B) Aggregation index of flotillin-depleted HaCaT cells after 4 hours. Bars represent the mean ± SD of three independent experiments. C) Aggregation index after 4 hours. EGTA was added to determine the cadherin dependency of the observed effects. Bars represent the mean ± SD of triplicates in one experiment. One-way ANOVA with Bonferroni`s multiple comparison test. ***, $p < 0.001$.

4.2 The role of flotillins in desmosome homeostasis

The absence of one of the two flotillins in HaCaT cells leads to a reduction of demoglein-3 protein level, alters the morphology of desmosomes and interferes with the epithelial adhesion strength. However, the molecular details for the observed interferences that are caused by the ablation of flotillins are missing so far. These issues were addressed next.

4.2.1 Membrane distribution of desmogleins is not affected upon flotillin depletion

Flotillins are able to cluster molecules such as the EGFR, within membranes prior to their endocytic uptake (Amaddii et al., 2012). Desmosomal cadherins are assumed to exist in two different plasma membrane associated pools (Windoffer et al., 2002). The desmosomal pool consists of mature desmosome-associated cadherins that are connected to the cytoskeleton, whereas the extra-desmosomal pool is composed of cadherins that are associated with the plasma membrane but not part of a mature desmosome. Lateral mobility and exchange between the two pools allows the replacement of cadherins and is important for the flexibility of desmosomes. As flotillins bear membrane organizing capacities, it was examined next whether the exchange between the two cadherin pools is hampered upon a flotillin knockdown. The distribution of desmosomal components in the two pools can be monitored by a sequential detergent extraction. Thereby, the cells are initially extracted with a Triton X-100-containing buffer to solubilize proteins of the extra-desmosomal pool, which are not associated with the cytoskeleton. Due to their strong association with the cytoskeleton, proteins of the desmosomal pool are insoluble in this buffer. In a second step, these insoluble proteins are liberated by an SDS and urea containing buffer. The protein composition of both pools can then be detected by Western blot.

The membrane distribution of desmosomal cadherins is not affected in flotillin-depleted HaCaT keratinocytes (Fig 4.13). Less desmoglein-3 is found in the desmosomal pool (Triton X-100 insoluble) of the flotillin depleted cells as compared to the control cells (Fig. 4.13 A), but this is due to the overall diminished desmoglein-3 protein level in these cells (Fig 4.10). The percentage of desmoglein-3 in the desmosomal pool was calculated for each cell line (Fig 4.13), revealing that most of the desmoglein-3 in the cells (> 80%) is located in the desmosomal pool, and that the distribution of the available desmoglein-3 is not changed in the different cell lines. Interestingly, the distribution of desmoglein-1 is converse to desmoglein-3 (Fig 4.13 A). Most of the desmoglein-1 in HaCaT cells is associated with the extra-desmosomal pool, which suggests that desmosomes are composed mainly of desmoglein-3 and not of desmoglein-1 in HaCaT keratinocytes.

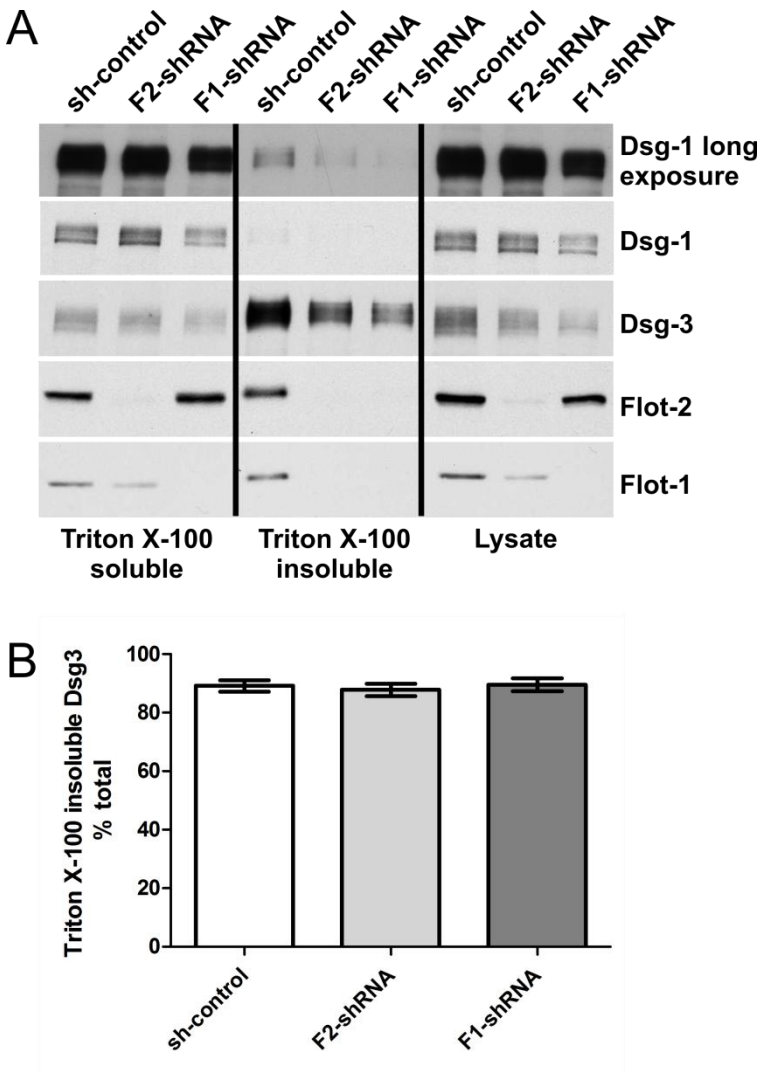

Figure 4.13: Flotillin knockdown results in an overall decreased level of desmoglein-3 in the desmosomal pool but does not change the membrane distribution of desmogleins per se.
Sequential detergent extraction of HaCaT control and flotillin knockdown cells. Cells were initially treated with a Triton X-100 buffer followed by extraction of the Triton X-100 insoluble proteins with a SDS-urea buffer. A) Extracted proteins were analyzed by Western blot. Please note that due to technical reasons, the total volume of the insoluble pool is twice that of the soluble one. (B) Desmoglein-3 distribution in control and flotillin-depleted HaCaT cells. Western blot bands of proteins were quantified by scanning densitometry and the percentage of desmoglein-3 in the Triton X-100 insoluble pool was calculated by taking Triton X-100 soluble plus Triton X-100 insoluble proteins as 100 %. Bars represent the mean ± SD of three independent experiments. Fig 4.13 was modified from Völlner et al., 2016.

4.2.2 Raft association of desmogleins is not affected upon flotillin knockdown

Flotillins are membrane raft associated proteins. Several studies found adhesion proteins associated with membrane rafts (Chartier et al., 2011; Roitbak et al., 2005). Also desmosomal cadherins were reported to reside in membrane rafts (Brennan et al., 2011; Resnik et al., 2011). To determine the raft association of desmosomal cadherins in HaCaT cells and to elucidate if the knockdown of flotillins has an impact on the distribution of these proteins, raft isolations were performed. Detergent extraction and density gradient centrifugation (according to Harder et al., 1998) was used to monitor the association of adhesion proteins with lipid rafts in flotillin depleted HaCaT and control cells (Fig 4.14). No major alterations in density gradient distribution and raft association were observed in the flotillin knockdown HaCaT cells. A major part of desmoglein-3 is associated with the raft fractions 1-4, whereas desmoglein-1, y-catenin and E-cadherin are mainly associated with the non-raft fractions. As described previously, the raft association of one flotillin was impaired in the absence of the other flotillin. However, the raft association of the tested adhesion proteins remained intact in the flotillin knockdown cells. The assay was performed three times, and the results for desmoglein-1 and -3 were quantified by scanning the densitometry of the Western blot bands (Fig 4.14 D-E). This revealed some variations between the different experiments, which are due to technical reasons. To confirm the results, a second raft isolation approach that was recently published for HaCaT cells was tested (McGuinn and Mahoney, 2014). Consistent with the former results, no alterations in the density gradient distribution of desmoglein-1 and -3 were observed in the flotillin knockdown cells with this second approach (Fig 4.15).

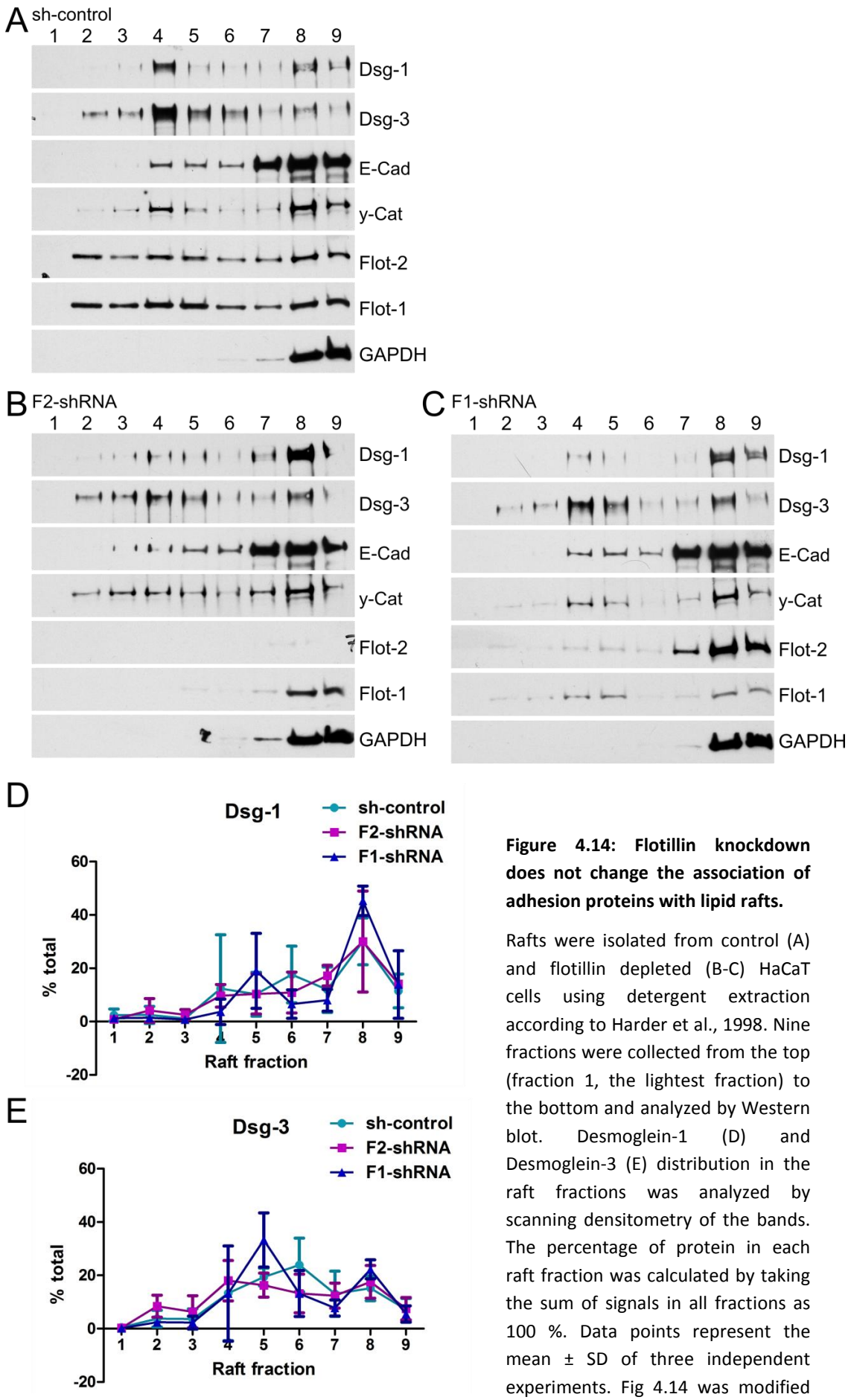

Figure 4.14: Flotillin knockdown does not change the association of adhesion proteins with lipid rafts.

Rafts were isolated from control (A) and flotillin depleted (B-C) HaCaT cells using detergent extraction according to Harder et al., 1998. Nine fractions were collected from the top (fraction 1, the lightest fraction) to the bottom and analyzed by Western blot. Desmoglein-1 (D) and Desmoglein-3 (E) distribution in the raft fractions was analyzed by scanning densitometry of the bands. The percentage of protein in each raft fraction was calculated by taking the sum of signals in all fractions as 100 %. Data points represent the mean ± SD of three independent experiments. Fig 4.14 was modified from Völlner et al., 2016.

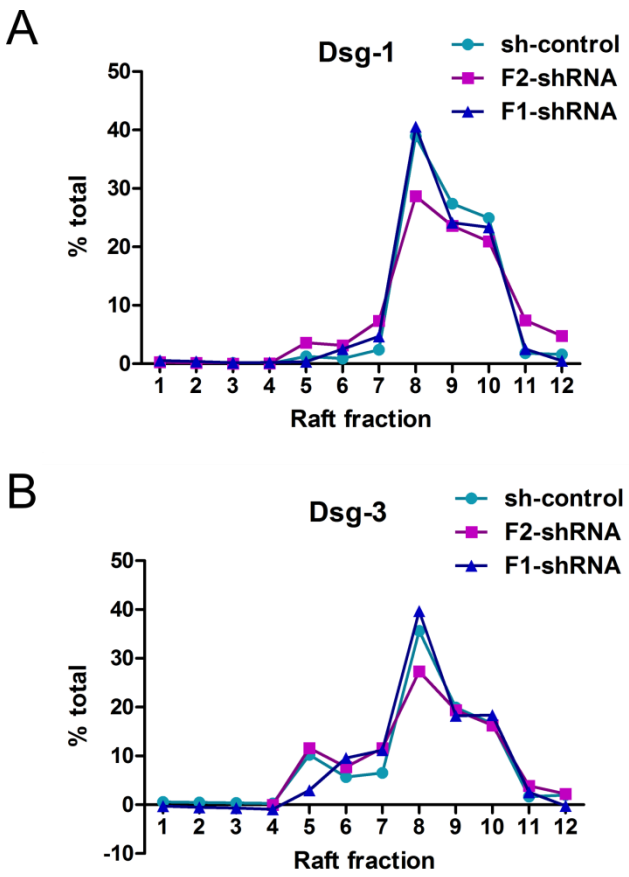

Figure 4.15: The association of desmogleins with lipid rafts is not affected in HaCaT keratinocytes.
Rafts were isolated from control and flotillin depleted HaCaT keratinocytes according to a protocol that was established for HaCaT cells (McGuinn and Mahoney, 2014). Cells were lysed and a sucrose gradient was applied before ultracentrifugation. 12 fractions were afterwards collected from the top (lightest fraction) to the bottom. Fractions were separated by Western blot and the distribution of desmoglein-1 (A) and desmoglein-3 (B) within the fractions was analyzed by scanning the densitometry of protein bands. The percentage of protein in each raft fraction was calculated by taking the sum of signals in all fractions as 100 %. Data points are the results of a single experiment, n=1.

4.2.3 Derailed endocytosis of desmoglein-3 upon flotillin knockdown

Flotillin knockdown has no impact on the distribution of desmogleins within membranes. Thus, the observed alterations in the flotillin knockdown HaCaT cells (altered morphology, decreased desmoglein-3 protein level) have to be ascribed to other functions of flotillins. Flotillins are involved in endocytic processes but their exact role there is still under debate (Meister and Tikkanen, 2014). In addition, they are reported to participate in endosomal sorting (John et al., 2014; Saslowsky et al., 2010). Hence, a possible involvement of flotillins on the endocytosis of desmoglein-3 was addressed next.

The internalization of desmoglein-2 is a dynamin dependent process (Chen et al., 2012). Dynamin is a GTPase that is involved in endocytic processes like vesicle fission but also in other trafficking events. Inhibition of dynamin can be achieved with the inhibitor dynasore. Flotillin knockdown and control HaCaT cells were treated for two hours with dynasore and immunostained for desmoglein-3. This revealed that the altered/ disturbed membrane

staining of desmoglein-3 in the flotillin knockdown cells that was observed before (Fig 4.6) was restored when dynamin and thus desmoglein-3 endocytosis was inhibited. Upon dynasore treatment, desmoglein-3 localizes at the plasma membrane in a defined and sharp manner in the flotillin knockdown cells, comparable to its appearance in the control cells (Fig 4.16). A second dynamin inhibitor MiTMAB was used to verify the results. When flotillin knockdown cells were treated with MiTMAB, the disturbed membrane staining of desmoglein-3 was restored as well (Fig 4.17). These data suggest that inhibition of desmoglein-3 endocytosis enhances the amount and/or availability of desmoglein-3 at the plasma membrane, which then normalizes the morphological alterations caused by a knockdown of flotillins. Therefore, it can be postulated that flotillins are required to stabilize desmoglein-3 at the plasma membrane and that their ablation may result in an enhanced endocytic uptake of desmoglein-3.

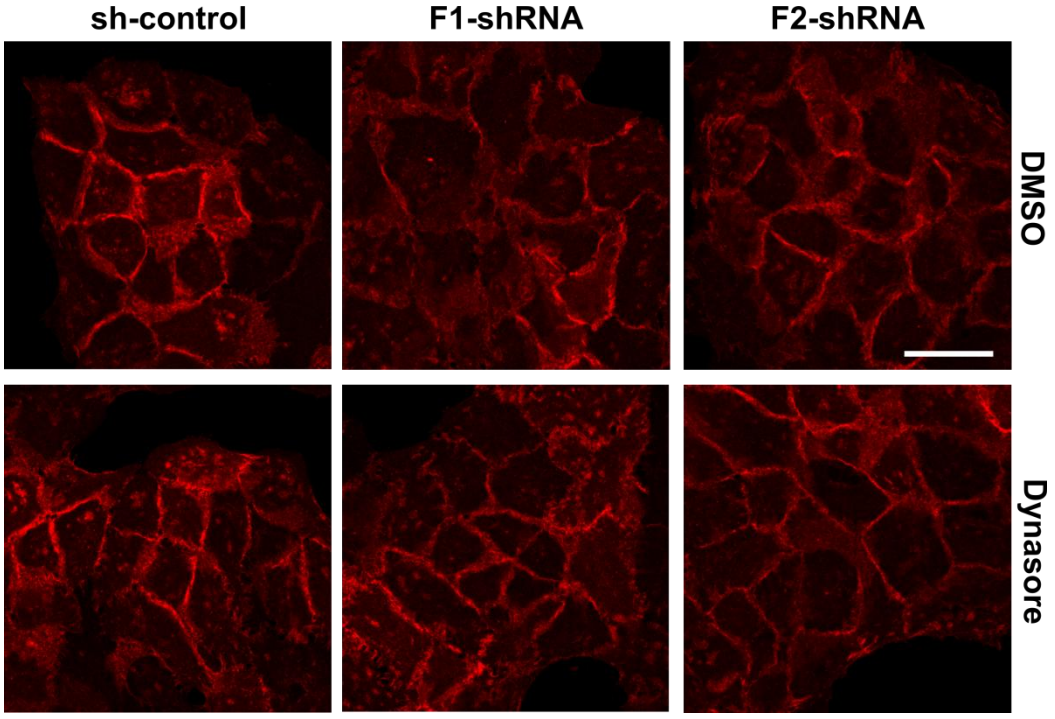

Figure 4.16: Dynasore treatment restores the altered membrane localization of desmoglein-3 in flotillin knockdown HaCaT keratinocytes.
HaCaT keratinocytes were grown on glass coverslips for three days, serum-starved over night and treated with 80 µM dynasore or DMSO for two hours. Cells were fixed and stained with a desmoglein-3 antibody. The disturbed membrane staining observed in flotillin knockdown cells (upper panel) was restored in cells where the endocytic uptake of desmoglein-3 was inhibited by dynasore (lower panel). Scale bar: 20 µm. Fig 4.16 was modified from Völlner et al., 2016.

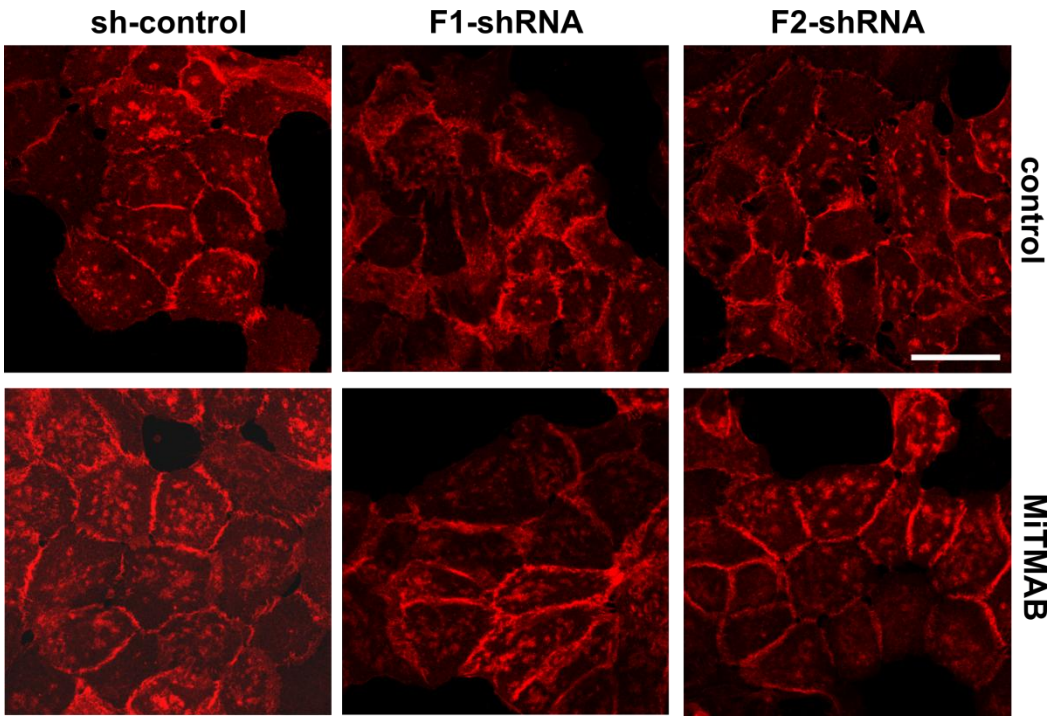

Figure 4.17: MiTMAB treatment restores the altered membrane localization of desmoglein-3 in flotillin knockdown HaCaT keratinocytes.
HaCaT keratinocytes were grown on glass coverslips for three days, serum-starved overnight and treated with 30 µM MiTMAB for 30 minutes. Cells were fixed and stained with a desmoglein-3 antibody. The disturbed membrane staining observed in flotillin knockdown cells (upper panel) was restored in cells where the endocytic uptake of desmoglein-3 was inhibited by MiTMAB (lower panel). Scale bar: 20 µm. Fig 4.17 was modified from Völlner et al., 2016.

4.3 Flotillins in the autoimmune disease pemphigus vulgaris

Pemphigus vulgaris is a potentially life-threatening disease that causes a blistering of skin and mucosal areas. Auto-antibodies against desmosomal components, mainly desmoglein-3, lead to the dissociation of epidermal keratinocytes, a process that is called acantholysis. Several hypotheses how Pemphigus auto-antibodies (PV Ig) may trigger acantholysis are discussed. There is evidence that the binding of PV Ig to desmoglein-3 activates its endocytic uptake from the plasma membrane and subsequent lysosomal degradation (Jennings et al., 2011).

HaCaT keratinocytes were incubated with PV IgG, isolated from the blood of Pemphigus vulgaris patients, and immunostained for desmoglein-3 and flotillin-2 (Fig4.18). As described previously, the desmoglein-3 staining was affected and became scattered and dash-like. This pattern is typical and has been described before as “linear arrays” (Jennings et al., 2011), which are assumed to be sites of endocytic uptake of desmoglein-3. Surprisingly, the localization of flotillin-2 was affected as well in response to PV IgG. The membrane staining was almost completely lost and the typical intracellular staining became diffuse and disordered. This, together with the similar morphology of desmoglein-3 in response to PV IgG and in flotillin knockdown keratinocytes (Fig 4.6), argues for a

role of flotillins not only in physiological processes in the skin but also in the disease pemphigus.

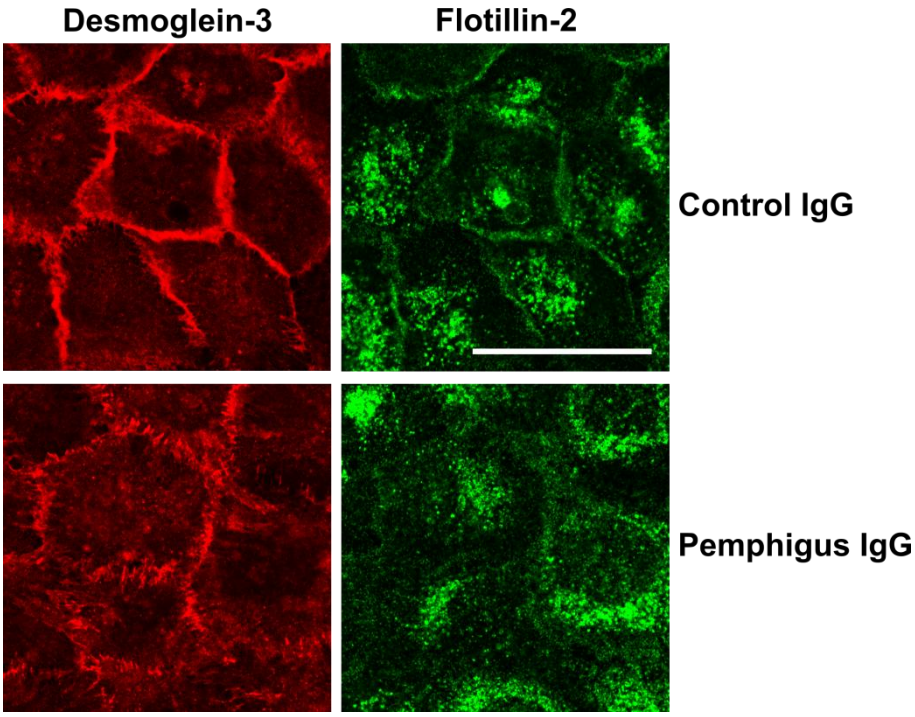

Figure 4.18: Pemphigus vulgaris IgG influence the cellular localization of flotillin-2 in HaCaT keratinocytes.
HaCaT keratinocytes were grown for two days on glass coverslips, serum-starved for 16 hours and treated with 150 µg/ml of either control IgG (healthy person) or Pemphigus vulgaris IgG (Immuno-apherisate mix) for 20 hours. Cells were fixed and immunostained for desmoglein-3 (red) and flotillin-2 (green). Scale bar: 20 µm. Fig 4.18 was modified from Völlner et al., 2016.

4.4 Flotillins and desmosomes *in vivo*

Flotillin-2 knockout mice (Flot-2-KO) are available in the Tikkanen lab (Banning et al., 2014a). The status of adhesion protein level in the skin of these mice was addressed before, but due to a limited number of animals, no final conclusion could be drawn (PhD thesis N. Kurrle). Therefore, in this study the skin of the Flot-2-KO mouse was examined further.

The skin of adult Flot-2-KO and wild type (WT) mice was lysed and the protein amount of desmoglein-1 and -3, E-cadherin and y-catenin was analyzed by Western blot (Fig 4.19 A). Quantitative analysis revealed that the expression of none of these proteins was significantly affected in the skin of the Flot-2-KO mice (Fig 4.19 B-E). Desmoglein-3 showed a tendency to be up-regulated, but due to large variations between the mice, the data failed to become significant. An up-regulation of desmoglein-3 in the Flot-2-KO mice is contradictory to the reduced desmoglein-3 level observed in the flotillin-2 knockout HaCaT keratinocytes (Fig 4.10). However, it is possible that mechanisms like

compensation account for the differences observed in mice, which represent a more physiological model.

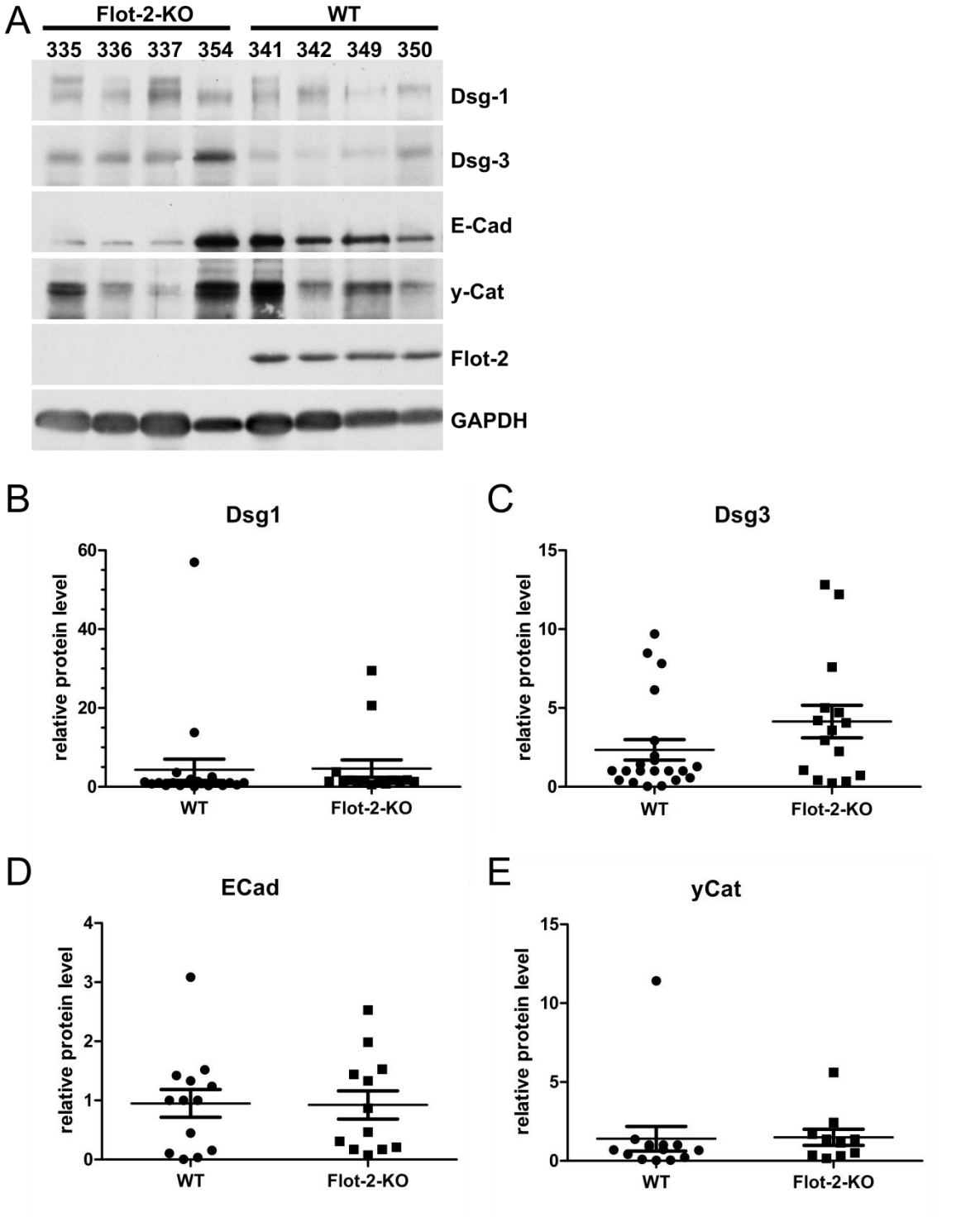

Figure 4.19: Level of adhesion proteins in flotillin-2 knockout mouse skin.
(A) Skin samples of adult wildtype (WT) and flotillin-2 knockout (Flot-2-KO) mice were lysed, and the levels of several adhesion proteins were analyzed by Western blot. (B-E) Western blot bands for desmoglein-3, desmoglein-1, E-cadherin and y-catenin were quantified by scanning densitometry and normalized against GAPDH. Data points represent the relative protein amount in one mouse skin lysate, normalized to one wildtype skin lysate. The mean ± SD is indicated for each protein. Fig 4.19 B-E were modified from Völlner et al., 2016.

Mouse skin samples were also immunostained for desmoglein-1 and desmoplakin (Fig 4.20). A staining of desmoglein-3 and both flotillins would have been very interesting, but was not feasible due to technical reasons. Desmoglein-1 is found at the cell borders in the epidermis of WT and Flot-2-KO mice. No remarkable alterations between the WT and the flotillin-2 knockout were observed (Fig 4.20 A). Desmoplakin is mainly localized at the cell borders in the epidermis and no difference in the staining pattern was detected either (Fig 4.20 B).

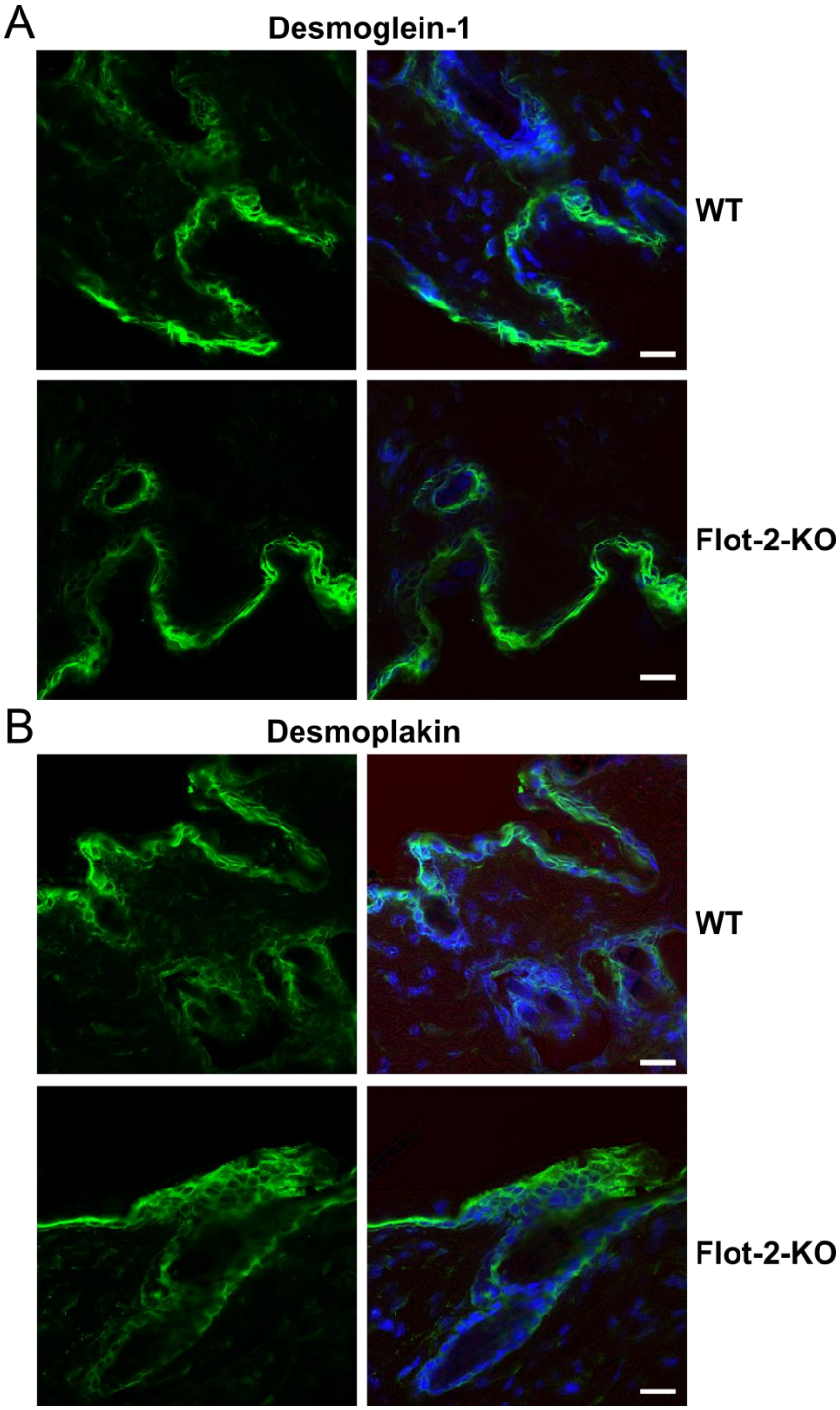

Figure 4.20: Desmoglein-1 and desmoplakin localization is not altered in the skin of Flot-2-KO mice. Cryo-sections of the skin of a wildtype (WT) and a flotillin-2 knockout (Flot-2-KO) mouse were stained in green for desmoglein-1 (A) and desmoplakin (B). Nuclei were stained in blue with DAPI. Scale bar: 20 µm. Fig 4.20 was modified from Völlner et al., 2016.

5 Discussion

So far, the role of membrane microdomains in adhesion processes was examined by several research groups. Some studies linked flotillins to cell-cell adhesion, but mainly focused on adhesion that is mediated by adherens junctions (Bodin et al., 2014). Furthermore, there is emerging evidence that normal desmosomal adhesion as well as the pathomechanism of the desmosome-associated disease pemphigus are linked to functional membrane microdomains (Brennan et al., 2011; Stahley et al., 2014). However, the possible role of flotillins in the regulation of desmosomal adhesion was so far not investigated. Recently, we were able to show that flotillins directly bind to the armadillo protein γ-catenin which can be part of both the adherens junction and the desmosome, albeit its affinity for desmosomes is much greater than for adherens junctions (Chitaev et al., 1996; Kurrle et al., 2013). Hence, it was reasonable and interesting to investigate the role of flotillins in desmosomal adhesion.

5.1 Flotillins are associated with desmosomes

It is known that desmosomal cadherins colocalize with CD59 and caveolin-1, which are components of membrane microdomains (Brennan et al., 2011; Delva et al., 2008). Flotillins are proteins that are associated with membrane rafts distinct from caveolin-1 enriched membrane microdomains. Flotillins were found to colocalize with several classical cadherins at the plasma membrane of epithelial and non-epithelial cells (Guillaume et al., 2013; Kurrle et al., 2013; Solis et al., 2012). In addition, we were able to show for the first time that flotillin-2 also colocalizes with the desmosomal cadherin desmoglein-3 in MCF10A mammary epithelial cells, as well as with γ-catenin, a protein that is found in adherens junctions and in desmosomes (Kurrle et al., 2013). As HaCaT keratinocytes were the main focus of this study, the localization of flotillins and desmoglein-3 was investigated. Flotillin-2 and desmoglein-3 colocalize at the plasma membrane of HaCaT cells. As the flotillin-1 staining overlaps almost completely with the flotillin-2 staining, it can be assumed that both flotillins colocalize with desmoglein-3. However, a substantial pool of flotillins is found in intracellular vesicles in HaCaT keratinocytes, whereas desmoglein-3 is not found in these structures. This indicates that only a fraction of the flotillin proteins found in these cells might be associated with desmosomes.

The results of the present study show that flotillins are found in an endogenous complex with desmogleins 1-3 in HaCaT keratinocytes. This association was verified and further examined by pulldown experiments. Flotillins are associated with the cytosolic domain of desmosomal cadherins, which was to be expected as flotillins do not exhibit either extracellular or transmembrane domains. The pulldown assays revealed that, in addition to desmogleins, flotillins are associated with the second class of desmosomal cadherins, the desmocollins. Interestingly, they only precipitated with the long isoforms of desmocollins 1-3, but did not associate with the shorter isoforms. The shorter desmocollin isoforms lack the ICS sequence that is found in the longer isoforms, as well as in the desmogleins. The

ICS sequence is the binding motif for γ-catenin in cadherins. We could recently show that flotillins directly bind to γ-catenin (Kurrle et al., 2013). Therefore, it was possible that flotillins bind to desmosomal cadherins only indirectly via γ-catenin. However, in the pulldown assay, γ-catenin was pulled down with the short isoform desmocollin-2b, whereas neither flotillin was detectable in that sample. This indicates that the interactions that were detected with that assay might be direct and not facilitated by γ-catenin or some other protein.

The binding of flotillins to desmosomal cadherins was investigated further with *in vitro* pulldown assays using purified recombinant proteins. The interaction of both flotillins with desmoglein-3 and desmocollin-1a is direct and not facilitated by γ-catenin which was absent in these assays. Therefore, it is assumed that flotillins are able to directly bind to desmosomal cadherins, albeit the direct association with the other isoforms was so far not tested. A good candidate for the binding motif of flotillins within cadherins is the ICS sequence that is present in all cadherins that are bound by flotillins. In addition, the long and short isoforms of the desmocollins are identical in their sequence but the short isoforms, which do not bind flotillins, lack the ICS. Hence, it can be postulated that flotillins bind to the ICS sequence in desmosomal cadherins (Fig 5.1).

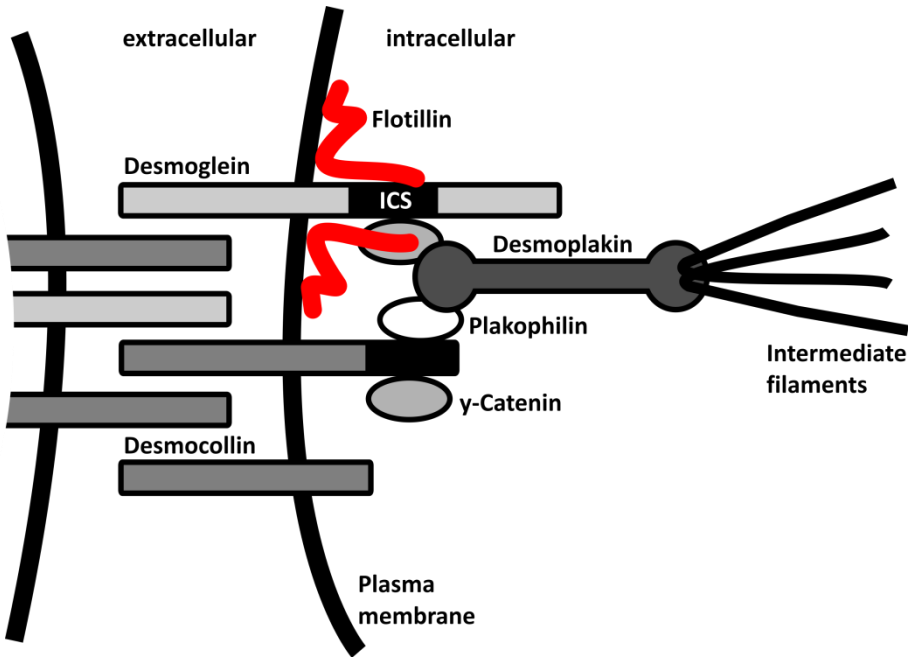

Figure 5.1: Flotillins are associated with the desmosomal plaque.
The membrane raft associated flotillins are able to directly bind to the ICS sequence in desmosomal cadherins. In addition, they bind the plaque protein γ-catenin. Hence, flotillins can be considered to be part of the desmosomal plaque in keratinocytes, where they are required to stabilize the desmosome.

5.2 Flotillin depletion interferes with desmosomal function

It has been reported that the depletion of flotillin-2 affects the morphology of classical cadherins and adherens junctions. In diverse epithelial cell lines, flotillin-2 depletion

results in an altered staining of E-cadherin and γ-catenin at the plasma membrane in a way that the signal becomes diffuse and/ or scattered. Depletion of flotillin-1 resulted in a diminished E-cadherin staining at the plasma membrane (Guillaume et al., 2013; Kurrle et al., 2013; Solis et al., 2012). The desmosomal cadherin desmoglein-2 displayed a disturbed localization when its binding to the lipid raft component caveolin-1 was disrupted (Brennan et al., 2011). In line with these observations, the knockdown of flotillins affected the morphology of desmogleins in HaCaT keratinocytes. The plasma membrane staining of desmoglein-3, desmoglein-1 and desmoglein-2 (J. Ali, MD thesis) was disturbed and blurred in the stable flotillin-1 and flotillin-2 knockdown HaCaT cells. Furthermore, the staining pattern of desmosomal plaque proteins was also affected by flotillin knockdown, implicating that flotillins are necessary for the molecular organization of the desmosome.

The protein expression of desmoglein-3 was significantly reduced upon knockdown of flotillins in HaCaT cells. Other desmosomal proteins (desmoglein-1 and γ-catenin) show large variations of expression levels in flotillin-1 knockdown cells and exhibit a tendency to be downregulated as well. In addition, the expression of desmoglein-2 and desmoplakin was not affected in the flotillin depleted HaCaT keratinocytes (Völlner et al., 2016; J. Ali, MD thesis). It is unclear why the ablation of flotillins affects only the desmoglein-3 protein levels. The cells used for these experiments were grown confluent for 2 days and 5 days in total. Upon formation and maturation of desmosomes, the protein expression of desmoglein-1 is detectable early in HaCaT cells, prior to the appearance of desmoglein-3 (data not shown). It is possible that under other circumstances (e.g. longer culture of cells, subconfluent cells), the expression of other desmosomal proteins is impaired as well.

Interestingly, desmoglein-3 showed a tendency to be up-regulated in the skin of the Flot2 KO mice, although the data were not significant. In contrast, the protein amounts of desmoglein-1, γ-catenin and E-cadherin were not altered in the Flot2 KO mice. This result seems somewhat contradictory to the reduced expression of demoglein-3 observed in the flotillin knockdown HaCaT cells. However, it is known that flotillin ablation in cell culture and mouse models can result in opposite effects due to compensatory mechanisms in the mouse model. For instance, flotillins are known enhancers of the EGFR and MAPK signaling, but in the Flot2 KO mouse the MAPK signaling cascade is hyperactive due to an upregulation of EGFR target genes (Amaddii et al., 2012; Banning et al., 2014a). In addition, the expression status of flotillins in the Flot2 KO mouse differs from the HaCaT cell culture model, as both flotillin proteins are absent in the mouse skin, whereas only one flotillin is knocked down in each cell line (Banning et al., 2014a). However, the fact that only desmoglein-3 showed a tendency to be upregulated may imply that the observed reduction of desmoglein-3 level in the HaCaT cells is actually a specific effect and that the other desmosomal proteins are not regulated by flotillins in the same way.

In line with the mislocalization of desmosomal components and the reduction in desmoglein-3 protein level, flotillin depletion in HaCaT cells interferes with the epithelial strength in cell monolayers. In a DBDA, the monolayer of the flotillin depleted HaCaT cells dissociated into numerous fragments upon mechanical stress, whereas the monolayer of the control cells remained basically intact. The addition of ETA (which cleaves desmoglein-1) had no impact on the fragment number, indicating that desmoglein-1 is not

or only to a minor extent implicated in the cell-cell adhesion strength in these cells. However, almost all of desmoglein-1 in HaCaT cells is found in the nonjunctional pool of desmosomal proteins, whereas desmoglein-3 is largely associated with the desmosomal pool. This would strengthen the hypothesis that desmoglein-3 is more important for the desmosomal adhesion in HaCaT keratinocytes than desmoglein-1. The weakened epithelial strength upon flotillin ablation is in accordance with the data obtained in A493 cells, where the depletion of flotillin-2 resulted in an enhanced fragmentation in a DBDA (Solis et al., 2012). It is also known that destruction of lipid rafts per se (by means of cholesterol-depletion) weakens epithelial adhesion and results in more fragments in a DBDA (Stahley et al., 2014). However, ablation of membrane cholesterol is a rough method that destroys the organization of membranes and affects the entire lipid rafts in the cell. In contrast, the depletion of a single protein component is a more precise experiment. It is thus likely that the effects observed for cholesterol depletion are at least in part due to the destruction of flotillin microdomains. Nevertheless it remains open whether other membrane microdomain associated proteins, like caveolin-1, have the same impact on cell-cell adhesion strength.

Another assay to measure the adhesive strength of cells is a re-aggregation assay. The main difference to a DBDA is that intact cell layers are destroyed and a single cell suspension is produced. The assay measures the capacity of these single cells to re-aggregate over a certain, usually rather short, time. In contrast, a DBDA measures the strength of an intact cell monolayer to withstand mechanical stress. The fast aggregation is supposed to be dependent on classical cadherins rather than on desmosomal cadherins. Nevertheless, it was surprising that the re-aggregation capacity of flotillin-depleted HaCaT keratinocytes was more than 4-fold enhanced compared to the control cells, although E-cadherin expression is downregulated in these cells. The re-aggregation was clearly dependent on the action of cadherins as upon addition of EGTA, which chelates calcium ions, no difference in the formation of aggregates was observed compared to control cells. These data suggest that the desmosomal adhesion in HaCaT cells is weakened upon loss of flotillins, whereas that mediated by adherens junctions may even be enhanced. This result is contradictory to data obtained by Guillaume and colleagues who reported that the re-aggregation capacity of MCF7 cells and myoblasts is reduced upon flotillin-1 knockdown (Guillaume et al., 2013). In addition, our own data for MCF10A cells show that flotillin ablation had no impact on the re-aggregation capacity in this cell line, which points towards a cell type specific regulation of the process (data not shown).

5.3 Flotillins as regulators of desmosome dynamics

Flotillin depletion in keratinocytes results in the mislocalization of desmosomal proteins at the plasma membrane, weakens the epithelial strength and affects the desmoglein-3 protein level (see above). A general feature of lipid rafts and flotillin microdomains is their ability to move freely within the membrane and to cluster to large platforms. In addition, flotillins recruit and interact with several proteins of the catenin family, like p120-catenin and γ-catenin and thus may help to strengthen cis-interactions of cadherin complexes at the plasma membrane (Chartier et al., 2011; Kurrle et al., 2013). It was shown that flotillin-

cadherin complexes are associated with F-actin rich regions, and disruption of F-actin enhances the lateral mobility of flotillin-2, suggesting that flotillin microdomains stabilize cadherins at cell contact sites (Guillaume et al., 2013; Langhorst et al., 2007). Hence, it was addressed here whether flotillins are also important for the stabilization of desmosomal proteins.

5.3.1 Lateral dynamics

Recently, it was shown in human keratinocytes that a fraction of desmogleins and other desmosomal components is associated with membrane rafts defined by caveolin-1 and flotillin-1 (Delva et al., 2008; Stahley et al., 2014). This raft association of desmosomal proteins even increased when the cells were shifted to a higher calcium concentration to induce the formation of desmosomes. Furthermore, when rafts were disrupted by a cholesterol perturbing agent, a dose-dependent shift of desmoglein-3 from the junctional to the nonjunctional desmosomal pool was observed (Stahley et al., 2014). Depletion of membrane cholesterol resulted in a shift of desmoglein-2 from the raft to the non-raft fraction (Brennan et al., 2011). Therefore, a model was suggested where membrane microdomains serve as platforms for the formation of desmosomes (Stahley et al., 2014). As flotillins define their own class of membrane microdomains, it was plausible that they are the components that link desmogleins to membrane rafts and thereby regulate the formation of desmosomes. However, a flotillin knockdown in the HaCaT keratinocytes neither influenced the distribution of desmogleins in the different pools, nor was the association of adhesion proteins with membrane rafts altered. However, as the expression of Dsg3 is much lower in the flotillin knockdown cells compared to the control cells, the absolute amount of Dsg3 is reduced in both the raft fractions and in the desmosomal pool. This diminished protein amount may explain the weaker desmosomal adhesion in the absence of flotillins. The association of desmosomal proteins with membrane rafts and with the junctional pool was studied using a calcium-shift to induce desmosome formation (Stahley et al., 2014). In contrast, in the experiments used for our study, the steady state situation (confluent stable knockdown cells) was examined (Völlner et al., 2016). Thus, it is still likely that flotillin microdomains influence cadherin dynamics in the process of desmosome formation. This could for example be studied by using a calcium-shift in primary keratinocytes with a transient flotillin-knockdown.

5.3.2 Endocytosis and membrane stabilization

Flotillins have been described as regulators of endocytic processes, although their definite function is under discussion. It was proposed that they may define a new clathrin and dynamin-independent endocytic pathway (Frick et al., 2007; Glebov et al., 2006). On the other hand, they also participate in clathrin-mediated endocytosis by clustering cargo molecules at the plasma membrane prior to their endocytic uptake (Amaddii et al., 2012; Schneider et al., 2008; Sorkina et al., 2013). In addition, their own endocytic uptake requires dynamin-2 and their recycling back from endosomes to the plasma membrane is dependent on clathrin (Meister et al., 2014). Only recently, it was stated that flotillins are implicated in the endocytosis and trafficking of E-cadherin. Solis and colleagues showed that flotillin-2 has an indirect impact on the internalization of E-cadherin by means of

macropinocytosis. They found that deletion of flotillin-2 in EGF stimulated A431 cells interferes with the endocytosis of the EGFR and results in an increased EGFR signaling, which in turn enhances the macropinocytosis of E-cadherin (Solis et al., 2012). These results are still under debate, as we were able to show that flotillins do not directly affect the endocytosis of the EGFR (Amaddii et al., 2012), and others found that flotillin-1 was not important for the endocytosis of cadherins, nor was E-cadherin macropinocytosis observed in flotillin-1 knockdown cells (Guillaume et al., 2013). In the study of Solis et al., the effects of flotillins on EGFR endocytosis and on cadherin macropinocytosis were studied in the epidermoid carcinoma cell line A432 that overexpresses the EGFR. It is likely that the observed effects are cell type specific and that the regulation of the studied processes differs between pathogenic and non-pathogenic situations.

In this study, it was shown that flotillin depletion in HaCaT keratinocytes results in diminished desmoglein-3 level due to an increase in desmoglein-3 endocytosis followed by lysosomal degradation. When lysosomal degradation was inhibited with the chemical Bafilomycin A, desmoglein-3 accumulated to a large extent in LAMP1 positive vesicular structures (lysosomes) in HaCaT keratinocytes. Furthermore, treatment of the flotillin-depleted HaCaT cells with Bafilomycin A rescued the diminished desmoglein-3 protein level in these cells to an extent comparable to the control cells (Völlner et al., 2016). This indicates that increased lysosomal turnover of desmoglein-3 accounts for the reduced protein levels observed in the flotillin knockdown cells.

Lysosomal degradation of desmoglein-3 requires its endocytosis from the plasma membrane. The endocytosis of desmosomal cadherins seems to be a clathrin-independent process (Chen et al., 2012; Delva et al., 2008). Several studies found that the internalization of different desmogleins is dependent on membrane cholesterol, and it is assumed that membrane rafts play a role in the endocytic uptake of desmogleins under physiological as well as PV IgG induced conditions (Brennan et al., 2011; Chen et al., 2012; Delva et al., 2008). Nevertheless, the raft protein caveolin-1 is presumably not required (Chen et al., 2012; Delva et al., 2008). Differing results were obtained concerning the importance of dynamin for the endocytosis of desmogleins. Delva and colleagues stated that the internalization of desmoglein-3 that is induced by PV IgG is a dynamin-independent process (Delva et al., 2008). They used overexpression of a dominant negative dynamin-2 mutant. In contrast, Chen and colleagues found that the endocytosis of desmoglein-2 is dependent on dynamin (Chen et al., 2012). In their study they addressed the physiological turnover of desmoglein-2 by using the chemical dynamin inhibitor dynasore, which is, compared to the overexpression of a dominant negative mutant, a more direct and efficient way to study dynamin dependency.

In this study, we could show that the physiological internalization of desmoglein-3 in HaCaT cells is dependent on dynamin. When dynamin-dependent endocytosis was blocked with dynasore in flotillin-depleted HaCaT keratinocytes, the altered morphology of desmoglein-3 was restored and became comparable to the control cells. These findings were confirmed by using MiTMAB, another inhibitor of dynamin. This suggests that in the absence of flotillins, enhanced endocytosis and turnover of desmoglein-3 take place, which results in diminished protein levels and, subsequently, in reduced cell-cell adhesion.

Furthermore, the morphological alterations observed in the flotillin knockdown HaCaT cells are similar to the localization of desmoglein-3 that is observed upon the treatment with PV IgG. These structures, described as linear arrays, are sites of endocytic uptake (Delva et al., 2008). This strengthens the hypothesis that enhanced endocytosis of desmoglein-3 occurs in the absence of flotillins in HaCaT keratinocytes, or that flotillins are required to stabilize desmoglein-3 in desmosomes.

Flotillins may even be of importance for the pathomechanism of pemphigus vulgaris, as treatment of HaCaT cells with PV IgG alters the cellular localization of flotillin-2, away from the plasma membrane towards the cytoplasm. Loss of flotillins at the plasma membrane may contribute to an enhanced desmoglein-3 internalization and thus to acantholysis, which is the cause of the blistering in pemphigus vulgaris.

Taken together, our data depict a new model of the function of flotillins in cell-cell adhesion mediated by desmosomes. By binding to the ICS sequence of desmosomal cadherins and to the plaque protein γ-catenin, flotillins stabilize and cluster desmosomal components at the plasma membrane, which results in stabilization of their plasma membrane localization (Fig 5.1 and Fig 5.2 upper part).

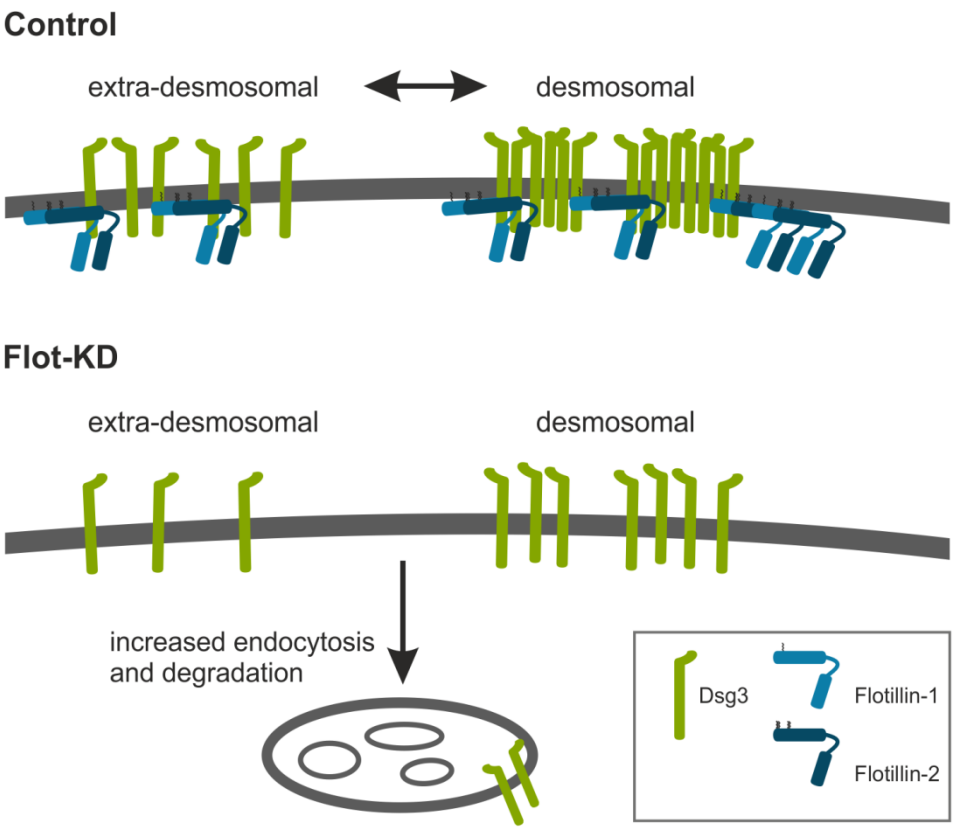

Figure 5.2: Model for the function of flotillins in desmosomal adhesion in keratinocytes.
Desmoglein-3 exists in two different plasma membrane associated pools. The extra-desmosomal pool consists of cadherins that are plasma membrane associated but not part of mature desmosomes. Cadherins belonging to the desmosomal pool are considered to be associated with the cytoskeleton and incorporated in mature desmosomes. It is assumed that lateral exchange takes place between both cadherin pools. Flotillins are associated with desmoglein-3 at the plasma membrane, where they are required to stabilize desmoglein-3. In the absence of flotillins, desmoglein-3 is exposed to an increased dynamin-dependent endocytosis followed by its lysosomal degradation. As a consequence, the amount of total desmoglein-3 becomes diminished and the desmosomal adhesion is reduced. Fig 5.2 was taken from Völlner et al., 2016.

Flotillins do bear such a clustering function in the process of EGFR signaling and uptake (Amaddii et al., 2012). It is currently unknown if flotillins interact with the desmosomal or non-desmosomal pool of desmosomal cadherins or with both. In the absence of flotillins, desmoglein-3 is not longer efficiently clustered and stabilized at the plasma membrane, which increases its endocytic uptake and degradation. Finally, this results in a weaker desmosomal adhesion (Fig 5.2 lower part).

5.4 Outlook

In this study, co-immunoprecipitation and pulldown experiments were used to show that flotillins are novel binding partners of desmosomal cadherins. The ICS sequence is presumably the binding motif of flotillins within cadherins. However, a detailed binding map of the interaction of flotillins with desmosomal cadherins is missing so far, and the binding motif within flotillins is completely unknown. Furthermore, flotillins bind to the desmosomal plaque component γ-catenin (Kurrle et al., 2013), and it is thus possible that they interact with other desmosomal plaque proteins like desmoplakin and the plakophilins as well. These issues can be easily addressed by further pulldown assays and coimmunoprecipitation experiments. Mapping the exact interaction domains will also help to find out which features of flotillins (e.g. oligomerization, phosphorylation, heterodimerization, membrane and/ or raft-association) are required for their role in the regulation of desmosomes. It is of importance to keep in mind that the HaCaT keratinocytes used for this study carry a single flotillin-1 or flotillin-2 knockout. Double knockouts have not yet been analyzed, and it is very likely that the effects on adhesion are drastically potentiated when no flotillin is available in the cells. As a CRISPR/Cas9-based knockout model is now available in our laboratory, it should be used to study this. In addition, the CRISPR/Cas9 method has the advantage that it generates genetic knockouts, whereas the shRNA-based knockdown system that was used here bears the risk of residual protein expression. Hence, expression of flotillin or cadherin-mutants in these knockout cells (or even in desmoglein-knockout cells) will reveal which protein domains and/ or post-translational modifications are involved in the flotillin-desmosome interactions and how they affect desmosomal assembly, morphology, and function without any endogenous protein background.

Several studies found desmosomal components associated with membrane rafts, and also in this study, we could show that a part of desmoglein-3 resides in membrane rafts. However, it is currently unknown whether flotillins are associated with desmosomal proteins that reside in the raft or in the non-raft fraction, or whether they interact with desmosomes irrespective of their membrane localization. It would be interesting to find out the composition of flotillin-desmosome complexes in raft versus non-raft fractions, or in the junctional vs. nonjunctional pool, respectively. However, desmosomal proteins are hard to solubilise, and experiments like raft isolations and sequential detergent extraction may destroy weak protein interactions. Hence, standard co-immunoprecipitation experiments are not feasible. Therefore, a method that uses enzymatic labeling to study protein interactions that is now available can be used (Roux, 2013). The protein of interest is genetically fused to a biotin ligase (BirA) and co-expressed together with its potential

interaction partner that carries a short biotinylation target sequence. Upon addition of biotin to the cells, the biotin ligase will biotinylate proteins in its close vicinity that carry this target sequence. The proteins can then be fully solubilised and isolated by means of streptavidin beads. It is not necessary to keep the interaction of the complex partners, as only proteins that have been in close contact are biotinylated.

The stabilization and formation of desmosomes and their association with rafts seem to be cholesterol-dependent processes (Resnik et al., 2011; Stahley et al., 2014). Our results here indicate that a flotillin-knockdown has no impact on the raft association of desmosomal components or on the distribution of desmogleins with membrane pools. However, until now, we focused on the steady state situation in stable flotillin knockdown cells. It is possible that flotillins are involved in dynamic processes within the membrane. Thus, it would make sense to address the question whether the raft-association of desmosomal components is required for processes like desmosome formation and whether flotillins are involved. The use of primary human keratinocytes where desmosome formation can be induced by a simple calcium switch would be needed to answer these open questions. It is not possible to use HaCaT cells for these experiments. However, primary keratinocytes only grow a limited number of passages and are thus not suitable for a proper flotillin knockdown and for experiments like raft isolations, where a rather large amount of cell material is required. Induced pluripotent stem cells (iPS) which can be differentiated to keratinocytes could serve as another source of keratinocytes (Itoh et al., 2011). A stable knockdown or knockout of flotillins in these cells can be achieved with siRNAs/shRNAs or the CRISPR/Cas9 method. Such a cell system would combine a flotillin knockout with the benefits of a more natural keratinocyte cell system that allows experiments like a calcium switch.

In this study, a novel role for flotillins in the stabilization of desmoglein-3 at the plasma membrane was revealed. Upon flotillin knockdown, the internalization of desmoglein-3 is elevated and dependent on dynamin. Nevertheless, it is possible that flotillins are directly involved in the endocytic uptake and further transport processes of desmoglein-3 and that in their absence, this normal takeover is hampered and occurs in a fast and uncoordinated way. Therefore, it is of importance to elucidate the molecular mechanism of desmoglein-3 endocytosis further.

The single components that are involved can be discovered by systematic inhibition or knockdown of known elements of classical endocytic and trafficking routes in wildtype as well as flotillin knockdown cells. In addition, the fate of PV IgG induced endocytosis and involvement of flotillins in this pathomechanism can be studied the same way. It is of importance to dissect whether PV IgG induced and “normal” endocytosis follow the same routes. The disappearance of flotillins form the plasma membrane in response to PV IgG may indicate that flotillins play a role in the pathomechanism of pemphigus. It is possible that the absence of flotillins at the membrane promotes the destabilization of desmoglein-3 and its internalization. The detailed chronology of events that are triggered by PV IgG can be determined using fluorescent fusion proteins and live microscopy. More quantitative data about the internalization of desmogleins in the presence or absence of flotillins and/or in response to PV IgG can be obtained by experiments that use reversible surface

biotinylation. In these experiments, cell surface proteins are biotinylated and the amount of internalized proteins can be determined by streptavidin-precipitation after stripping of the cell surface biotin. This kind of experiments can also help to find out whether flotilins are solely associated with plasma membrane bound desmogleins or whether they also accompany them on their way through other compartments (like endosomes and lysosomes).

We were able to show that desmoglein-3 is targeted for lysosomal degradation after its uptake from the plasma membrane (Völlner et al., 2016). In addition we could show that flotillins play a role in the trafficking of endocytic cargo like the EGFR and BACE1 (John et al., 2014; Meister et al., 2014). Flotillins interact with protein complexes that are required for endosomal sorting, like the GGA (Golgi-associated, gamma-ear containing) and ESCRT (endosomal complex required for sorting) proteins. Thereby, they seem to be important for the recycling of certain cargo molecules (John et al., 2014) as well as for the degradative sorting (M. Meister, PhD thesis). Thus, it is likely that they influence the trafficking/recycling/degradative sorting of desmoglein-3 (and other desmosomal proteins) as well. Live cell imaging or a time course with fixed cells using fluorescent fusion proteins can help to discover the endocytic route of desmogleins and colocalisation with different markers of endosomal subclasses can reveal the single steps of endosomal sorting. A potential interaction of desmoglein-3 with components of the sorting machinery can be achieved by pulldown or co-immunoprecipitation experiments. In the process of degradative sorting, ubiquitination is required to tag proteins. Desmoglein-3 contains several lysines residues that are putative ubiquitination sites (our unpublished data), but it is unknown yet whether it becomes ubiquitinated during endocytosis. It should be examined whether desmoglein-3 becomes ubiquitinated and if so, which lysine residues are implicated.

Finally, it is open at the moment whether flotillins are also implicated in the stabilization of other desmosomal components. The observed mislocalization of other desmosomal components may be restored as well when the endocytic uptake from the plasma membrane is inhibited by dynasore or MiTMAB. The above mentioned experiments and questions may be adopted to ascertain the whole interplay between flotillins and desmosomal proteins (cadherins and plaque proteins).

In general, it would be interesting to investigate the role of fotillins in processes that require desmosome dynamics in more detail. The putative skin phenotype of the flotillin knockout mice that are available in our lab (F2KO, F1KO, double knockout) has not yet been analyzed under conditions of mechanical or biological stress. Here we just analyzed in a first step whether or not the skin of these mice has profound differences in the morphology of desmosomes. Due to technical reasons, it was only possible to determine the staining pattern of desmoglein-1 and desmoplakin. No significant differences in the localization or morphology of these two desmosomal proteins were observed. Hence, it would be interesting to examine whether these mice have problems in regenerating processes that require dynamic desmosomes (e.g. wound healing). The mice could also be used to study if flotillins are implicated in the pathology of pemphigus in an animal model. These experiments may reveal a hidden phenotype that is only seen under specific

conditions, and help to elucidate the function of flotillins in adhesion in a more physiological context.

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Deutsche Zusammenfassung

Einleitung

Zell-Zell-Adhäsion

Die Adhäsion zwischen Zellen ist eine Voraussetzung für das Entstehen und die Beständigkeit von Zellverbänden, wie z.B. Epithelien. Zell-Zell-Adhäsion wird gewährleistet durch das Zusammenspiel verschiedener Zell-Adhäsionsmoleküle (engl. CAMs) und spezieller Zellverbindungen (engl. cell junctions). Bei Säugern haben sich vier verschiedene Zellverbindungen mit unterschiedlichen Aufgaben entwickelt. Zwei von ihnen gehören zu den sogenannten haftenden Verbindungen oder Ankerverbindungen (engl. anchoring junctions), die *Zonula adhaerens* (engl. adherens junction) und das Desmosom (auch *Macula adhaerens*). Beide Strukturen haben gemeinsame strukturelle Merkmale und gehören zu den Cadherin-basierten Zellverbindungen.

Cadherine sind große Transmembranproteine, die im extrazellulären Raum an Cadherine von benachbarten Zellen binden können. Diese Bindung ist abhängig von der Anwesenheit von Calcium-Ionen. Die Cadherine der Desmosomen werden als desmosomale Cadherine bezeichnet. Im Zytoplasma sind Cadherine mit unterschiedlichen Plaque-Proteinen, wie z.B. den Armadillo Proteinen, assoziiert. Darunter ist das Armadillo Protein γ-Catenin das einzig momentan bekannte Protein, das sowohl mit klassischen als auch mit desmosomalen Cadherinen assoziert ist. Des Weiteren werden Cadherine von Adaptorproteinen gebunden, welche ihrerseits eine Bindung zu Strukturen des Zytoskellets eingehen können. Auf diese Weise sind die Zellverbindungen mit dem Zytoskelett verbunden, was für eine stabile Adhäsion essentiell ist.

Desmosomen

Desmosomen sind von immenser Bedeutung für Gewebe und Organe, die einer permanenten mechanischen Belastung ausgesetzt sind, wie z.B. die Blase, das Herz oder die Haut. Desmosomen sind punktförmige Strukturen, die mit den Intermediärfilamenten von Zellen verbunden sind und quasi als Ankerpunkte eines Filament-Netzwerkes in Geweben dienen. Obwohl Desmosomen unerlässlich für die Stabilität von Zellverbänden sind, müssen sie auch flexibel und schnell veränderbar sein. Physiologische Prozesse wie Wundheilung oder die Proliferation von basal nach apikal in mehrschichtigen Epithelien erfordern, dass Desmosomen rasch moduliert und umgebaut werden können.

Es gibt zwei Gruppen von desmosomalen Cadherinen, Desmogleine und Desmocolline, die jeweils als verschiedene Isoformen existieren. Ihr extrazelluläre Aufbau ist sehr ähnlich, jedoch gibt es einige Unterschiede in den zytoplasmatischen Domänen. Durch alternatives Splicing existieren alle Desmocolline als kurze oder lange Isoform. Die kurzen Isoformen haben eine sehr trunkierte zytoplasmatische Struktur. Die langen Isoformen besitzen die sogenannte ICS Sequenz (engl. intracellular cadherin-like sequence), die sich auch in den Desmogleinen sowie den klassischen Cadherinen findet. Desmogleine haben im Vergleich zu Desmocollinen weitere intrazelluläre Domänen. Desmosomale Cadherine sind mit γ-

Catenin und den Plakophilinen assoziiert. Plakophiline sind vermutlich wichtig für die laterale Vernetzung innerhalb des desmosomalen Plaques. γ-Catenin bindet sowohl an die ICS Sequenz in Cadherinen als auch an Desmoplakin. Desmoplakin wiederum ist mit den Intermediärfilamenten der Zelle assoziiert und ist damit die entscheidende Komponente, welche die Verbindung des Desmosoms zum Zytoskelett herstellt.

Cadherine, die für den Aufbau der Desmosomen benötigt werden, werden kontinuierlich in der Zelle synthetisiert und zur Plasmamembran transportiert. Dabei folgen sie einer klassischen sekretorischen Route von der Synthese ins endoplasmatische Retikulum (ER), gefolgt vom Transport über den Golgi-Apparat, zur Plasmamembran. Es wird angenommen, dass die Cadherine in der Membran in zwei verschiedenen Pools vorliegen. Der desmosomale (engl. auch junctional) Pool besteht aus allen Cadherinen, die fester Bestandteil von gereiften Desmosomen sind. Dagegen gehören Cadherine, die mit der Plasmamebran assoziiert aber (noch) nicht fest in Desmosomen eingebaut sind, zum extra-desmosomalen (engl. auch nonjunctional) Pool. Beide Pools stehen in einem dynamischen Gleichgewicht zueinander. Es wurde angenommen, dass Cadherine aus dem extra-desmosomalen Pool schnell endozytiert werden können und dann entweder lysosomal abgebaut oder recycelt werden. Generell ist über den Abbau von desmosomalen Cadherinen jedoch wenig bekannt.

Pemphigus

Pemphigus umfasst eine Gruppe von blasenbildenden Autoimmundermatosen. Bei der schweren Form Pemphigus vulgaris (PV) sind sowohl die Haut als auch die Schleimhaut großflächig von der Blasenbildung betroffen. Unbehandelt ist die Krankheit sehr schmerzhaft und kann zum Tod führen. Die Blasenbildung ist immer das Resultat aus der Dissoziation von Epithelzellen, ein Prozess, den man Akantholyse nennt. Die Akantholyse wird ausgelöst durch Autoantikörper (PV IgG), die im Zellzwischenraum an die extrazellulären Domänen von Desmogleinen binden, beim Pemphigus vulgaris ist hauptsächlich Desmoglein-3 betroffen. Die Bindung der Antikörper bewirkt letztendlich eine Zerstörung der Desmosomen, wobei der genaue Pathomechanismus noch nicht vollständig aufgeklärt ist.

Eine Haupt-Hypothese geht davon aus, dass die Bindung der Autoantikörper die Endozytose von Desmoglein-3 auslöst, darauf folgt der lysosomalen Abbau des Proteins. Die Internalisierung von Desmoglein-3 wurde als ein koordinierter Prozess beschrieben, bei dem zunächst der extra-desmosomale Pool von Desmoglein-3 betroffen ist, gefolgt von einer Umordung der verbleibenden Cadherine in Strukturen die *linear arrays* genannt werden. Sie erscheinen als strichartige Strukturen, senkrecht zur Plasmamembran und sind sehr wahrscheinlich Stellen, an denen Endozytose stattfindet. Des Weiteren scheint die Endozytose von der Anwesenheit von Cholesterol in der Membran abhängig zu sein, was auf eine Beteiligung von Membran Mikrodomänen am Pathomechanismus von Pemphigus vulgaris schließen lässt.

Membranmikrodomänen und Zell-Adhäsion

Membranmikrodomänen oder *lipid rafts* ist die Bezeichnung für spezielle Bereiche in der Plasmamembran, die besonders reich an Sphingolipiden und Cholesterol sind. Als Antwort auf verschiedenste Stimuli können *lipid rafts* zu großen Membranstrukturen fusionieren und besitzen damit die Fähigkeit, Membranen zu kompartmentalisieren. Membranmikrodomänen können mit einer Vielzahl von Proteinen assoziiert sein, welche durch die laterale Mobilität der *rafts* gezielt an bestimmten Bereichen von Membranen angehäuft werden können, um dort als Funktionsplattformen zu fungieren. *Lipid rafts* sind an vielen zellulären Funktionen beteiligt, wie z. B. Signaltransduktion, Endozytose oder der Ausknospung von Viren (engl. *virus budding*).

Mittlerweile ist durch eine Vielzahl von Studien belegt, dass *lipid rafts* an der Zell-Adhäsion in epithelialen aber auch nicht-epithelialen Zelltypen beteiligt sind. So weiß man zum Beispiel, dass M-Cadherin und N-Cadherin, aber auch ß-Catenin und p120-Catenin in Myoblasten mit *lipid rafts* kolokalisieren und dass die Zellfusion in diesen Zellen *raft*-abhängig stattfindet. In Endothelzellen ist VE-Cadherin mit *rafts* assoziiert und eine Reduktion des Cholesterollevels in diesen Zellen beeinträchtigt die Funktion von VE-Cadherin. Des Weiteren wurde gezeigt, dass N-Cadherin, E-Cadherin und diverse Catenine in verschiedensten Epithelzelllinien mit *rafts* assoziieren.

Auch desmosomale Proteine wurden in Membranmikrodomänen gefunden und es wurde postuliert, dass der Zusammenbau von Desmosomen und/oder die Endozytose von desmosomalen Bestandteilen in Abhängigkeit von Cholesterol stattfinden. Allerdings wurden widersprüchliche Studien publiziert und es ist bislang unklar, welche Proteine an diesen Vorgängen beteiligt sind. So wurde z. B. gefunden, dass das *raft*-assoziierte Protein Caveolin-1 an Desmogleine bindet, die Endozytose von Desmoglein-2 und -3 scheint aber Caveolin-unabhängig stattzufinden. Auch die Bedeutung von Dynamin für die Endozytose von Desmogleinen ist umstritten.

Flotilline

Flotilline sind hochkonservierte Proteine, die mit Membranmikrodomänen assoziiert sind. Dabei handelt es sich um spezielle *lipid rafts*, die kein Caveolin enthalten. Die beiden homologen Proteine Flotillin-1 und Flotillin-2 sind auf Proteinebene zu 44% identisch und es wird angenommen, dass sie eine sehr ähnliche Struktur haben. Der N-Terminus beider Proteine umfasst eine Stomatin/Prohibitin/Flotillin/HflK/C (SPFH) Domäne, die hauptsächlich für die Membranverankerung der Proteine wichtig ist. Weiter C-terminal befindet sich die sogenannte Flotillin-Domäne, die für die Oligomerisierung der Proteine von Bedeutung ist.

Flotilline werden ubiquitär exprimiert, wobei ihre zelluläre Lokalisation stark vom Zelltyp abhängig ist. Tendenziell befinden sich Flotilline in differenzierten oder konfluenten Zellen eher an der Plasmamembran als in intrazellulären Kompartimenten. Flotilline sind an einer Vielzahl von zellulären Prozessen beteiligt. Sie spielen eine Rolle bei der Endozytose sowie Transport- und Sortierungsprozessen. Zudem reagieren sie auf Wachstumsfaktorstimulation und fungieren bei der Signaltransduktion über den EGFR-MAPK-Weg als Gerüstproteine.

Flotilline wurden auch im Zusammenhang mit Zell-Adhäsionsprozessen beschrieben. Für eine Vielzahl von Adhäsionsmolekülen wurde gezeigt, dass sie (partiell) mit Flotillin-*rafts* assoziiert sind. Zudem interagieren verschiedene klassische Cadherine und Catenine mit Flotillinen, wobei die von uns beschriebene Interaktion mit γ-Catenin die einzig direkte Bindung ist, die bislang gefunden wurde. Eine Depletion von Flotillinen wirkt sich auf die Morphologie der *Zonula adhaerens* aus und beeinträchtigt die Zelladhäsion. Es wird diskutiert, ob Flotilline für die Endozytose und/ oder die Stabilität von klassischen Cadherinen von Bedeutung sind. Indes wurde der Einfluss von Flotillinen auf die Endozytose bislang nur in einer Studie untersucht, welche keinen Zusammenhang zeigen konnte.

Zielsetzung dieser Arbeit

Bislang hat man sich hauptsächlich auf die Funktion von Flotillinen in solchen Adhäsinsprozessen konzentriert, an denen klassische Cadherine beteiligt sind. Allerdings gibt es zahlreiche Hinweise, dass *lipid rafts* an der Regulation der „normalen" desmosomalen Adhäsion, sowie am Pathomechanismus der Desmosomen-assoziierten Autoimmunerkrankung Pemphigus beteiligt sind. Ziel dieser Arbeit war es herauszufinden, ob und wie Flotilline an der desmosomalen Zelladhäsion beteiligt sind.

Ergebnisse und Diskussion

Flotilline assoziieren mit Desmosomen

Es ist bekannt, dass Flotilline mit verschiedenen Cadherinen kolokalisieren, wenig ist allerdings über desmosomale Cadherine bekannt. In dieser Studie wurden HaCaT Keratinozyten als Zellsystem eingesetzt, weshalb zunächst die Lokalisation von Desmoglein-3 in diesen Zellen untersucht wurde. Desmoglein-3 befindet sich in konfluenten HaCaT Keratinozyten hauptsächlich an der Plasmamembran, wo es mit Flotillin-2 kolokalisiert. Die Kolokalisation zweier Proteine weist darauf hin, dass sie sich in Nähe zueinander befinden. Um zu untersuchen, ob Flotilline tatsächlich mit desmosomalen Komponenten assoziiert sind, wurden Koimmunopräzipitationen durchgeführt. Es zeigte sich, dass beide Flotilline die Desmogleine 1-3 präzipitieren können. Diese Interaktion wurde mit aufgereinigten GST-gekoppelten Konstrukten genauer untersucht. Diese Experimente bestätigten, dass die zytoplasmatischen Domänen von Desmoglein-1-3 mit beiden Flotillinen interagieren. Zudem konnte gezeigt werden, dass die langen Isoformen der zweiten Gruppe der desmosomalen Cadherine, der Desmocolline, ebenfalls mit beiden Flotillinen assoziiert sind. Die kurzen Isoformen zeigten dagegen keine Interaktion. Desmogleine und die langen Isoformen der Desmocolline enthalten, im Gegensatz zu den kurzen Isoformen, die ICS Sequenz, welche auch die Bindestelle von γ-Catenin in Cadherinen ist. Da bereits bekannt ist, dass Flotilline direkt mit γ-Catenin interagieren, wurde untersucht, ob sie auch direkt an desmosomale Cadherine binden oder ob die Interaktion über γ-Catenin stattfindet. Direkte Interaktions-Assays zeigten, dass Flotillin 1 direkt an Desmoglein-3 und Desmocollin-1a, nicht jedoch an Desmocollin-1b binden kann. Flotillin-2 bindet direkt an Desmoglein-3, eine direkte

Interaktion mit Desmocollinen konnte jedoch nicht nachgewiesen werden. Deshalb wird angenommen, dass Flotilline direkt mit desmosomalen Cadherinen interagieren können und dass die Bindung sehr wahrscheinlich über die ICS Sequenz stattfindet.

Die Depletion von Flotillinen beeinträchtigt die desmosomale Funktion

Es ist bereits bekannt, dass die Depletion von Flotillin-2 die normale Morphologie klassischer Cadherinen sowie der *Zonula adhaerens* beeinflusst. In dieser Arbeit wurde der Einfluss eines Flotillin Knockdowns auf desmosomale Strukturen mittels stabilen Flotillin-1 und Flotillin-2 Knockdowns HaCaT Keratinozyten untersucht. Es zeigte sich, dass die Lokalisation von verschiedenen desmososmalen Proteinen in den Zellen gestört ist. Zwar befindet sich Desmoglein-3 in den Knockdown Zellen nach wie vor an der Plasmamembran, allerdings ist die Membranfärbung sehr diffus und verstreut und zeigt sich teilweise in Strukturen, die vertikal zur Membran verlaufen. Diese Strukturen ähneln den bereits erwähnten *linear arrays*, die als Stellen, an denen Desmoglein-3 Endozytose stattfindet, beschrieben wurden. Neben Desmoglein-3 ist auch die Lokalisation von Desmoglein-1 sowie die der beiden Plaque-Proteine Desmoplakin und γ-Catenin in den Flotillin-knockdown-Zellen verändert. In allen Fällen ist die Membranfärbung der Proteine diffuser und ungeordneter als in den Kontrollzellen. Aus diesem Grund kann angenommen werden, dass die Depletion von Flotillinen in Keratinozyten zu einer Störung der Struktur von Desmosomen führt. Des Weiteren zeigte sich, dass das Proteinlevel von Desmoglein-3 in den Flotillin Knockdown HaCaT Zellen signifikant vermindert ist. Andere getestete Adhäsionsproteine zeigten teilweise große Schwankungen in den Proteinmengen, jedoch konnten keine signifikanten Unterschiede zu den Kontrollzellen festgestellt werden. Lediglich die Menge von E-Cadherin ist nach Flotillin-2 Knockdown vermindert.

Analog zu der gestörten desmosomalen Struktur und zu dem verminderten Proteinlevel von Desmoglein-3 nach Flotillin Knockdown, zeigte ein Dispase-basiertes Fragmentierungsassay (DBDA), dass die epitheliale Stärke in HaCaT Keratinozyten beeinträchtigt ist. In Folge von mechanischem Stress zerfallen Flotillin-depletierte Zellverbände zu zahlreichen Fragmenten, wohingegen die Kontrollzellen als intakter Zellverband erhalten bleiben. Diese Ergebnisse decken sich mit Daten aus A493 Zellen, bei denen gezeigt wurde, dass ein Knockdown von Flotillin-2 zu einer vermehrten Fragmentierung führt. Über den Einfluss von anderen *raft*-assoziierten Proteinen ist bislang wenig bekannt. Generell resultiert die Zerstörung von Membranmikrodomänen *per se* zu einer Verminderung der epithelialen Stärke. Allerdings ist dies eine relativ grobe Methode, welche die gesamte Membranorganisation und möglicherweise Zellhomöostase angreift.

Flotilline stabilisieren Desmosomen

Um herauszufinden, auf welche Weise Flotilline die Morphologie von Desmosomen sowie die Zell-Adhäsion beeinflussen, wurde zunächst die Assoziation von Adhäsionsproteinen mit Membranmikrodomänen in HaCaT Kerationozyten untersucht. Es ist bereits bekannt, dass ein Teil der desmosomalen Cadherine mit *rafts* assoziiert ist und dass diese Assoziation gesteigert wird, wenn die Reifung von Desmosomen in Zellen angeregt wird. In dieser Studie konnte gezeigt werden, dass ein großer Teil von Desmoglein-3 in HaCaT

Keratinozyten mit Membranmikrodomänen assoziiert ist. Desmoglein-1 sowie weitere Adhäsionsproteine befinden sich dahingegen nur zu einem kleinen Teil in diesen Membranstrukturen. Die *raft*-Assoziierung der Adhäsionsproteine ändert sich mit dem Knockdown von Flotillin-1 oder Flotillin-2 nicht. Des Weiteren zeigten Löslichkeitsstudien, dass auch die Verteilung der desmosomalen Cadherine in den Membran-Pools durch einen Flotillin Knockdown nicht verändert wird.

Flotilline sind an Endozytoseprozessen beteiligt und die Endozytose von Desmoglein-3 ist abhängig von intakten Membranmikrodomänen. Als nächstes wurde untersucht, ob Flotilline die Endozytose von Desmoglein-3 beeinflussen. Die Endozytose vieler Membranproteine kann durch Inhibierung der GTPase Dynamin verhindert werden. Es ist bereits bekannt, dass die Endozytose von Desmoglein-2 dynaminabhängig abläuft, weshalb eine Beteiligung an der Desmoglein-3 Endozytose ebenfalls wahrscheinlich ist. Dynamin wurde mit chemischen Inhibitoren in Flotillin Knockdown HaCaT Zellen gehemmt. Dabei zeigte sich, dass die gestörte Lokalisation von Desmoglein-3 in diesen Zellen durch Hemmung der Endozytose wiederhergestellt werden kann. Dies lässt den Rückschluss zu, dass es auf Grund eines Flotillin Knockdowns zu einer gesteigerten Endozytose von Desmoglein-3 kommt, wodurch weniger Desmoglein-3 an der Membran vorhanden ist, was wiederum die Adhäsionsfähigkeit der Keratinozyten beeinträchtigt.

Flotilline sind möglicherweise ebenfalls am Pathomechanismus von Pemphigus beteiligt. Eine Behandlung von HaCaT Keratinozyten mit PV IgG führte, wie erwartet, zu einer Anordnung von Desmoglein-3 in *linear arrays*. Zudem war aber auch die Lokalisation von Flotillin-2 deutlich verändert. Flotillin-2 befindet sich nach PV IgG Inkubation nahezu ausschließlich in intrazellulären Strukturen. Es ist möglich, dass die Abwesenheit von Flotillinen an der Membran zu einer Destabilisierung von Desmoglein-3 führt, was letztendlich die Akantholyse der Epithelzellen begünstigt.

Fazit

In dieser Arbeit wurde erstmals ein Zusammenhang zwischen desmosomaler Adhäsion und Flotillinen beschrieben. Die Rolle von Membranmikrodomänen für Desmosomen wurde weiter untersucht und Flotilline als *raft*-assoziierte Proteine wurden als wichtige Regulatoren der desmosomalen Funktion beschrieben. Mit dieser Studie konnte gezeigt werden, dass Flotilline direkt an desmosomale Cadherine binden. Diese Bindung führt zu einer Stabilisierung der Cadherine in der Membran und ist wichtig für die Adhäsionsfähigkeit in Keratinozyten. In Abwesenheit von Flotillinen kommt es zu strukturellen Änderungen in der Morphologie von Desmosomen. Des Weiteren werden Desmogleine vermehrt Dynamin-abhängig endozytiert und abgebaut, was zu einer Beeinträchtigung der Adhäsion in Keratinozyten führt. Flotilline wurden damit als neue Regulatoren für die desmosomale Adhäsion in Keratinozyten identifiziert.

List of original publications

Journal articles

Völlner, F., Ali, J., Kurrle, N., Exner, Y., Eming, R., Hertl, M., Banning, A. and Tikkanen, R. (2016) Loss of flotillin expression results in weakened desmosomal adhesion and Pemphigus vulgaris-like localisation of desmoglein-3 in human keratinocytes. Sci. Rep. 6, 28820; doi: 10.1038/srep28820

Kurrle, N., **Völlner, F**., Eming, R., Hertl, M., Banning, A., and Tikkanen, R. (2013). Flotillins Directly Interact with γ-Catenin and Regulate Epithelial Cell-Cell Adhesion. PLoS ONE 8, e84393; doi:10.1371/journal.pone.0084393

Kurrle, N., Ockenga, W., Meister, M., **Völlner, F**., Kühne, S., John, B.A., Banning, A., and Tikkanen, R. (2013). Phosphatidylinositol 3-Kinase dependent upregulation of the epidermal growth factor receptor upon Flotillin-1 depletion in breast cancer cells. BMC Cancer 13, 575; doi:10.1186/1471-2407-13-575

Poster and other presentations

Exner, Y., Schmidt, T., Dittmar, L., **Voellner, F**., Spindler, V., Waschke, J., Tikkanen,R., Hertl, M and Eming, R. (2015): Desmoglein 3-specific autoantibodies recognizing a membrane-proximal epitope induce loss of keratinocyte adhesion. Experimental Dermatology. 42nd Annual Meeting of the Arbeitsgemeinschaft-Dermatologische-Forschung. Volume: 24. (Conference paper)

Debus, F., Kurrle, N., Eming, R., Hertl, M., Banning, A. and Tikkanen, R. (2013). A role for flotillins in cell-cell adhesion. Molecular Life Sciences. International Symposium of the German Society for Biochemistry and Molecular Biology (GBM). Frankfurt am Main, Germany. (Poster)

Kurrle, N., **Debus, F.**, Eming, R., Hertl, M., Banning, A. and Tikkanen R. (2013): Flotillins regulate cell-cell adhesion by interacting with γ-catenin. Membranes in Motion. 64. Mosbacher Kolloquium of the German Society for Biochemistry and Molecular Biology (GBM). Mosbach, Germany. (Poster)

Kurrle, N., **Debus, F.**, Eming, R., Hertl, M., Banning, A. and Tikkanen R. (2012): Flotillins regulate cell-cell adhesion by interacting with γ-catenin. Molecular concepts in epithelial differentiation, pathogenesis and repair. International Meeting of the German Society for Cell Biology (DGZ). Leipzig, Germany. (Poster)

Erklärung und eidesstattliche Versicherung

Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung im mathematisch-naturwissenschaftlichen Bereich unterzogen habe.

Gießen, den

(Frauke Völlner)

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation über „Flotillins as novel regulators of desmosome dynamics“ selbstständig angefertigt habe und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.

Ich versichere, die Grundsätze der guten wissenschaftlichen Praxis beachtet und nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Gießen, den

(Frauke Völlner)