Drebrin preserves endothelial integrity by stabilizing nectin at adherens junctions

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II INTRODUCTION

1 The endothelium

The closed cardiovascular system of mammals is a complex network of vessels, which distributes blood and its components from the heart to all tissues of the body. Starting in major arteries with diameters of two centimeters, blood carries soluble nutrients, gases, hormones and cells towards the remote capillaries, which permit interchange of nutrients and waste products between vessels and tissue[1,2]. Every blood vessel, independent of size, has in common that the interior surface is covered by a monolayer of specialized cells, the endothelium. In small vessels with high interchange rates of substances the *Tunica intima,* consisting of the endothelium and its basement membrane, is the only layer that separates the bloodstream from the tissue. In larger blood vessels two more layers exist, the *Tunica media,* which provides structural anchorage through its elastic tissue and smooth muscle cells and the *Tunica externa,* which consists of collagen and connects blood vessels to organs, increasing the stability of large vessels[3] (Figure 1).

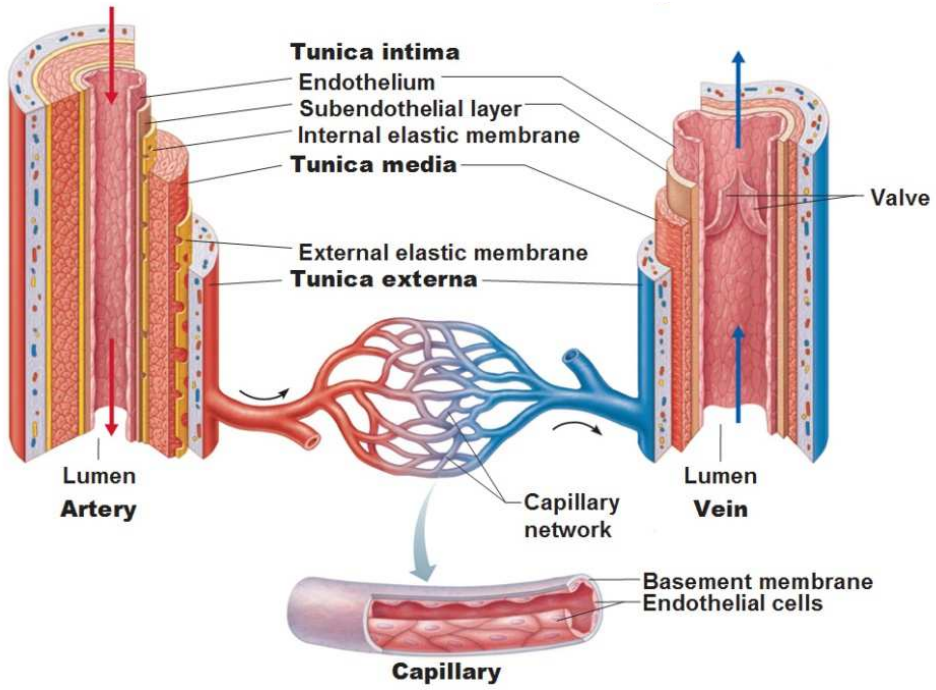


Figure 1 Blood vessels and the endothelium
Schematic view of the vascular tree with structural details of arteries, veins and capillaries. In all cases, the endothelium forms the interface between lumen and the surrounding tissue. Capillaries only exist of a basement membrane and the endothelium, whereas arteries and veins have two additional layers, the *Tunica media* and *Tunica externa,* which provide stability for larger vessels. Picture was reproduced with permission of Antranik Kizirian[4].

1.1 Function and organization of the endothelium

One of the most obvious functions of the endothelium is to serve as a semi-selective barrier between the bloodstream and the surrounding tissue, controlling the passage of leukocytes and solutes. Depending on the location of the endothelium in the vascular tree, its permeability is quite variable. Sites with high infiltration rates, such as glomeruli, mucosa or glands form an endothelium, which allows high transcellular transport. This is enabled through *fenestrae*, pores in the endothelial cells that allow regulated passage of metabolites. In organs, which require a well-established barrier function between bloodstream and tissue such as brain, skin, lung and heart, the endothelium is continuous and non-fenestrated, only allowing passage of < 3 nm solutes[2,5]. Of major importance for maintaining endothelial monolayer integrity is an elaborate anchorage system of the individual cells to their adjacent neighbours, which will be described in detail in chapter II.2.

In specific situations when frequent passage of blood components across the barrier is required, the permeability of the endothelium can be increased – for example during acute inflammation, agents like histamine and thrombin lead to leakage of plasma into the underlying tissue[6]. Inevitably, this results in a higher concentration of blood cells, thus enabling leukocytes to adhere to the activated endothelium and extravasate into the inflamed tissue. This process involves either highly regulated opening of cell-cell junctions to let leukocytes pass paracellularly in between cells or the involvement of transport vesicles for using a transcellular pathway[7-9]. Agents that promote leukocyte transmigration can induce opening of junctions with different velocity: quick effects are results of treatment with histamine or thrombin, whereas inflammatory cytokines take hours before showing an effect[9,10]. Obviously, dysregulation of cell-cell junction opening - and vascular damage in general - compromises endothelial integrity by uncontrolled increase in permeability and is considered as a highly thrombogenic event[11,12].

Another interesting function of endothelial cells is their constant production of NO (nitric oxide), a gas that diffuses rapidly towards the underlying smooth muscle cells, where it activates signaling cascades that lead to vasodilation, thus contributing to an atheroprotective phenotype[13-15].

Additionally, the endothelium is involved in processes of blood coagulation, via secretion of thrombin[16] and angiogenesis, where new blood vessels arise from existing endothelial cells after stimulation with VEGF (**V**ascular **E**ndothelial **G**rowth **F**actor)[17]. Corresponding to its presence in all parts of the body and its importance for the organism, endothelial dysfunctions lead to a variety of diseases, such as a rheumatoid arthritis, thrombosis or potential tumor invasion into blood vessels[18].

1.2 Shear stress and cell-matrix adhesion

During blood flow through arteries and veins, two hemodynamic forces are applied to the vascular wall: shear stress, frictional force acting at the interface between flowing blood and the endothelium, and hydrostatic pressure, which imposes circumferential stretch to the vessel[19,20]. The endothelium sensitively responds to this mechanical stress through adaptations of its cell metabolism, gene expression, and cell morphology (Figure 2)[21]: cultured endothelial cells undergo a rapid change in cell shape from polygonal to ellipsoidal while they uniformly orient with the direction of flow to decrease shear stress[22,23].

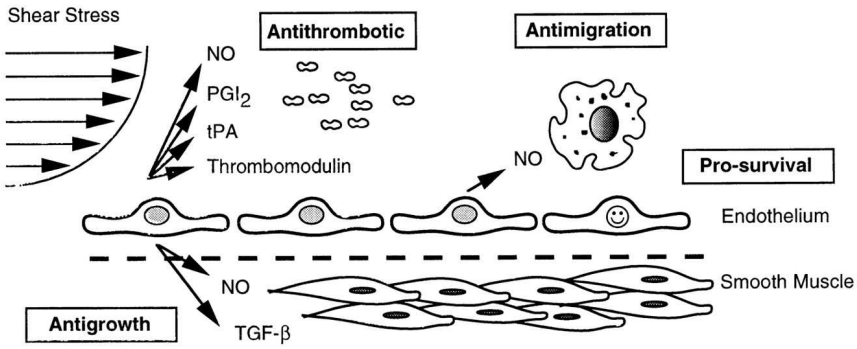


Figure 2 Effects of shear stress on the endothelium
Steady laminar flow and the resulting shear stress induce the release of inhibitory agents from the endothelial cells. They show antimigratory effects on leukocytes, antigrowth effects on smooth muscle cells and generally promote an antithrombotic phenotype. Altogether, this results in survival of the endothelial cells, showing that shear stress is crucial for the maintenance of the endothelium. NO= **n**itric **o**xide. PGI_2= prostacyclin, effective vasodilator. tPA= **T**issue **p**lasminogen **a**ctivator, enzyme that dissolves blood clots. Thrombomodulin, reduces blood coagulation. TGF-β= **T**ransforming **g**rowth **f**actor beta, antiproliferative factor.[24]

The transduction of signals, which leads to these cellular changes in response to shear stress, is dependent on the attachment of endothelial cells to an underlying matrix, the basement membrane. Among others, integrins are prominent transmembrane adhesion receptors, which bind to collagen, fibronectin, laminin or other extracellular matrix components, linking them to intracellular F-actin through adaptor proteins (e.g. talin, α-actinin)[25-27]. Simultaneously, integrins are sensors of their environment serving as transducers of physical stress into signaling pathways that affect cellular physiology, proliferation, migration and transcriptional activity[28,29]. In endothelial cells, it could be shown that integrins are able to transduce shear stress into signaling cascades, being one of the main players in the respond to flow induced physical stress[30,31].
Integrins always consist of one α and one β subunit; at least 18 types of α and eight types of β subunits can be combined to 24 different functional integrins with distinct - and overlapping - binding specificities[32]. In HUVEC (**H**uman **U**mbilical **V**ein **E**ndothelial **C**ells), the functional integrin-dimers α5β1 and αvβ3 are most common. αvβ3 expression was shown to be upregulated upon shear stress leading to increased Rho-signaling and thus F-actin rearrangements that result in changes of cell shape, accounting as adaptations of the exposure to flow[33-35].

2 Cell-cell junctions

Cell-cell junctions are essential for the development of multicellular organisms, since they connect singular cells of the same or different types, into higher organs. They are most typical for epithelial cells as found in skin, kidney or bladder, but also occur in other cell types, for example fibroblasts and smooth muscle cells. Cell-cell junctions consist of multiprotein complexes that provide anchorage and enable signaling between cells. They are formed by the intercellular interaction of different transmembrane proteins (Figure 3) that are linked to a variety of intracellular cytoskeletal and signaling proteins. A dysfunction of cell-cell adhesion leads to a multitude of diseases, reaching from various skin disorders to pronounced cancer metastasis[36-38].

2.1 Endothelial cell-cell junctions

Epithelial cell-cell junctions are the best studied system, with the different junctional complexes of gap junctions, adherens junctions, tight junctions and desmosomes. These follow a well-defined distribution along the intercellular cleft with the tight junction being the most apical component, followed by adherens junctions and desmosomes. However, in endothelial cells, the junction types are less organized and relatively intermingled along the contact zone[39]. Desmosomes are missing and instead one can find typical endothelial proteins such as PECAM-1 (**P**latelet **E**ndothelial **C**ell **A**dhesion **M**olecule-1) and ICAM-2 (**I**nter**c**ellular **A**dhesion **M**olecule-2), which contribute to cell-cell adhesion, but also have other functions, for example in angiogenesis or leukocyte extravasation[40-42]. To fulfill their functions of letting plasma constituents and blood cells pass through upon demand, the endothelial junctions are highly dynamic and can re-organize within minutes[43]. Altogether, junctions of the epithelium and endothelium are highly homologous, which might allow a careful transfer of observations made in epithelial cell models to endothelial cells.

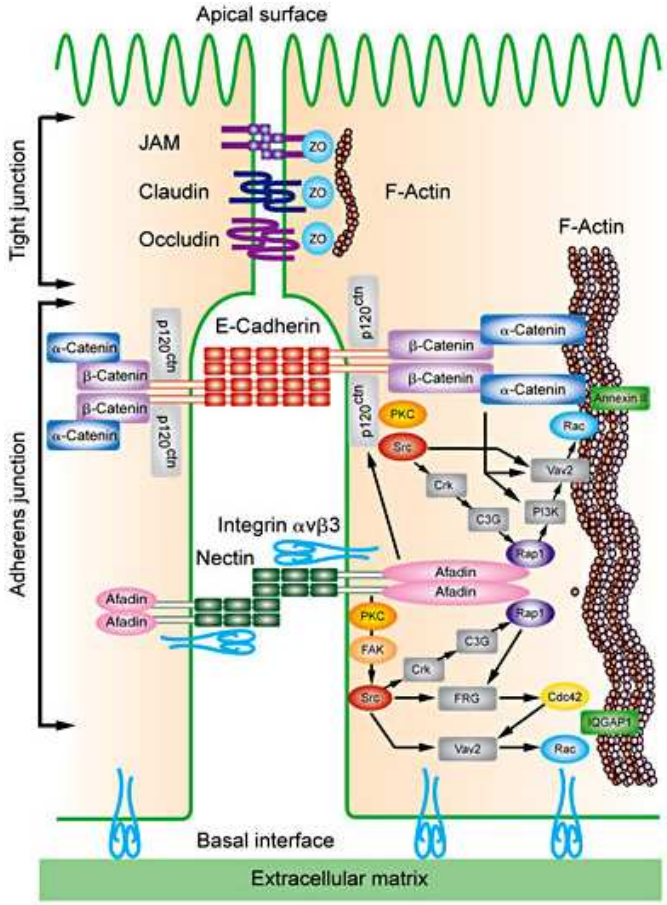


Figure 3 Typical cell-cell adhesion molecules and their cytosolic effector proteins
In epithelial cells, tight junctions are located in the apical part of the intercellular cleft. They comprise JAM (**J**unctional **A**dhesion **M**olecules), claudins and occludins, all linked to F-actin through ZO (**Z**onula **O**ccludentes)-proteins.

Adherens junctions are formed through cadherin and nectin dimers, both linked to a variety of cytoplasmic proteins. The most direct binding partners at cadherin junctions are β-catenin and p120-catenin. Regarding the second type of adherens junction proteins it is most prominently afadin, which binds to nectin. The two systems are closely interconnected through signaling cascades (not explained in detail here) that influence the interplay between the junctional complexes depending on the different stages of adhesion.

Integrins link the cells to the underlying matrix forming focal adhesion sites, thus providing anchorage to a basement membrane[44].

2.1.1 Tight junctions

In epithelial cells, where the different junctional complexes are arranged in a precise manner along the intercellular cleft, tight junctions can be found at the most apical position[45]. Their main function is to form an impermeable barrier for soluble molecules. In endothelial cells,

varying degrees of necessity for permeability control exist, depending on the location in the vascular tree[46]. In larger vessels or the brain, tight junctions are very frequent whereas 30 % of the small capillaries with high infiltration rates lack tight junctions, thus facilitating the passage of leukocytes[47]. The most prominent components of tight junctions are occludins, claudins and JAMs (**J**unctional **A**dhesion **M**olecule), with the latter also occurring in cells that do not form junctions[48]. Occludins have four membrane-spanning regions with their N- and C-termini both being intracellular, where they bind to ZO-proteins (**Z**onula **O**ccludentes)[49,50]. Some splice variants of the canonical occludin, which is expressed in endothelial cells, have been identified but their exact functions need to be investigated[51]. Occludin expression throughout the endothelium remarkably correlates with the permeability of the vessels, with highest expression levels and thus least permeability in brain vessels[52].
The 15 claudins, which are identified so far, show similar architecture as occludins, with claudin-5 being the endothelial-specific member[53]. According to its characteristic distribution, mainly in the brain, knockout of claudin-5 in mice is lethal due to impaired barrier function of brain vessels[54]. Occludin and claudin can bind to different ZO-proteins (ZO-1, ZO-2, ZO-3) as their cytosolic adaptors, which serve as linkers to the F-actin cytoskeleton and to other proteins, besides having functions in signaling (Figure 3)[55].

2.2.2 Gap junctions

In addition to the junctional complexes that serve as anchoring structures, gap junctions mediate communication between neighboring cells[56]. They comprise clusters of a few to hundreds of intercellular channels, which are permeable for small ions and metabolites excluding those molecules that exceed 1 kDa in size[57]. Each channel comprises transmembrane proteins belonging to the connexin (Cx) family, which consists of 20 members in humans, with Cx43, Cx40, and Cx37 being expressed in the endothelium. Connexins assemble into hexameric clusters, forming a hemichannel (connexon) in the plasma membrane, which aligns with another connexon of an adjacent cell, thus forming a pore that connects the cytoplasms[46]. This way, adjoined cells can share for example second messengers or metabolites and consequently give coordinated responses to certain stimuli, which is especially important during embryogenesis, where groups of cells simultaneously develop into different tissues. Gap junctions are regulated in many ways, for example through their composition of different connexins, to gain selectivity towards the metabolites that can pass. Their importance is underlined by their expression in all chordate tissues[58] and the variety of human diseases linked to mutations in connexin genes[59,60].

2.2.3 Adherens junctions

Adherens junctions are molecular ensembles of intercellular proteins, which mainly provide anchorage of cells to each other. Historically, they were discovered by developmental biologists, who realized that they are responsible for homophilic cell-sorting during embryogenesis[61] (Figure 4). In the 1970s, the major component of adherens junctions was described, the cadherin-catenin system[62].

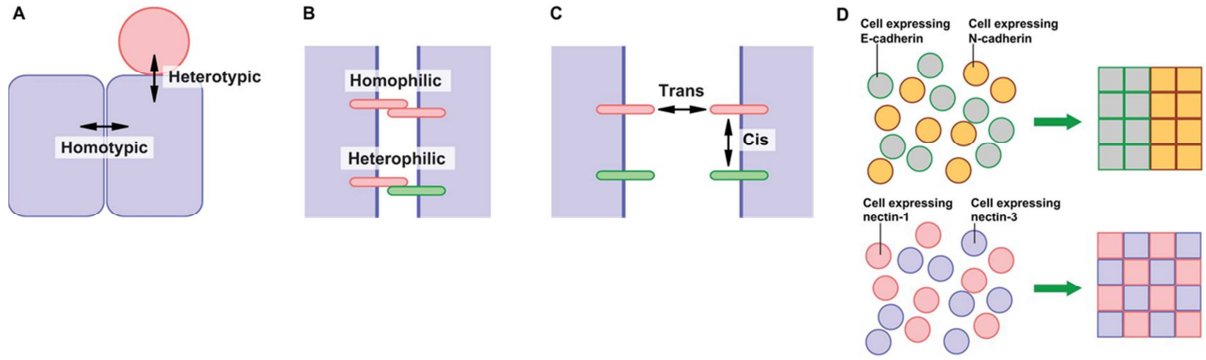


Figure 4 Different types of cell-cell adhesion
A Interactions between cells can be homotypic (between the same kind of cells) or heterotypic (between different cell types). **B** Concerning the adhesion proteins, an interaction is homophilic, when two proteins of the same type interact, and heterophilic if different types are connected. **C** Trans interactions occur between proteins of opposing cells, whereas cis-interactions take place at the same cell surface. **D** The result of homophilic interactions is a segregation of cells, while they form a mosaic when they tend to establish heterophilic trans-interactions.
[63], with alterations.

2.2.3.1 The cadherin-catenin system

The most prominent members of the adherens junction complex belong to the superfamily of cadherins, with VE-(**V**ascular **E**ndothelial) cadherin being the one of over 350 cadherins that is expressed only in endothelial cells[64]. It belongs to the subfamily of the classical cadherins, which all share six conserved extracellular cadherin domains responsible for their calcium-dependent dimerization[65]. After forming lateral homo-cis-dimers, they homophilically trans-dimerize with other cadherin dimers of adjacent cells, followed by lateral clustering of many cadherins, which leads to increasing adherence[66,67].
Besides their main function of mediating adhesion, cadherins play a role in intracellular signaling, requiring their cytoplasmic tail region that interacts with a variety of proteins. Of major importance is p120-catenin, which binds to the juxtamembrane region of cadherins, preventing their clathrin-related endocytosis through stabilizing them at the membrane[68,69]. Upon release of p120-catenin, it is able to translocate into the nucleus to regulate transcription, displaying a dual role in the cell that is common for many junction-associated proteins[67,70].
The most prominent cadherin binding partner is β-catenin, which was thought to stably link cadherins to F-actin through α-catenin[71]. Since 2005, this model has been challenged by the Nelson group, for example through FRAP (**F**luorescence **r**ecovery **a**fter **p**hotobleaching, see chapter III.2.2.7) experiments showing that F-actin has a more dynamic behavior than α-catenin, which excludes the possibility of being present in a stable complex[72,73]. Accordingly, Drees *et al.* showed that α-catenin either binds to β-catenin/cadherin, or to F-actin, but never both at the same time (Figure 5A)[73,74]. Nevertheless, disruption of the cortical F-actin network leads to defects in adherence and loss of the cadherin complex at the surface creating the assumption that there should be other ways of linking cortical F-actin to adherens junction complexes (for functions of F-actin, see also II.3.1)[75]. Possible scenarios are chains of interactions through the variety of proteins that bind to α-catenin, such as ZO-1, α-actinin and spectrin - or a direct link of F-actin to different adherens junction components, for example the nectins (see chapter II.2.2.3.2)[71].

As mentioned above, endothelial cells undergo rapid remodeling of junctions, for example in context of leukocyte extravasation. In some cases, VE-cadherin is specifically targeted through phosphorylation or internalization to increase vascular permeability (Figure 5B). Lymphocyte adhesion has been shown to induce tyrosine phosphorylation of residues 645, 731 or 733 resulting in their successful transmigration[76]. Also the clathrin-dependent internalization of VE-cadherin leads to an increase in permeability, just like the cleavage of its extracellular domain through metalloproteinases secreted by leukocytes[67,77,78]. In general, these mechanisms enable a high turnover of junctional cadherin and are thus a way to control the adhesive properties of the adherens junctions[79,80].

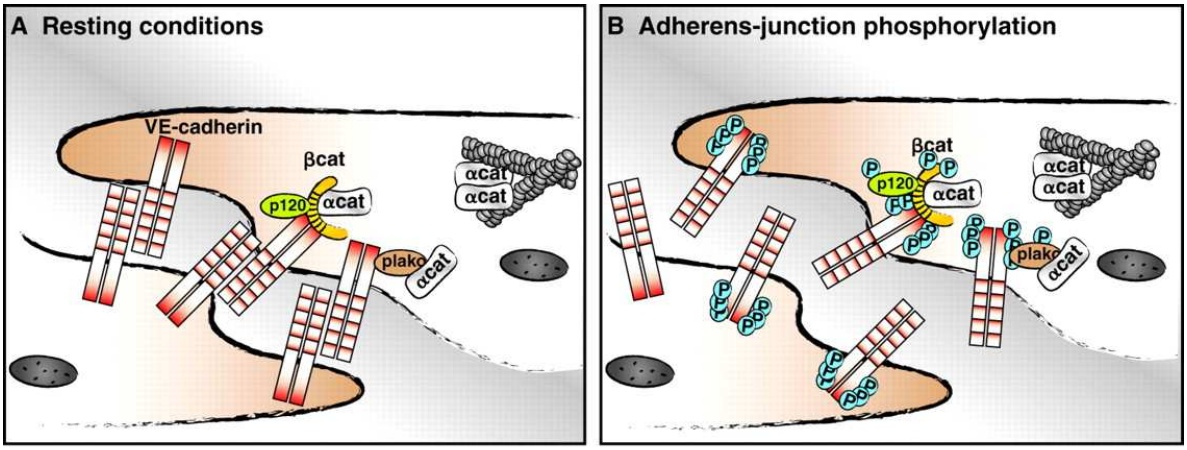


Figure 5 VE-cadherin stability at adherens junctions
A VE-cadherin forms cis- and trans-dimers between adhering cells. Various cytoplasmic proteins interact with the C-terminal part of cadherins. p120= p120-catenin. Plako= plakoglobin. β-, α-cat= β-, α-catenin.
B One way to increase endothelial permeability is the specific phosphorylation (P) of cadherins, leading to reduced adherence, for example to facilitate lymphocyte transmigration. Other ways to reduce adherence involve endocytosis of cadherins or cleavage of their extracellular domain through metalloproteinases. [81]

2.2.3.2 The nectin-afadin system

Roughly 15 years after the identification of the cadherins, another group of transmembrane proteins localizing to adherens junctions was discovered by the Takai group. These were immunoglobulin(Ig)-like, calcium independent adhesion molecules, the nectins[82]. They consist of a cytoplasmic tail region, a single transmembrane region and three immunoglobulin-like loops that exert extracellular binding. Their cytoplasmic tail contains the motif E/A-X-Y-V, which binds the PDZ (**P**ost synaptic density protein, **D**rosophila disc large tumor suppressor, **Z**onula occludens-1 protein) domain of nectin's typical binding partner, the F-actin binding protein afadin[83] (Figure 6).
Four members of the nectin family have been identified so far (nectin-1,2,3,4), each having several splice variants[84,85]. Nectin-1 and nectin-2 were first discovered and shown to serve as a receptor for α-herpes virus[86]. Nectin-4 is mainly expressed in the placenta, whereas the others are ubiquitously expressed in many cell types, including fibroblasts, epi- and endothelia as well as nervous tissue[87,88]. Nectin-2 and -3 are also expressed in cells, which lack cadherins, for example monocytes and spermatids[89]. Just like cadherins, nectins first form lateral homo- (or hetero-) cis-dimers before they interact with a dimer of the adjacent cell. A

specific characteristic, which clearly separates them from the cadherins, is that they do not only form homo-trans-dimers – they are able to trans-dimerize with different kinds of cis-dimers. Indeed, the trans-interaction of different nectin-dimers is usually stronger than between identical ones[84,85]. This enables nectins to not just connect cells of the same type, but also different cell types, expressing different nectins. This situation occurs for example in the adhesion between spermatids, expressing only nectin-3, and their supporting sertoli cells, which express nectin-2. Since this is one of the few cell types only expressing one family member, knockout of nectin-2 is not compensated and leads to a phenotype displayed in these organs - mice show male infertility due to defects in sperm morphogenesis[90,91]. In endothelial cells, such as HUVEC, nectin-2 was mainly described, potentially interacting with nectin-3[89].

Another important function of nectins, besides contributing to adhesion, is their role during the assembly and formation of junctions, interacting with their prominent binding partner afadin (see II.2.2.4).

Afadin (or l-Afadin) has a variety of functional domains (two Ras associated domains (RA), one dilute (DIL) domain, a forkhead-associated (FHA) domain, a PDZ domain, three proline rich regions (PR) and an F-actin binding C-terminus, which allow its binding to many other proteins, serving as an adaptor[94,95] (Figure 6). Its shorter splice variant, s-Afadin (or AF-6), is mainly expressed in neurons and its knockdown does not affect intercellular adhesion[96]. Afadin is thought to serve as a connector between the two main adhesion systems, linking nectins and cadherins through different interacting protein systems. Among others, it can bind to α-catenin that is mainly localized at cadherin based junctions and to ponsin, which then binds vinculin[97,98].

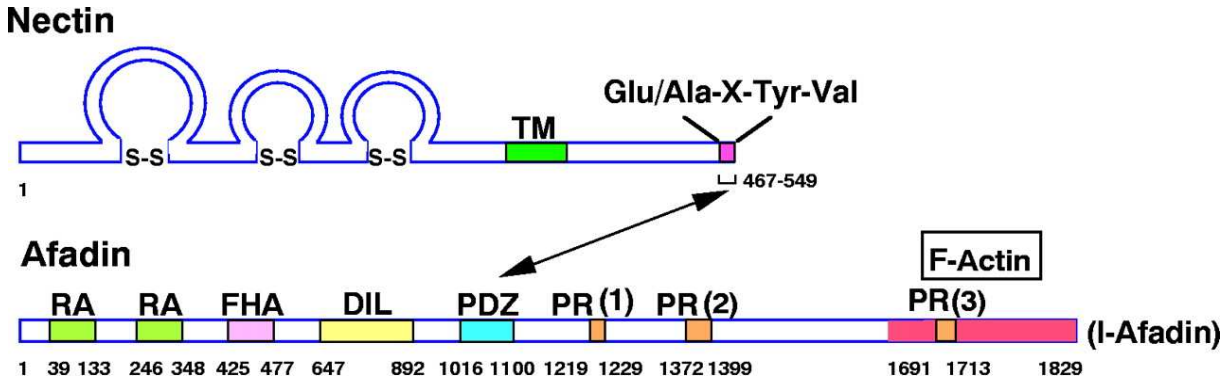


Figure 6 Nectin and afadin

Nectins belong to the superfamily of Ig(immunoglobulin)-like proteins since they comprise three Ig-like loops in their extracellular region. The first loop is important for the trans-dimerization of nectins, the second for lateral cis-dimerization[92]. TM= transmembrane segment. The cytoplasmic part has a 4 amino acid consensus sequence, which enables binding to afadin's PDZ region.

Afadin has many functional domains: two Ras associated domains (RA), a forkhead-associated (FHA) domain, one dilute domain (DIL), one PDZ domain (post synaptic density protein, Drosophila disc large tumor suppressor, zonula occludens-1 protein), three proline rich regions (PR) and an F-actin binding C-terminus. They mainly allow the interaction with other proteins, e.g. Rap1, Ponsin and ZO-1. [93]

In migrating cells, afadin is located at lamellipodia of the leading edge where it interacts with active Rap1, a small GTPase involved in cell adhesion mechanisms. Knockdown of afadin leads to impaired leading edge formation and directional migration, often going along with disturbed junction assembly[99].

2.2.4 Formation of cell-cell junctions

There are several different theories about how the initial formation of junctions is accomplished in different cell types, with the most widely accepted one being shortly discussed here. Takai *et al.* hypothesize that all adhesion molecules are randomly distributed along lamellipodia of migrating cells, with their directional movement influenced through Necl5 (**Nec**tin-**l**ike) and afadin being located at the leading edge[99,100]. Upon encountering another cell, the first loose contact is made through nectin-dimerization, because they kinetically dimerize most rapidly. It is their interaction with afadin, which is indispensable for the proper positioning of the nectins. Subsequently, the nectin-afadin interaction leads to small GTPase (Rap1, Cdc42, Rac; explained in chapter II.3) dependent signaling that re-organizes the underlying F-actin cytoskeleton, stabilizing the initial junction[101-103]. Through chains of interactions, for example afadin-ponsin-vinculin-α-catenin, the cadherin proteins are successively recruited to the initial nectin-based contact sites, eventually forming mature adherens junctions[93,95,104]. Of major importance is the arrest of the permanent cadherin monomer endocytosis, which is only blocked after nectin dimerization in an afadin/p120-catenin-dependent manner, underlining the idea that nectin interaction is the first step of junction assembly[105]. Afadin is indispensable for many steps of junction formation – consequently its depletion in cell lines or mice leads to problems in junction association, just like inhibition of nectin trans-dimerization blocks the assembly of cadherin based adherens junctions[106,107]. The most convincing point of evidence that initial junction formation depends on nectins and not cadherins, is derived from experiments in a cancer cell line (HSC-39). These cells express cadherins and could in theory form cadherin-based adhesion sites - however, they show no adherence to each other. Impressively, upon overexpression of nectin-2, they suddenly start forming adherens junctions, which are comprised of nectins and cadherins[108,109].

Once the adherens junctions are formed, tight junction proteins occludin, claudin and ZO-1 are recruited to the sites of adhesion, again mediated through the nectin-afadin system[110,111]. Evidence is provided by inhibiting nectin trans-interaction, which led to improper tight junction formation in epithelial cells[112,113]. The two adhesion systems are connected to each other very closely, which becomes obvious through knockout of VE-cadherin resulting in defective tight junctions. Absence of VE-cadherin leads to accumulation of β-catenin in the nucleus, where it forms a repressor complex of the *claudin-5* promoter - influencing junction formation on a transcriptional level[114]. After the complex of adherens and tight junctions has been formed, also the maintenance of these mature junctions requires frequent crosstalk between the different components. Junctions are very dynamic, especially in the endothelium, and thus the adhesion proteins are continuously recycled to keep the adhesion machinery in a dynamic equilibrium[46] (see also II.3.1).

3 The F-Actin cytoskeleton and its regulators

The cytoskeleton of cells is essential to determine their shape, for movement of vesicles within cells, migration, endo- and exocytosis, as well as cytokinesis, muscle contraction and a variety of other cellular processes[115]. It is comprised of three systems, microtubules, intermediate filaments and F(**F**ilamentous)-actin, each associated with numerous specific accessory and regulatory proteins[116] (Figure 7).

Microtubules are the largest filaments (25 nm diameter), arising from the MTOC (**m**icro**t**ubule **o**rganizing **c**enter) besides the nucleus, stretching out towards the cell periphery. They are build up by constant assembly and disassembly of tubulin dimers at their (+)-end, and have major functions in cell division and organelle localization[117].

Intermediate filaments play mainly structural roles and are formed through different sets of proteins, depending on the cell type. Exhaustively studied are the keratins, which are important structural components in epithelial cells and associated with many epidermal diseases[118]. Vimentin filaments are present in endothelial cells, but the physiological functions of intermediate filaments in the endothelium are not well understood[119].

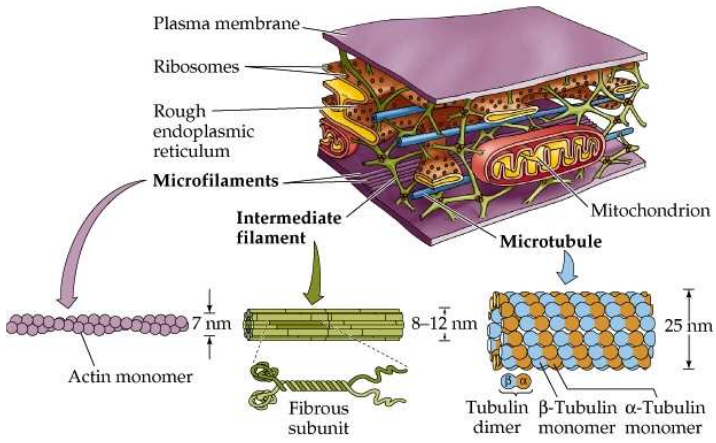


Figure 7 The cytoskeleton of cells
Schematic side view of a cell and the different filament systems.
Microfilaments (F-actin) are the smallest, mainly localized at the cell cortex of resting cells and build up by the assembly of actin monomers.
Intermediate filaments have fibrous subunits and predominantly play structural roles.
Microtubules form hollow tubes consisting of tubulin dimers and are usually anchored to the MTOC (Microtubule Organizing Center). Among others, they fulfill functions during cell division and vesicle transport. [120]

Actin is a central cytoskeletal element of endothelial cells, which comprises up to 15 % of total protein content, either being present as a monomer or forming polymeric structures[121]. With the help of nucleating proteins, such as the ARP2/3-complex (**A**ctin-**R**elated **P**rotein2/3) or formins, monomeric ATP-bound G(**g**lobular)-actin assembles into nucleation seeds, consisting of G-actin dimers or trimers[122]. Once nucleated, the elongation of actin filaments, which is its functional conformation, starts through the addition of G-actin to the dimers/ trimers. All G-actin subunits assemble in the same orientation, thus creating polar filaments: at the barbed, or (+) end of the filament, addition of monomers occurs 10 times faster than at the pointed, or (–) end[123]. However, the assembly of monomers into filaments is a reversible process, also regulated through a variety of actin-binding proteins, which will not be discussed in detail. Generally, actin binding proteins create a balance between polymerization and depolymerization of F-actin via stabilizing filaments (tropomyosin), severing (gelsolin) or capping (CapZ) them, which allows rearrangement of the cytoskeleton upon cellular stimuli[124-126]. Of major importance for the different F-actin organizations are the nucleating complexes, which do not just initiate F-actin assembly, but can moreover influence the shape of the

emerging filament network. Depending on the type of nucleation complex, F-actin is organized either in unbranched networks through formins, or branched networks via the ARP2/3-complex, thus enabling the formation of different structures within the cell (Figure 8)[127-129]. In addition, filaments can be crosslinked (α-actinin) or bundled (fascin), also supporting different cell shapes[130,131].

Activators of the nucleation complexes - and thus indirect regulators of F-actin assembly - are primarily Rho-GTPases. GTPases are small cytosolic proteins, often referred to as "molecular switches" since they can be activated through binding of GTP and quickly inactivated through hydrolysis of the bound GTP to GDP. Once activated, they can influence actin dynamics through engaging downstream effector proteins[132]. Prominent Rho-GTPases are Cdc42, RhoA and Rac1, which control the formation of filopodia, stress fibers and lamellipodia, respectively[133].

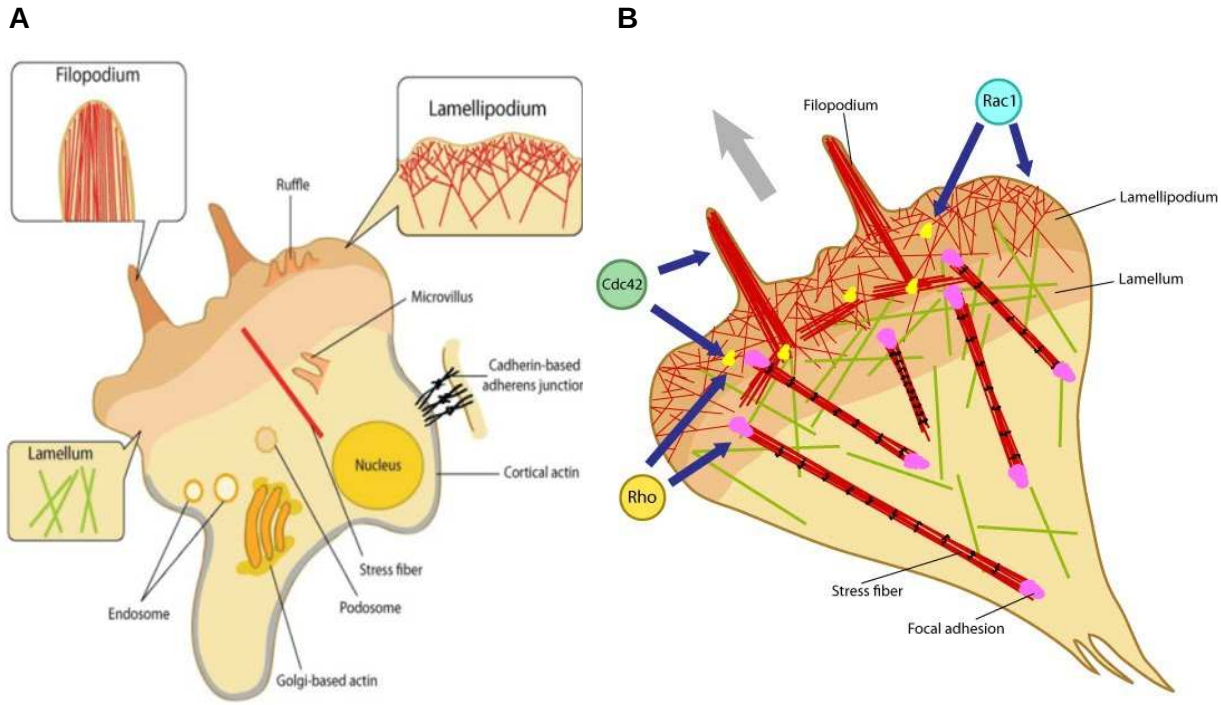


Figure 8 Forms of F-actin and its regulating Rho GTPases
A Different actin nucleating complexes shape different actin structures. The ARP2/3-complex produces branched F-actin networks, as present in lamellipodia. Formins lead to the formation of straight bundles of F-actin, as for example present in filopodia.
B Members of the family of Rho-GTPases control the formation of different F-actin structures. RhoA is mainly responsible for stress fiber assembly, Cdc42 for filopodia and Rac1 for lamellipodia formation, which is important for movement of migrating cells.[134]

3.1 Cortical F-actin and junction integrity

In confluent endothelial cells, F-actin is found primarily beneath the plasma membrane, forming the "cortical F-actin", where it interacts with cell-cell adhesion complexes through adaptor proteins. This pool of long filamentous actin is essential for junction integrity - when cortical F-actin is disrupted, endothelial integrity is lost[135,136]. However, not just F-actin's presence beneath the plasma membrane, but especially its interconnection to the junctional

structures is essential for monolayer integrity. This becomes obvious, when loss of linker-proteins leads to compromised integrity. An example is the tight-junction associated protein ZO-1 – its phosphorylation upon VEGF-treatment leads to instability of junctions and increased permeability[137]. Another protein, which is not *per se* responsible for intercellular adhesion, but nevertheless has effects on barrier integrity, is VASP (**VA**sodilator-**S**timulated **P**hospho-protein), binding to α-catenin at adherens junctions. Its knockdown leads to enhanced permeability of the endothelium, showing that not only the transmembrane proteins and the cortical F-actin are needed for maintaining junction integrity, but also the adaptor proteins connecting these two structures[138,139]. Another example for these linker proteins is EPLIN (**E**pithelial **P**rotein **L**ost **I**n **N**eoplasm), which is known to bundle and stabilize cortical F-actin and can simultaneously interact with cadherin-bound α-catenin[140,141].

Some scenarios, for example passage of leukocytes during inflammation, require the formation of gaps between the cells, accompanied with compromised monolayer integrity. This gap formation is induced through a cascade of events: inflammatory agonists (e.g. histamines) lead to Rho-dependent signaling. Redistribution of F-actin from the cortical rim into stress fibers, mediating retraction of cell borders, is the result of RhoA GTPase activity[142,143]. Simultaneously, RhoA activity leads to enhanced phosphorylation of MLC (**M**yosin-**L**ight-**C**hain), leading to contraction of stress fibers and the concomitant loss of barrier integrity (Figure 9)[135,144,145].

Stress fibers consist of 10-30 actin filaments that are cross-linked by α-actinin and span throughout the cells mostly either being dorsal, ventral or perinuclear[146]. They are associated with bipolar bundles of nonmuscle myosin II, which is responsible for their contractile nature[146-148]. In order to transmit force, stress fibers can be attached to matrix adhesion structures, which fix them at the membrane, acting as an anchor. It has also been shown that stress fibers can be anchored to adherens junctions during wound healing in epithelial cells, thus forming a contractile ring around the wound edge with the force directed along the wound axis[149]. Another novel idea about how the actomyosin system interacts with adhesion structures claims that the contractile fibers can attach directly to VE-cadherin/β-catenin/α-catenin complexes, forming a novel endothelial type of junctions – the focal adherens junctions[150]. Especially during an early phase of junction formation, their disassembly or during junctional turnover, this type of junction has been described (see also Figure 43)[151].

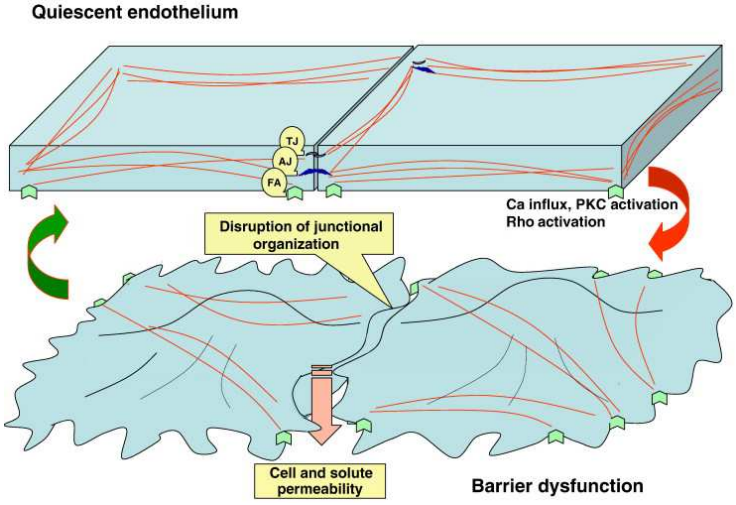


Figure 9 Distribution of F-actin in the quiescent and activated endothelium
In resting cells, F-actin is mainly localized at the cell periphery, forming the F-actin-rich cortex. Upon certain stimuli, Rho-GTPase signalling leads to a reorganization of cortical F-actin into stress fibers that span through the cell and are enriched in myosin II. Phosphorylation of myosin II leads to actomyosin contractility, retracting the cell borders, and thus to disruption of junction integrity. PKC= protein kinase C, TJ= tight junction, AJ= adherens junction, FA= focal adhesion.
[152], with alterations.

In general, an increase of junction permeability is accompanied by diminished levels of junctional adhesion proteins at the surface; in endothelial cells preferentially of adherens junction components[153]. Their levels can be modulated through transcriptional regulation, and thus expression levels of proteins, or through internalization and degradation. Upon certain stimuli (for example growth factors, calcium depletion[154]) cadherins undergo rapid endocytosis in early endosomes and subsequent degradation in lysosomes – or recycling to regions where new adhesion sites are formed[155]. During these processes, a variety of other proteins are involved: protein tyrosine kinases label cadherin through phosphorylation at its cytoplasmic tail, which is a signal recognized by ubiquitin ligases, inducing its endocytosis[156,157]. After internalization, again members of the small GTPases, most prominently Rab-GTPases, mediate the intracellular trafficking of cadherins either into lysosomes for degradation or back to the plasma membrane via recycling endosomes[158,159]. As opposed to the well described degradation and recycling processes of cadherins, the trafficking of other cell-cell junction components, such as nectins, is still poorly understood and needs to be investigated.

4 Drebrin

Drebrin (**D**evelopmentally **re**gulated **br**ain prote**in**) belongs to the family of actin binding proteins. It is well studied in the brain where it maintains the dynamic nature of neurons; primarily through interfering with other F-actin binding proteins. Recently, it became clear that drebrin's activity is not restricted to neuronal cells, which is underlined by the growing list of functions that it exerts in non-neuronal cell types[160-164]. In our group, drebrin was identified through a screening for actin regulating proteins involved in formation of endothelial cell protrusions, and elucidating its functions in HUVEC formed the center of this study.

4.1 Drebrin in the brain

Drebrin is an F-actin binding protein originally identified in chick brain and was first thought to be neuron-specific[165]. The three existing isoforms of drebrin (drebrin A (**A**dult), E1 and E2 (**E**mbryonic)), all splice variants from a single gene (*DBN1*), are spatially and temporally regulated in the brain. The embryonic isoform drebrin E1 is expressed first while neurons still migrate, later being replaced by drebrin E2, which localizes to neuronal cell processes[166]. Besides low expression levels of drebrin E2, it is predominantly drebrin A that is expressed in the mature adult brain and involved in maintaining post-synaptic dendritic spine plasticity through its F-actin regulating capabilities[167-169] (Figure 10). Mammals express only one embryonic isoform orthologous to chick drebrin E2, which is missing 46 amino acids compared to drebrin A. The predicted molecular weight is 71 kDa, but due to its acidic nature and post-translational modifications, it shows a slow SDS-PAGE mobility corresponding to ~120 kDa[170].

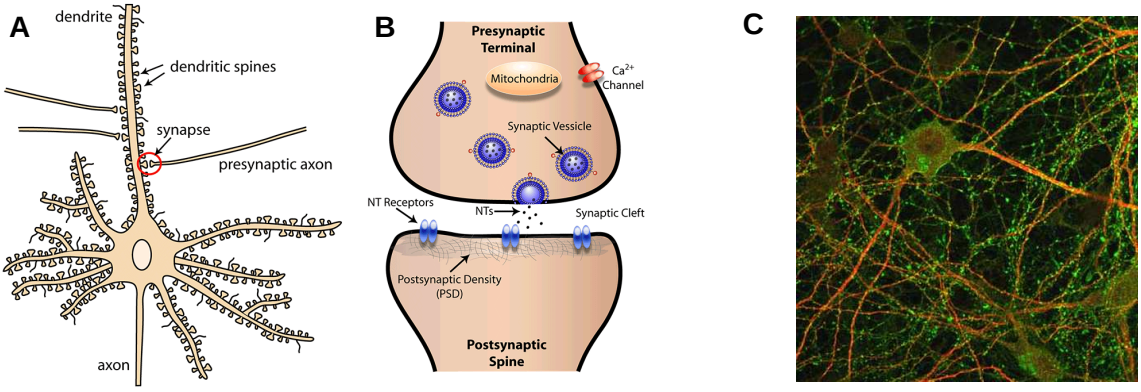
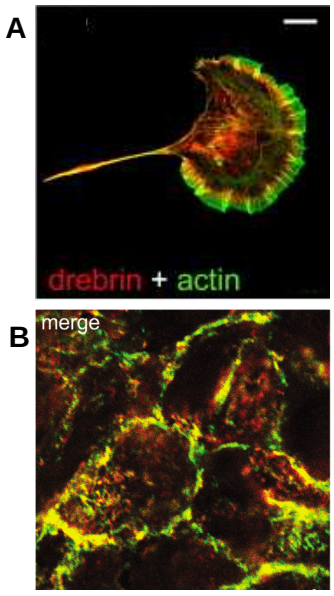


Figure 10 Drebrin localizes to dendritic spines of neurons
A Scheme of a typical neuron. The dendrites show numerous protrusions, called dendritic spines forming the contact (synapse) between other neurons. **B** Dendritic spines are important for signal transmission of neurons. Drebrin localizes to the postsynaptic spine region where it interacts with F-actin[171]. NT= neurotransmitters **C** Drebrin (Alexa-488 - green) in dendritic spines of mouse hippocampal neurons. Picture was reproduced with permission of Dr Britta Eickholt[172].

Binding F-actin with high affinity leads to a competition with other F-actin binding proteins such as the actin-bundling protein fascin, the crosslinking protein α-actinin and tropomyosin, which stabilizes filaments[173,174]. Drebrin facilitates the recruitment of the actin-severing protein gelsolin and directly interacts with profilin, which stimulates F-actin polymerization[167,175]. Through its capability of binding to myosin II, drebrin can also influence the actomyosin system. Moreover, it can also bind to microtubule-(plus)-tip binding protein EB3 (**e**nd-**b**inding) and might thus coordinate actin-microtubule interactions[176]. Altogether, these possible interactions enable drebrin to influence F-actin dynamics in many different ways and at different stages even though it does not sever, cap, nucleate or crosslink actin by itself[177].
Of major importance are drebrin's different functional domains. Due to its N-terminal ADF-H (**A**ctin **D**epolymerizing **F**actor **H**omology) domain, drebrin has been included in the ADF-H family of actin-binding proteins, even though this domain does probably not exert functions in drebrin[178]. Drebrin changes the twist of actin filaments opposite to the "overtwisted" filament conformation evoked through the actin-severing ADF/cofilins and actively competes with cofilin for actin filament binding[179,180]. Its central MAR (**M**inimal **A**ctin **R**emodeling) region (residues 233-317) is sufficient for the binding to F-actin and drebrin's actin remodeling characteristics[181]. It partly overlaps with a CC (**C**oiled-**C**oil) region important for dimerization of proteins and for drebrin binding to golgi membranes[182]. Opposite to the well-studied N-terminal regions, the C-terminus is poorly conserved and just contains a proline-rich stretch that might be involved in profilin binding[175] (Figure 11C).
Drebrin's importance in the adult brain becomes obvious, when drebrin levels are decreased: this leads to Alzheimer's disease through loss of dendritic spines, and was correlated to Down Syndrome[183-185].
Also in non-neuronal cell types such as cultured fibroblasts or HUVEC, overexpression of drebrin affects F-actin dynamics, as thick bundles of F-actin are formed[186] in addition to the formation of dendritic-like cell processes[187]. Kidney epithelial MDCK (**M**adin-**D**arby **C**anine **K**idney) cells form long protrusions after overexpression of drebrin[162], supporting the idea that drebrins also exert functions on microfilament systems of non-neuronal cells.

4.2 Drebrin in non-neuronal cell types

In 1987, messenger RNA of drebrin has initially been identified in non-neuronal cell types[188,189]. Seven years later, Fisher *et al.* reported the existence of pre-mRNA for drebrin E2 in heart, placenta, lung, skeletal muscle, kidney, pancreas, fibroblasts and bone-derived cells[190], but the presence of drebrin protein was not investigated until 1999, when Keon *et al*. co-precipitated drebrin with the tight junction associated protein symplekin[162]. This interaction was never confirmed, but raised the idea that drebrin might have more functions - also in non-neuronal cells - than previously considered. Already in the same year, this idea could be supported by Peitsch *et al*. who described drebrin E2 in a variety of cell types and tissues, including smooth muscle, stomach, kidney and a variety of epi- and endothelial cells[191]. In stomach and kidney, drebrin shows cell-type specific patterns with an expression in acidic-secreting cells[162]. Butkevich *et al.* found drebrin at gap junctions of green monkey kidney epithelial cells, with its loss leading to impaired cell-cell coupling and internalization of gap junction protein connexin-43[192]. Also in the human epidermis, which contains very small amounts of drebrin, it is mainly expressed in secreting sweat gland cells and in hair follicles. Interestingly, drebrin levels are strongly increased in epidermal skin tumors, where it localizes to junctional areas and the little amount that is expressed in cultured keratinocytes is also enriched at adherens junctions (Figure 11)[160]. Confluent endothelial (HUVEC) monolayers show a high expression of drebrin E2, which is organized into different subcellular pools: a low amount of drebrin can be found in cytosolic dot-like arrangements, while the majority is enriched at the cell cortex, associating with F-actin filaments preferably near adherens junctions[191].



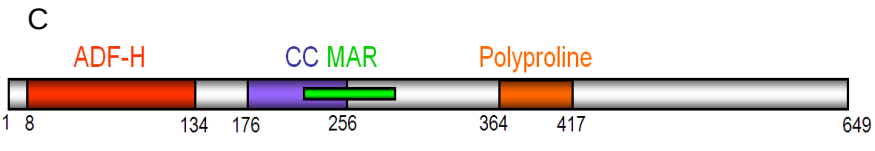


Figure 11 Drebrins localization in non-neuronal cells
A In migrating cells (here, SV80 (**S**imian **V**irus 80) cells), drebrin often localizes to lamellipodia and filopodia[193].
B In confluent cells, drebrin colocalizes with F-actin at the submembraneous cell cortex. In skin tumours, expression levels of drebrin are increased. Basal cell carcinoma cryostat sections, stained for drebrin (red) and F-actin (green). Note: These localizations of drebrin to lamellipodia and junctional areas are also typical for HUVEC[160].
C Domain structure of drebrin: ADF-H (**A**ctin **D**epolymerizing **F**actor **H**omology) domain, a central MAR (**M**inimal **A**ctin **R**emodeling) domain (residues 233-317), overlapping with a CC (coiled-coil) region. The unconserved C-terminus contains a PP (**P**oly**p**roline) region.

Migrating cells show a different distribution of drebrin: in oculomotor neurons, drebrin is necessary for the formation of leading processes and their migration and it has been shown that migratory neuroblasts in adult brain are positive for drebrin E[194,195]. In subconfluent endothelial cells, which have not established intercellular junctions yet, drebrin E2 has a characteristic localization to filopodia tips and leading edges of membrane ruffles or

lamellipodia. In motile cultured cells, drebrin's occurrence at lamellipodia could be defined more precisely as being present in the posterior region of actin microspikes[193].
Recently, drebrin has also been shown to be involved in the formation of the immune synapse of T-cells by binding to the chemokine receptor CXCR4 via its N-terminal region[164].

On the one hand, it has been shown that there are similar functions of drebrin in different cells, for example its ability to interact with F-actin - but on the other hand, it can fulfill specialized and enormously variable functions in diverse kinds of cells. However, the necessity of drebrin's presence at cell-cell junctions remains largely unknown so far. To elucidate drebrin's functions in endothelial cells and to clarify the molecular basis of its localization to cell-cell junctions was therefore the central aim of this thesis.

III Material and Methods

1 Material

1.1 Devices

Device	Type, Provider
Balance	440-47N, Kern, Balingen-Frommern (G)
Benches	Hera Safe, Thermo Scientific, Rockford (USA)
Film-cassette	Hartenstein, Würzburg (G)
Cell counter	Neubauer chamber, Hartenstein, Würzburg (G)
NEON Transfection System	Life Technologies, Carlsbad (USA)
Centrifuges	Sorvall RC-5B, RC28S, Thermo Scientific, Rockford (USA) 5417R and 5810R, Eppendorf, Hamburg (G)
Ibidi Pump System	Pump, Fluidic Units, Lenovo ThinkPad (Software: PumpControl v.4.0.2-5.0.1), Ibidi, Martinsried (G)
iblot	Life Technologies, Carlsbad (USA)
Incubator	CB Series, Binder, Tuttlingen (G); BBD 6220, Heraeus, Hanau (G)
Incubator (shaking)	Certomat BS-1, Sartorius, Göttingen (G)
µMACS TM Separator	Miltenyi Biotech, Bergisch Gladbach (G)
Microplate reader	Infinite M200, TECAN, Männedorf (CH)
Mr. Frosty	5011 cryo 1℃ freezing container, Nalgene/Thermo Scientific, Rockford (USA)
NanoDrop® ND-1000	PeqLab, Erlangen (G)
Photometer	Ultrospec 3100 pro, Amersham/GE Healthcare Europe, Munich (G)
Pipettes	2, 10, 20, 100, 200, 1000 µl, Eppendorf, Hamburg (G); Accu-jet pro, Brand, Wertheim (G)
PCR-cycler	Primus 25 advanced, PeqLab, Erlangen (G)
Gel Electrophoresis Chamber	PeqLab, Erlangen (G)
Scanner	CanoScan 4400F, Canon, Amsterdam (NL)
Sonifier	Digital Sonifier 250-D, Branson, Danbury (USA)
Transilluminator	Vilber Lourmat, ETX, Eberhardzell (G)
UV-Transilluminator + detector	ChemiDoc XRS, BioRad, Munich (G)
Voltohmmeter	Millicell® ERS-2, Millipore, Billerica (USA)
Vortex	REAX top, Heidolph Instruments, Schwabach (G)

Table 1 Devices

1.2 Microscopic devices

Live cell spinning disk confocal	Type, Provider
Provider	Improvision, Coventry (UK)
Microscope	Axiovert 200M, Zeiss, Jena (G)
Objective	Plan-Apochromat 63x / 1.4 Ph3 oil immersion
Confocal unit	Spinning disk CSU22, Yokogawa, Tokyo (J)

Camera	EM-CCD C9100-02, Hamamatsu (J)
Laser	Cobolt Calypso CW 491 nm, Cobolt Jive 561 nm, Stockholm (S)
Laser Combiner	LMM5, Spectral Applied Research, Richmond Hill (CAN)
Emission Filters	ET 525/50 (green), ET 620/60 (red), Chroma Technology, Rockingham (USA)
UV lamp	X-cite series 120 W with Hg-lamp, EXFO, Mississauga (CAN)
Halogen lamp	Standard housing 100 W, Zeiss, Jena (G)
Incubation chamber	Temperature / humidity / CO_2 control, Solent Scientific, Regensworth (UK)
Equipment	Motorized BioPrecision inverted XY stage and PiezoZ stage, Ludl Electronic Products, Hawthorne (USA)
Software	Volocity versions 4.2-6.1, Perkin Elmer, Waltham (USA)

Table 2 Microscopic devices

Confocal laser scanning microscope	Type, Provider
Provider	Leica, Wetzlar (G)
Stand	Leica DM IRE2, Leica, Wetzlar (G)
Objective	Plan-Apochromat 63x / 1.4 Ph3 oil immersion
	Plan-Apochromat 63x/1.3 Ph3 water
Confocal unit	Leica TCS SP2 AOBS confocal point scanner
Laser)	Ar, Ar/Kr (488 nm, 514 nm), HeNe (543 nm, 594 nm, 633 nm
Emission Filters	Filtersystems: I 3, blue ecx. (BP 450-490, LP515); N 2.1, green exc. (BP 515-560, LP590); A, UV exc. (BP 340-380, LP425)
UV lamp	Standard housing, 50 W HBO mercury
Halogen lamp	Standard housing, 100 W, 12 V
Equipment	POC perfusion chamber, CO_2: PeCon CTI-Controller 3700 digital, Temp.: PeCon tempcontrol 37-2 digital; Z-drive: Piezo focus drive
Software	Leica LCS version 2.61.1537, Leica, Wetzlar (G)

Table 3 Confocal laser scanning microscopes

Epifluorescence microscope	Type, Provider
Provider	Visitron Systems, Puchheim (G)
Stand	Axioplan (upright), Zeiss, Jena (G)
Objective	Plan-Neofluar 10x / 0.30 Ph1
	Plan-Neofluar 40x / 0.72 Ph2
Camera	CCD SPOT Pursuit 1.4MP monochrome, Diagnostic Instruments, Sterling Heights (USA)
Emission Filters	Filter set 02 (blue) / 09 (green) / 15 (red), Zeiss, Jena (G)
UV lamp	HBO 50 W with HG lamp, Zeiss, Jena (G)
Halogen lamp	Standard housing 100 W, Zeiss, Jena (G)
Software	SPOT version 4.6 Diagnostic Instruments, Sterling Heights (USA)

Table 4 Epifluorescence microscopes

Transmitted light microscopes	Type, Provider
Provider	Nikon, Tokyo (J)
Stand	Eclipse TS100
Objective	Plan Fluor 4x / 0.13 PhL
	LWD 10x / 0.25 Ph1
	LWD 20x / 0.4 Ph1
	LWD 40x / 0.55 Ph1
Camera	Nikon D5000 digital camera
Emission Filters	Filter set HQ EGFP (green) / HQ Calcium Crimson, Chroma Technology, Rockingham (USA)
UV lamp	Nikon Intensilight 130 W with Hg lamp
Halogen lamp	Standard housing 30 W

Table 5 Transmitted light microscopes

For processing of images, which were acquired with these microscopic devices, Volocity (Perkin Elmer, Waltham, USA), ImageJ (Bethesda, USA), and Adobe Photoshop CS5 (Adobe Systems GmbH, Munich, G) were used.

1.3 Disposables

Disposables	Type, Provider
µMACS Protein A/G beads	MACS, Milteny Biotec GmbH, Bergisch Gladbach (G)
Amylose Resin	New England Biolabs, Ipswich, (USA)
Conical centrifuge tubes	Sterile 15 ml/50 ml, Sarstedt, Nümbrecht (G)
Cell culture dishes	Sarstedt, Nümbrecht (G)
Cell culture flasks	T-25 nunclon, Thermo Scientific / Nunc, Rockford (USA); T-75, Sarstedt, Nümbrecht (G)
Columns, polypropylene, 1 ml	Qiagen, Hilden, (G)
Glass coverslips	round 12 mm diam. No. 1, Hartenstein, Würzburg (G)
Glass bottom dishes, 12/22 mm	WillCo-dish®, WillCo Wells BV, Amsterdam (NL)
Glass Pasteur pipettes	230 mm, Heinz Herenz Medizinalbedarf, Hamburg (G)
Glutathione Sepharose 4B	GE Healthcare, Uppsala (S)
iBlot Gel Transfer Stacks Nitrocellulose, Mini/Regular	Life Technologies, Carlsbad (USA)
Inoculating loops	10 µl, Sarstedt, Nümbrecht (G)
Microscopy slides	76 x 26 mm, cleaned, frosted end, Karl Hecht, Sondheim (G); µ-Slides I $^{0.6\ Luer}$ (with perfusion set green/yellow), Ibidi, Martinsried (G)
Multiwell plates	6- / 12-well Sarstedt, Nümbrecht (G); Nunclon 96-well flat bottom black polystyrol, Thermo Scientific/Nunc, Rockford (USA)
Reaction tubes	0.2 ml, Biozym Scientific, Hessisch Oldendorf (G); 0.5, 1.5, 2 ml standard; 1.5 ml Protein LoBind, Eppendorf, Hamburg (G)
Parafilm M	Bemis®, Pechiney Plastic Packaging, Neenah (USA)
Pipette tips	Sterile Biosphere filter tips and non-sterile 10, 200, 1000 µl, Sarstedt, Nümbrecht (G); 5000 µl, Eppendorf, Hamburg (G)
Serological pipettes	Sterile 2, 5, 10, 25 ml, Sarstedt, Nümbrecht (G)

Scalpel	Sterile, B. Braun, Melsungen (G)
SYBR® Safe DNA Gel Stain	Life Technologies, Carlsbad (USA)
Syringes	Sterile 5, 20 ml, B. Braun, Melsungen (G)
Syringe filters	SFCA 0.2 µm, Thermo Scientific/Nalgene, Rockford (USA)
Transwell inserts	6.5 mm inserts, 0.4 µm pore size, tissue culture treated sterile polycarbonate membrane, Corning Incorporated, Corning (USA)
X-ray film	Super RX, Fuji medical X-ray film, Fujifilm, Tokyo (J)

Table 6 Disposables

1.4 Kits, enzymes and agents

Kit, enzyme, agent	Provider
α-Chymotrypsin	Sigma-Aldrich, St Louis (USA)
BioRad Protein Assay	BioRad, Munich (G)
DNA Clean and Concentrator-5	Zymo Research, Irvine (USA)
EndoFree Plasmid Maxi Kit	Life Technologies, Carlsbad (USA)
FastDigest® restriction enzymes	Fermentas, St. Leon-Rot (G)
Neon®Transfection System	Invitrogen/Life Technologies, Carlsbad (USA)
QuikChange™ Site-Directed Mutagenesis Kit	Stratagene, La Jolla, (USA)
SuperSignal West Femto/ Pico detection	Thermo Scientific, Rockford (USA)
T4 DNA Ligase	Roche, Mannheim (G)
Taq DNA Polymerase + buffers	Peqlab, Erlangen (G)
Trypsin 0.05 %, 0.53 mM EDTA x 4 Na with phenol red	Invitrogen/Life Technologies, Carlsbad (USA)
ZR Plasmid Miniprep Kit	Zymo Research, Irvine (USA)
Zymoclean Gel DNA recovery Kit	Zymo Research, Irvine (USA)

Table 7 Kits, enzymes and agents

1.5 Growth media, additives, antibiotics and collagen

Media	Provider
Endothelial Cell Growth Medium (ECGM)	Low-serum content (2 % v/v); for cultivation of endothelial cells, Promo Cell GmbH, Heidelberg (G)
LB-medium (Lennox)	for cultivation of *E. coli* 10 g/l tryptone 5 g/l yeast extract 5 g/l NaCl (15 g/l agar), pH 7.5

Table 8 Growth media

Additive/Antibiotics	Provider
Gentamicin (50 µg/ml)	Sigma-Aldrich, St. Louis (USA)
Ampicillin (100 µg/ml)	Sigma-Aldrich, St. Louis (USA)
Kanamycin (50 µg/ml)	Sigma-Aldrich, St. Louis (USA)
Supplement mix for ECGM	PromoCell GmbH, Heidelberg (G)

Table 9 Additives/Antibiotics

Coating agent	Provider
Collagen G from bovine calf skin	Biochrom, Berlin (G)

Table 10 Coating agents

1.6 Chemicals and buffers

Chemicals and antibiotics were obtained from Amersham/GE Healthcare, Munich (G), BD Biosciences, Heidelberg (G), Invitrogen/Life Technologies, Carlsbad (USA), Roche, Mannheim (G), Biozyme, Oldendorf (G), Dianova, Hamburg (G), Fermentas, St. Leon-Rot (G), Merck, Darmstadt (G), PAA, Pasching (A), PromoCell, Heidelberg (G), Roth, Karlsruhe (G) and Sigma-Aldrich, St. Louis (USA). Media and buffer were autoclaved for 20 min., 121 °C, and 1.4 bar. Complete Protease Inhibitor and PhosSTOP Phosphatase Inhibitor Cocktails were obtained from Roche, Mannheim (G).

Buffer	Composition	Concentration
Coomassie staining solution	Coomassie Brilliant Blue R-250 methanol glacial acetic acid ddH_2O	0.1 % w/v 25 % w/v 10 % w/v
Destain solution	methanol glacial acetic acid ddH_2O	25 % w/v 10 % w/v
DNA loading buffer (5x)	glycerol bromphenolblue xylenblue 1x TAE buffer	30 % v/v 0.25% w/v 0.25% w/v
Elution buffer	= SDS-PAGE loading buffer (4 x)	
GST-buffer I	PMSF protease tablette PBS ad 50 ml	1 mM
GST elution buffer II	Glutathione Tris pH 8,8 ddH_20	30 mM 50 mM
IP lysis buffer I	NaCl Tris/HCl, pH 7.4 EDTA, pH 8.0 Triton-X100	50 mM 20 mM 1 mM 1 %
IP lysis buffer II	NaCl Tris/HCl, pH 8.0 SDS Igepal CA-630 sodium deoxycholate	150 mM 50 mM 0.1 % 1 % 0.5 %
IPTG (isopropyl β-D-1-thiogalactopyranoside)	AppliChem, Darmstadt (G)	
MBP-buffer I	Tris pH 7,4 NaCl EDTA protease tablette H_2O ad 50 ml	20 mM 200 mM 1 mM
MBP elution buffer II	MBP-buffer I +	

	Maltose	10 mM
MBP-buffer III	NaCl MgCl2 protease tablette PBS ad 50 ml	150 mM 5 mM
PBS (10x)	KCl Na_2HPO_4 KH_2PO_4 ddH_2O	1.37 M 26.5 mM 0.1 M 17.6 mM adjust to pH 7.7
PBST	Tween20 1x PBS	0.05 %
Resolving buffer (SDS-PAGE)	Tris base SDS ddH_2O	1.5 M 0.004 % w/v adjust to pH 8.8
Stacking buffer (SDS-PAGE)	Tris base SDS ddH_2O	0.5 M 0.004 % w/v adjust to pH 6.8
SDS-PAGE loading buffer (4 x) reducing or (non-reducing)	Tris/HCl, pH 6.8 glycerol SDS bromphenolblue (β-mercaptoethanol) ddH_2O	250 mM 20 % w/v 8 % w/v 4 mg (4% v/v)
SDS-PAGE running buffer (10x)	Tris base glycine SDS ddH_2O	0.025 M 0.192 M 0.1 % w/v
TAE (50x)	Tris acetate, pH 8.3 EDTA ddH_2O	40 mM 10 mM adjust to pH 7.4
TBS (10x)	Tris base NaCl ddH_2O	20 mM 150 mM adjust to pH 7.4
TBST	Tween20 1x TBS	0.3 %
Tfb1 (4 ℃)	KAc $MnCl_2$ RbCl $CaCl_2$ glycerol ddH_2O	30 mM 50 mM 100 mM 10 mM 15 % w/v adjust to pH 5.8
Tfb2 (4 ℃)	Na-MOPS, pH 7.0 RbCl $CaCl_2$ glycerol ddH_2O	10 mM 10 mM 75 mM 15 % w/v adjust to pH 6.8

Table 11 Buffers

1.7 Protein and DNA ladders

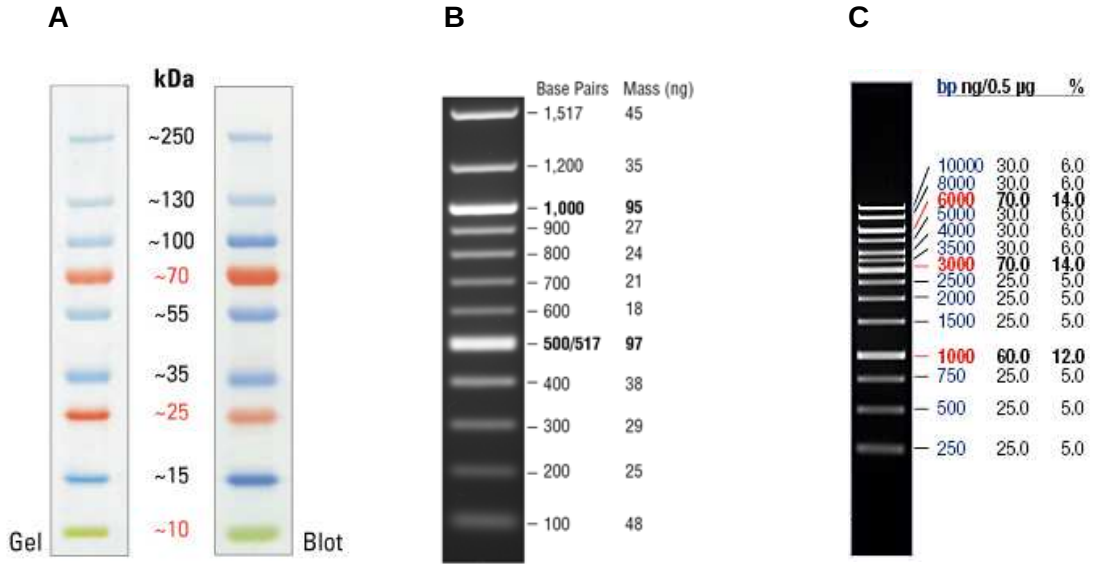


Figure 12 Protein and DNA ladders
A PageRuler Prestained PLUS Protein Ladder (Thermo Scientific, Rockford, USA). **B** Quickload 100 bp DNA Ladder (New England Biolabs, Ipswich, USA) **C** GeneRuler 1 kb DNA Ladder (Thermo Scientific, Rockford, USA).

1.8 siRNA

SiRNA (**s**mall **i**nterfering RNA)- based gene silencing is a commonly applied method, where short (19-21 nucleotides) RNA sequences are transfected into cells to degrade the mRNA containing their complement sequence. Thus, no protein can be translated, potentially revealing knockdown phenotypes related to its function. In general, knockdown experiments using siRNA were performed 72 hours before seeding cells on coverslips/µ-slides to yield a maximum suppression of the gene expression. Control experiments were performed by transfecting non-targeting siRNA against firefly luciferase from the Dharmacon siRNA collection (Dharmacon/Thermo Scientific, Rockford (USA)).

Target		Sequence (5'-3')	Company
Luciferase		„non-targeting siRNA #2"	Dharmacon
DBN1 (drebrin)	**-01**	GGAAACAGCAGACUUUAGA	Dharmacon
SMARTpool	**-02**	GAAGAGACCCACAUGAAGA	
	-04	GAUGUACCCUCGCCCUUCA	
	-17	GGUUCGAGCAGGAGCGGAU	
PVRL2 (nectin-2)		CGCUGAGCAGGUCAUCUUUtt	ambion
PVRL3 (nectin-3)		CCAUUGACUUUCAAUUAUUtt	ambion
MLLT4 (afadin))	**-01**	UGAGAAACCUCUAGUUGUA	Dharmacon
SMARTpool	**-02**	GUUAAGGGCCCAAGACAUA	
	-04	CAUCAGCGUUGGUAUGAGA	
	-17	CGAAAGUCUGAUAGUGAUA	

Table 12 siRNA sequences

1.9 Bacterial strains and eukaryotic cells

1.9.1 *Escherichia coli* strains

Strain	Characteristics	Reference
DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–	[196]
TOP10	F^- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	[197]

Table 13 *Escherichia coli* strains

1.9.2 Eukaryotic cells

Cells	Characteristics	Reference
HUVEC	Human Umbilical Vein Endothelial Cells, primary cells isolated from veins of human umbilical cords; passages P2-P6 were used. Grow in ECGM + Supplement Mix + Gentamicin	Self made isolations, umbilical cords were provided by the Marienkrankenhaus, Hamburg

Table 14 Eukaryotic cells

1.10 Plasmids

1.10.1 Prokaryotic expression

Vektor/ construct	Characteristics/ insert	Reference
pGEX-2T	Prokaryotic GST-fusion protein expression plasmid; empty MCS	Amersham/GE Healthcare, Munich (G)
pMAL-p2X	Prokaryotic MBP-fusion protein expression plasmid; empty MCS	NEB, Frankfurt (G)
MBP-drebrin-PP	pMAL-p2X with drebrins PP region, AA 327 - 411	Kerstin Rehm
GST-afadin-PR1-2	pGEX-2T with afadins PR1-2 region, AA 1212 - 1406	Kerstin Rehm

Table 15 Plasmids for prokaryotic expression

1.10.2 Eukaryotic expression

Vektor/ construct	Characteristics/ insert	Reference
pEGFP-C1	enhanced GFP-fusion protein expression; empty MCS; N-terminal tag	Clontech, Heidelberg (G)
pEGFP-N1	enhanced GFP-fusion protein expression; empty MCS; C-terminal tag	Clontech, Heidelberg (G)
pLifeActTagGFP2-N	LifeAct with TagGFP2-fusion protein expression	Ibidi, Martinsried (G)

Human drebrinE2 constructs:		
Drebrin-GFP	pEGFP-N1 with drebrinE2	[160]
GFP-drebrin	pEGFP-C1 with drebrinE2	[160]
GFP-drebrin-insensitive	pEGFP-C1 with drebrinE2, insensitive against drebrin siRNA pool	Kerstin Rehm
C-terminus-GFP	pEGFP-N1 with drebrin's AA 328-633	V. van Vliet (our lab)
PP-GFP	pEGFP-N1 with drebrin's polyproline region, AA 348-421	V. van Vliet (our lab)
CC-GFP	pEGFP-N1 with drebrin's coiled-coil region, AA 176-256	V. van Vliet (our lab)
Mem-like-GFP	pEGFP-N1 with drebrin's membrane-like region, AA 477-571	V. van Vliet (our lab)
Drebrin-RFP	pEGFP-N1 backbone, with GFP replaced through RFP, AA 1-633	V. van Vliet (our lab)
DrebrinΔPP	pEGFP-N1 with drebrin, deletion of AA 349-421	Kerstin Rehm
pAREK1-G^{PA}C-v2-drebrin	pArek1-G^{PA}C-zyxin (gift from Arkadiusz Welman) as backbone, zxin replaced through drebrin → photoactivatable drebrin	Kerstin Rehm
Mito-DrebrinPP-GFP	pEGFP-N1 backbone, with a mitochondrial-targeting sequence and drebrin's PP region (AA 348-421)	Kerstin Rehm [198]
Afadin constructs:		
GFP-Afadin	pEGFP-C1 and afadin's AA 1-1829	[105]
GFP-Afadin ΔRA	pEGFP-C1 and afadin's AA 352-1829	[105]
PR-1-2-flag	Afadin's PR1-2 AA 1219-1399	[199]
N-PDZ-flag	Afadin's N-PDZ AA 1-1100	[199]
PDZ-flag	Afadin's PDZ AA 1015-1100	[199]
CC-flag	Afadin's CC, AA 1532-1829	[199]
ΔPR1-2 -HA	Afadin fullength, with AA 1218 -1400 deleted	[199]
GFP-PDZ	pEGFP-C1 and afadin's PDZ AA 1000-1127	Kerstin Rehm
LifeAct-PDZ-TagGFP2	pLifeActTagGFP2-N and afadin's PDZ AA 1014-1179	Kerstin Rehm
DrebrinCC-PDZ-TagGFP2	pTagGFP2-N, Drebrin's CC and afadin's PDZ AA 1014-1179	Kerstin Rehm
Other constructs:		
Mito-GFP	pEGFP-N1 backbone, with mitochondrial-targeting sequence	Kerstin Rehm [198]
Mito-WASP-PP-GFP	pEGFP-N1 backbone, with mitochondrial-targeting sequence and WASP-Polyproline region, AA 313-408	Kerstin Rehm [198]
Flag-nectin-2	pFlag-CMV1 with nectin-2alpha	Yoshimi Takai
Nectin-2-GFP	pEGFP-N1 with nectin-2	Wayne Vogl [200]

LifeAct-ZO1-PDZ-TagGFP2	lifeact-TagGFP2 with the 1st PDZ region of ZO-1 (AA 14-14). pCDNA6-flag-ZO-1 was a gift from J. Kremerskothen	Kerstin Rehm
EEA1-GFP	gift from H. Stenmark	[201]
GFP-Rab22	gift from J. Donaldson	[202]

Table 16 Plasmids for eukaryotic expression

1.11 Primer

No.	Name	sequence (5' → 3')
1	DrebrinΔPP(1-348)-F	ccctcgagatggccggcgtcagcttcagcggccaccgcc
2	DrebrinΔPP(1-348)-R	atatatgaattcgacaggggtggaggcggtgctggagt
3	DrebrinΔPP(423-649)-F	tatattgaattcatgttcatggagtctgcagagcag
4	DrebrinΔPP(423-649)-R	ggggatccggcttatcaccaccctcgaagccctcctcct
5	mito-drebrin-PP-GFP-F	agctccaccgcgctagcggccgccatggcc
6	mito-drebrin-PP-GFP-R	gacggtatcgataagcttgatatcgaattc
7	mito -GFP-F	agctccaccgcgctagcggccgccatggcc
8	mito -GFP-R	gacggtatcgataagcttgatatcgaattc
9	mito-WASP-PP-GFP-F	agctccaccgcgctagcggcc gccatggcc
10	mito-WASP-PP-GFP-R	gaattcgatatcaagcttatcgataccgtc
11	GST-afadin-PR1-2-F	ataacatct gtctctactggatccctctgcactgaggag
12	GST-afadin-PR1-2-R	tgcagcagccacctgagaattctgggggagctgcttggtt
13	MBP-Drebrin-PP-F	agcccgtctgactccagcaccggatccacccctgtcgctgagcagata
14	MBP-Drebrin-PP-R	acgggagcagccaggacagcgtcgactgcagactccatgaacatcaa
15	plifeact-PDZ-TagGFP2-F	gacccagcctctgaggaaggatcctgaggtaatcactgtga
16	plifeact-PDZ-TagGFP2-R	ttgccacattggggctggaccggtgcgtcagctctgttt
17	GFP-Afadin-PDZ-F	gattatgaaagtcacctcgagcccgt gagaacaca
18	GFP-Afadin-PDZ-R	tggtctgggtttaccggatccacgacgatctgaa at
19	drebrinCC-afadinPDZ-GFP-F	tgcagctgtggaactcgagcggattaaccgagagcag
20	drebrinCC-afadinPDZ-GFP-R	ctcttcctcctcatcccgaggatccccaaagatagactgctc
21	plifeact-ZO-1PDZ-TagGFP2-F	gccaagagcacagcaatggaggatccagctata tgggaacaa
22	plifeact- ZO-1PDZ-TagGFP2-R	ctgttaaccacaccacaccggtgcacttcttggatc atgtat
23	Drebrin-mut-siRNA-01-F	g aaa cag cag aca tta gaa gcg g
24	Drebrin-mut-siRNA-01-R	c cgc ttc taa tgt ctg ctg ttt c
25	Drebrin-mut-siRNA-02-F	gatgaggag gag gag acc cac atg aaa aagtcagagtc
26	Drebrin-mut-siRNA-02-R	gactctgacttttcatgtgggtctcctcctcctcatc
27	Drebrin-mut-siRNA-04-F	ggcagctgtgat gtt ccc tcg ccg ttc aaccatcgacc
28	Drebrin-mut-siRNA-04-R	ggtcgatggttgaacggcgagggaacatcacagctgcc
29	Drebrin-mut-siRNA-17-F	aggctcagg ttt gag cag gag cga atg gagcaggagc
30	Drebrin-mut-siRNA-17-R	gctcctgctccattcgctcctgctcaaacctgagcct
31	RT-PCR nectin-2 F	cctcctgaagtgtccatct
32	RT-PCR nectin-2 R	gtgcagacgaaggtggtat

Table 17 Primer sequences

1.12 Antibodies

1.12.1 Primary antibodies

Antibody	Species	Provider	Dilution WB	Dilution IF
Actin	mouse	Millipore	1:10000	-
Afadin (AF6)	mouse	BD Biosciences	1:100	1:100
Drebrin	guinea-pig	Progen	1:500	1:100
Connexin-43	rabbit	Sigma	1:1000	1:100
Flag M2	mouse	Sigma	1:1500	1:100
GFP	mouse	Clontech	1:7000	-
GST	goat	GE Healthcare	1:1500	-
HA	rabbit	abcam	1:500	-
Integrin-alpha5	Rabbit	chemicon	-	1:100
LAMP-1	mouse	Santa Cruz	-	1:100
MBP	rabbit	abcam	1:2500	-
Nectin-2	rabbit	Y.Takai	1:1000	1:100
Nectin-2 (H108)	rabbit	Santa Cruz	1:500	-
Nectin-2 (R2.477.2)	mouse	Santa Cruz	-	1:100
Nectin-3	goat	Y.Takai	1:1000	1:100
Nectin-3 (C19)	goat	Santa Cruz	1:500	-
Occludin	mouse	Zymed	1:500	1:200
Paxillin	mouse	BD Biosciences	-	1:100
PECAM-1	rabbit	Santa Cruz	1:1500	1:100
VE-cadherin	mouse	Pharmigen	-	1:100
VE-cadherin	mouse	Chemicon	1:500	-

Table 18 Primary antibodies

F-actin was visualized using AlexaFluor® 488-,568- or 647-labeled phalloidin (Invitrogen) in a 1:200 dilution.

1.12.2 Secondary antibodies

Antibody	Provider	Dilution
Alexa-488 goat anti-mouse	Invitrogen	1:200
Alexa-568 goat anti-mouse	Invitrogen	1:200
Alexa-647 goat anti-mouse	Invitrogen	1:200
Alexa-488 donkey anti-goat	Invitrogen	1:200
Alexa-568 rabbit anti-goat	Invitrogen	1:200
Alexa-647 donkey anti-goat	Invitrogen	1:200
DyLight-488 donkey anti-guineapig	Jackson	1:200
TRITC goat anti-guineapig	Jackson	1:200
Alexa-568 goat anti-rabbit	Invitrogen	1:200
Alexa-647 goat anti-rabbit	Invitrogen	1:200
DyLight-488 goat anti-rabbit	Jackson	1:200

Anti-mouse IgG, HRP-conjugated	GE Healthcare	1:5000
Anti-rabbit IgG, HRP-conjugated	GE Healthcare	1:5000
Anti-guinea-pig IgG, HRP-conjugated	abcam	1:5000
Anti-goat IgG, HRP-conjugated	abcam	1:5000

Table 19 Secondary antibodies

1.13 Software and databases

Software	Provider
Adobe Photoshop CS5	Adobe Systems GmbH, Munich (G).
Ape-A Plasmid Editor v.1.17	M. Wayne Davis
BioEdit v7.1.7	Tom Hall Ibis Biosciences
Excel software	Microsoft, Redmond, WA
GraphPad Prism 5	La Jolla (CA)
ImageJ	Bethesda (USA)
PumpControl v.4.0.2-5.0.1	Ibidi, Martinsried (G)
Ultraview software	PerkinElmer Life Sciences, Boston, USA
Volocity 6.0	PerkinElmer Life Sciences, Boston, USA
Volocity DEMO	PerkinElmer Life Sciences, Boston, USA

Table 20 Software and databases

2 Methods

All experiments performed in this study were done in safety level 2 (S2) laboratories.

2.1 Molecular biology techniques

2.1.1 Cultivation of *E. coli*

In this study, *E. coli* DH5α were used for all experiments. *E. coli* were cultivated at 37 °C under aerobic conditions either in liquid (160-210 rpm) or on solid LB-medium. Antibiotics were added according to the particular plasmids that should be expressed (100 µg/ml ampicillin, 50 µg/ml kanamycin). Starter cultures with volumes of 2-10 ml were grown overnight, whereas the volume of the main cultures varied from 100-1000 ml with different cultivation times dependent on the subsequent experiment.

2.1.2 Preparation of chemically competent *E. coli*

The preparation of chemically competent bacterial cells was described by Hanahan *et al*.[203]. A 5 ml starter culture was inoculated with one *E. coli* DH5α colony and grown overnight. 500 µl of the overnight culture was used to further inoculate 500 ml LB medium, which was incubated until the culture reached an OD_{600} (optical density at 600 nm) of 0.3- 0.5. The culture was cooled down on ice, the bacteria were pelleted (3000 rpm, 10 min, 4 °C) and the pellet was resuspended in 15 ml Tfb1 and incubated on ice for 1.5 h. After pelleting the bacteria again (3.000 rpm, 10 min, 4 °C), the supernatant was removed, 20 ml Tfb2 was added and bacteria carefully resuspended through turning the tubes on ice. Aliquots of 100-200 µl were snap frozen in liquid nitrogen and stored at -80 °C.

2.1.3 Transformation of chemically competent *E. coli*

One Aliquot of 100 µl competent bacteria was thawn on ice and 1-100 ng of plasmid DNA (or 1-10 µl ligation reaction) were added. After incubating 30 min on ice a heat shock was performed at 37 °C for 5 min to introduce the DNA into the bacterial cells. After resting another 2 min on ice, 500 µl LB medium + glucose was added. Transformed bacteria were placed on a shaker for >1 h at 37 °C with 1400 rpm agitation and plated onto LB agar plates containing the antibiotics corresponding to the transfected plasmid to select for positive clones.

2.1.4 Isolation of plasmid DNA

Plasmid DNA was isolated from 5 ml bacterial cultures expressing the plasmid using the ZR Plasmid Miniprep-Classic Kit according to the manufacturers' protocol. To obtain pure, endotoxin-free plasmid DNA for transfection of mammalian cells the EndoFree Plasmid Maxi Kit was used and bacteria were grown in 100 ml cultures.

2.1.5 Polymerase chain reaction

The polymerase chain reaction (PCR) is a common technique for multiplying certain pieces of DNA by choosing oligonucleotides flanking the region of interest, which is then copied millions of times through a thermostable *Taq*-DNA polymerase[204]. Through introduction of enzymatic restriction site sequences at the 5' end of the oligonucleotide sequences, PCR is also suitable for generating DNA fragments that can be digested with the respective

endonucleases followed by subcloning them into expression vectors. Another commonly used application is the colony PCR, which allows a quick screen for plasmids containing a desired insert directly from *E. coli* colonies.
A typical PCR reaction setup is shown here:

reaction composition	**concentration**	**amount**
DNA template or colony	100 ng/µl	1 µl
PCR buffer	10x	5 µl
forward primer	10 pmol/µl	1 µl
reverse primer	10 pmol/µl	1 µl
dNTPs	10 mM	1 µl
***Taq* polymerase**	10 units/µl	0,3 µl
dH_2O		**ad 50 µl**

Table 21 PCR reaction mix

PCR program	**temperature**	**time**	**Cycles**
initial denaturation (colony PCR)	95 ℃	5 min (15 min)	1
denaturation	95 ℃	30 s	30-35
annealing	40-72 ℃	30 s	
extension	72 ℃	1 min/ 1 kb	
final elongation	72 ℃	10 min	1
storage	8 ℃	forever	1

Table 22 PCR program

2.1.6 Expression analysis by RT-PCR

The process of gene expression consists of several steps, where each part can be modulated resulting in different final expression levels of protein. Since almost all cells of an organism obtain the same DNA, the first regulatory step is to silence those genes that are not needed to be expressed in the respective cell type. This can be achieved by varying the chromatin accessibility, methylation of nucleotides and availability of regulatory proteins[205]. If a gene is active, transcription is initiated and pre-mRNA is produced by a RNA-Polymerase[25]. Subsequently, the pre-mRNA will undergo splicing, which will remove introns (intervening sequences) and can give rise to different protein isoforms depending on which exons are present in the mature mRNA[206]. Translation of the mRNA into protein is accomplished by ribosomes and can also be regulated via other proteins, as well as post-translational modifications that influence the activity of the mature protein[207]. Absence of a regulatory protein involved in any step of this chain of processes could lead to diminished expression levels of a protein. To check whether specific mRNA levels were altered in knockdown situations, RNA was extracted from HUVEC. Therefore, HUVEC (5×10^6) treated with drebrin siRNA (pool) or luciferase siRNA for 4 days were submitted to total RNA extraction using 8 ml TRIzol reagent following the manufacturer's instructions (Invitrogen, Darmstadt, Germany), to obtain all mRNA (**m**essenger RNA) present in the cell. The RNA quality was validated on a 2 % Agarose gel. Subsequently, the reverse transcription (**RT**) reaction was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Darmstadt, Germany). Using the obtained cDNA (**c**opy DNA) as a template, a PCR was performed using nectin-2 specific intron-spanning primers (Table 17,

No. 31, 32). The size of the PCR product was 212 bp for cDNA templates and 507 bp for genomic DNA, containing introns. The PCR products were analyzed by electrophoresis on a 1 % agarose gel containing SYBR Safe DNA Gel Stain (life technologies, Darmstadt, Germany) to visualize bands in UV-light. Marker: Quick-Load 100 bp DNA Ladder (Figure 12).

2.1.7 Restriction digest of DNA

For subcloning PCR-generated fragments of DNA into a vector of interest, specific endonuclease restriction sites were created at the end of the DNA fragment throughout the PCR. The same restriction sites were available in the vector of interest and both fragments were digested using FastDigest® restriction endonucleases according to the manufacturers' recommendations in a thermo block. Products were separated on agarose gels and the desired band was purified from the gel to avoid contaminations through the template using Zymoclean Gel DNA recovery Kit.

2.1.8 Ligation

Ligation reactions were performed using T4 DNA Ligase at 16 ℃ overnight or for 1 h at room temperature. The reaction was performed in the supplied T4 DNA ligation buffer using a molar ratio of at least 1:3 (vector:insert) and 50 ng vector-DNA.

$$ng_{(Insert)} = \frac{ng_{(Vector)} \times kb_{(Insert)}}{kb_{(Vector)} \times \text{molar ratio}_{(Vector/Insert)}}$$

2.1.9 Generation of constructs used in this study

Human drebrinE2-EGFP constructs were kind gifts of W. Ludwig. EGFP-tagged drebrinE2 domain constructs were created by cloning PCR-generated inserts into the *Hind*III and *Bam*HI sites of pEGFP-N1. To obtain a drebrin siRNA-pool insensitive mutant, 1-3 silent mutations were introduced (QuikChange Site-Directed Mutagenesis Kit, (Stratagene, La Jolla, CA)) according to the manufacturer's instructions (25 ng template) into all 4 regions complementary to the drebrin siRNAs -01, -02, -04, -17 contained in the siRNA pool (Table 17, No. 23-30). DrebrinΔPP was obtained by generating PCR-based inserts of aa 1-348, introducing *Xho*I/*Eco*RI sites (Table 17, No. 1, 2) and aa 423-649 introducing *Eco*RI/*Bam*HI sites and deleting the stop codon (Table 17, No. 3,4). After ligation of the fragments through their *Eco*RI-sites, the ligation product was subcloned into pGEM-T Easy (Promega, Mannheim, Germany), and subsequently into pEGFP-N1 using *Xho*I/*Bam*HI sites. Drebrin-Polyproline-GFP, drebrin-c-term-GFP and drebrin-CC-GFP domain constructs were subcloned into pEGFP-N1 using the *Hind*III and *Bam*HI sites. GFP-drebrin-mem-like was subcloned into pEGFP-C1 using *Hind*III and *Bam*HI sites. The mito-pCMV-Tag2B vector containing a mitochondrial targeting signal [198] was a kind gift of Britta Qualmann. The mito-drebrin-PP-GFP construct was created through inserting restriction sites for *Nhe*I (Table 17, No. 5, 6) via PCR into the mito-pCMV-Tag2B vector, and the mito-targeting sequence was subcloned into the drebrin-PP-GFP construct. To obtain the mito-GFP construct, the same primers were used to insert restriction sites for *Nhe*I and *Hind*III into the mito-pCMV-Tag2B vector, with subsequent subcloning into pEGFP-N1 (Table 17, No. 7,8). The polyproline domain (aa 313-408) of the WASP protein was cloned into the mito-pCMV-Tag2B vector using the *Bam*HI and *Eco*RI sites. The mito-WASP-PP-GFP construct was created by

cloning PCR-generated inserts of the mito-targeting signal + WASP-PP into the *Nhe*I and *Hind*III sites of pEGFP-N1 (Table 17, No. 9, 10). GST-afadin-PR1-2 was created by cloning a PCR-generated insert (Table 17, No. 11, 12) of afadin (aa residues 1212-1406) into the *Bam*HI and *Eco*RI sites of vector pGEX-2T. MBP-Drebrin-PP was created by cloning a PCR-generated insert (Table 17, No. 13, 14) of drebrinE2 (aa residues 327-411) into the *Bam*HI and *Sal*I sites of vector pMAL-p2X (New England Biolabs, Frankfurt am Main, Germany). plifeact-TagGFP2 was purchased from (Ibidi, Munich, Germany). plifeact-PDZ-TagGFP2 was created by cloning a PCR-generated insert (Table 17, No. 15, 16) of afadin (aa residues 1014-1179) into the *Bam*HI and *Age*I sites of vector plifeact-TagGFP2. A PCR-generated insert of afadin's PDZ (aa residues 1000-1127) was subcloned into the *Xho*I and *Bam*HI sites of pEGFP-C1 (Table 17, No. 17, 18) to create the afadinPDZ-GFP construct. The drebrinCC-afadinPDZ-GFP domain construct was created by replacing the lifeact situated in the plifeact-PDZ-TagGFP2-vector through drebrin's CC-region (aa residues 174-258), subcloning it into the *Xho*I and *Bam*HI sites with a PCR-generated insert (Table 17, No. 19, 20). The first PDZ region of ZO-1 (aa residues 22-107) was subcloned into the *Bam*HI and *Age*I sites of vector plifeact-TagGFP2 through inserting a PCR-generated insert (Table 17, No. 21, 22). Inserts of all constructs were fully sequenced.

2.1.10 Agarose gel electrophoresis

Agarose gels for the analysis of DNA contained 0,5 - 2 % agarose in 1 x TAE buffer. After heating the suspension in a microwave, 0,5 µl SYBR® Safe DNA Gel Stain per up to 200 ml gel was added and poured into agarose gel chambers. DNA samples, mixed with DNA loading dye, were separated on the gel using 80-150 V. DNA was visualized through ultraviolet light; images were taken using ChemiDOCs software. The size of the DNA molecules was determined by comparison to a 1 kb DNA ladder (Figure 12).

2.1.11 Measuring nucleic acids concentration

DNA concentrations were measured using the NanoDrop® ND-1000 spectrophotometer with a detection wavelength of 260 nm. For the measurement, the spectrophotometer was blanked using the reference solution (mostly ddH_20), then 1,1 µl of the sample was applied to the fiber optic cable. Using the ND-1000 V 3.1.0 software, the concentration and the purity of the sample were determined.

2.1.12 DNA sequencing

DNA sequencing was performed by Seqlab sequencing lab (Göttingen, G) and carried out according to their standard protocols. The sequences were analyzed with BLAST offered by NCBI (National Center for Biotechnology Information) website, BioEdit or Ape software.

2.2 Cell biological methods

2.2.1 Isolation and cultivation of HUVEC

All eukaryotic cells were cultured in incubators at 37 °C, 5 % CO_2 and 90 % humidity. HUVEC were prepared from human umbilical cord veins using α-chymotrypsin as adopted from Jaffe[208]. Cells were passaged every 4-6 days by trypsinizing flasks for 5 min, collecting the cells after they detached and centrifuging them at 1020 rpm for 5 min. Then, cells were

divided onto 2-5 new flasks and used until passage 6. HUVEC were always seeded on collagen G (diluted 1:40 in PBS) coated coverslips, flasks, transwells, microslides, live-cell dishes. Assessment of cell morphology was done under phase contrast on an inverted microscope (Nikon TS100).

2.2.2 Application of fluid shear stress

Endothelial cells are *in vivo* constantly exposed to mechanical stress (shear stress), evoked by the flow of blood through blood vessels. Effects of this constant shear stress are the rearrangement of the cell's cytoskeleton, changes in cell metabolism and gene expression compared to HUVEC cultured under static conditions. Physiological shear stress values vary from 0.5 dyne/cm² (small vessels) to 20 dyne/cm² (abdominal aorta). Experimentally, this *in vivo* situation can be mimicked by perfusing medium through microchannels with HUVEC cultured inside them, keeping the flow rate constant over time to examine HUVEC under more physiological conditions.
10^5 HUVEC in 100 µl endothelial cell growth medium were seeded at confluence in collagen-G coated flow-through microchannels (Ibidi µ-Slide I 0.4 Luer) according to the manufacturer´s instructions. 15 dyne/cm^2 of constant shear stress was applied to cells for 72 h using a peristaltic pump system (Ibidi, Munich, Germany). Unidirectional flow was maintained by switching of the valves of the fluidic unit, leading to constant pumping of media through tubes connecting the Ibidi µ-slide to a media reservoir. Morphology and rupturing of monolayers could be observed using an Axioplan (Visitron) upright microscope.

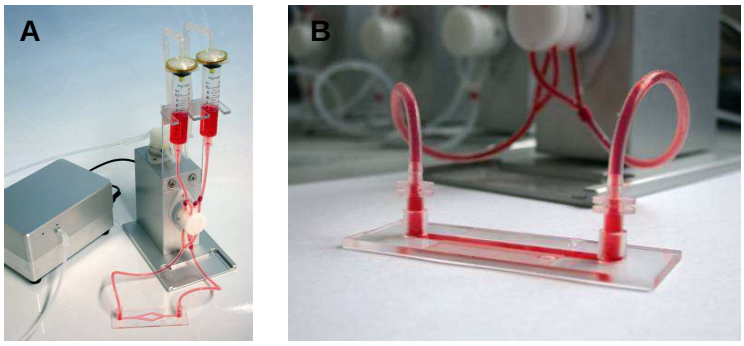


Figure 13 The Ibidi Pump System

A Ibidi Pump, Fluidic Unit, Perfusion Set. Not shown: notebook, PumpControl software

B Ibidi micro-Slide I 0.4 Luer. Here, the Perfusion Set YELLOW-and-GREEN was used.

2.2.3 Transendothelial electrical resistance measurement

For Transendothelial electrical resistance (TER) measurement, HUVEC were plated at confluent density (10^5 cells/transwell) on 6.5 mm diameter (transwell membrane area: 0.3 cm^2), 0.4 µm pore size Transwell Filters (Costar, New York, USA) coated with collagen G and cultured for 72 h in 24-well plates, with media replaced daily. TER was measured using a Millicell®-ERS ohmmeter according to the manufacturer's instructions. To obtain the resistance of the cell monolayer, independent of the size of the transwell membrane, the following calculation was used:

Resistance= (Resistance measured - resistance blank well (Ω)) × Membrane Area (cm^2)

2 mM EGTA was added for 1 h as a positive control leading to junction disruption.

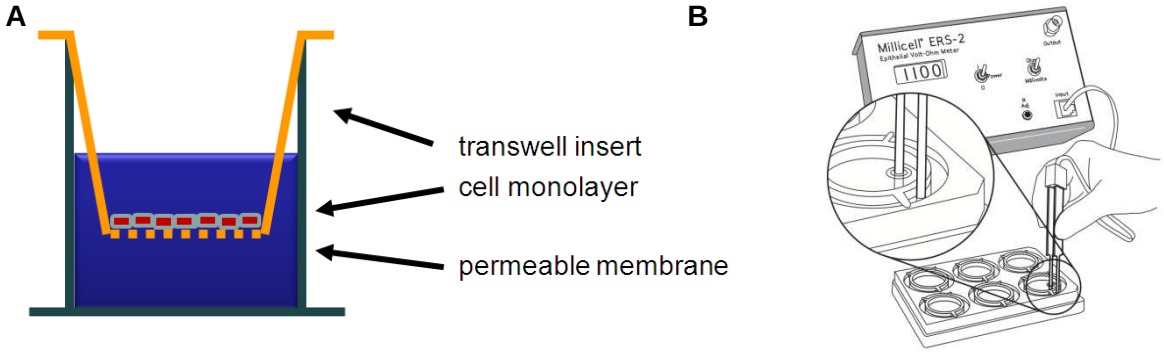


Figure 14 Transendothelial electrical resistance measurement
A Setup of the experiment: HUVEC monolayers are grown on permeable transwell filters.
B Ohm-meter with electrodes, measuring the resistance (from Millipore manual)

2.2.4 Cell-matrix adhesion assay

Cell adhesion assays were performed as described in[209]. Basically, $2x10^4$ control or drebrin knockdown cells were seeded in each collagen-coated 96-well for 20 minutes at 37 ℃. Plates were then shaken for 30 seconds at 400 rpm and cells were fixed afterwards. After washing, remaining cells were stained with crystal violet, and after 20 minutes and exhaustive washing, cells were lysed in SDS and the crystal violet amount was measured. Absorbance was measured at 550 nm with a Tecan infinite M200 plate reader (Tecan Group Ltd., Switzerland), taking the mean of 9 values/well. Cell-matrix adhesion capabilities of HUVEC without shaking the plate were set as 100 %. Cell-matrix-adhesion was also examined by measuring antibody-based fluorescence intensities of integrin-α5. HUVEC monolayers, transfected with drebrin siRNA or control siRNA were fixed and stained with integrin-α5. Fluorescence intensities were measured using Volocity Version 6.0 software (Perkin Elmer, Massachusetts USA).

2.2.5 Inhibition of protein degradation

An inhibitor of lysosomal activity (chloroquine)[210] and a specific inhibitor of the 26S proteasome (lactacystin)[211] were purchased from Sigma (Deisenhofen, Germany). Cells were transfected with drebrin siRNA for 3 days, seeded on coverslips for 16 hours and then treated with chloroquine at a final concentration of 100 µM or lactacystin at a final concentration of 10 µM for 0 h, 5 h and 12 h at 37 ℃.

2.2.6 Cell transfection and siRNA experiments

HUVEC were transfected using microporator technology (Peqlab), according to the manufacturers´ guidelines using 1350 V, 30 ms and 1 pulse. Knockdown of drebrinE2 was performed using siGENOME SMARTpool DBN1 (Dharmacon), or single siRNAs siGENOME DBN1 -01, -02, -04, or -17. Afadin knockdown was performed using siGENOME SMARTpool MLLT4 (Dharmacon). SiRNAs against nectin-2 and-3 were obtained from Santa Cruz. For siRNA sequences see Table 12. Luciferase control siRNA was obtained from Eurofins MWG Operon. For all experiments, HUVEC were cultured for 3 days after siRNA transfection, and re-seeded on collagen-G-coated coverslips after knockdown was established.

2.2.7 Collection of cell lysates

Cells were washed with PBS, scraped off the flask in 500 µl lysis buffer and vortexed thoroughly. After 30 min incubation on ice, lysates were sonicated and cleared by centrifugation at 4 ℃ for 15 min at 10.000 rpm.

2.2.8 Antibodies and immunofluorescence staining.

To visualize proteins in fixed cells using a confocal microscope, cells were stained for immunofluorescence as described by Linder *et al.*[212], with antibodies listed in table 18 and 19. Fixation was done for 10 min in 3,7 % formaldehyde/PBS, permeabilisation in 0,5 % Triton/PBS for 3 min. Coverslips were mounted in Mowiol (Calbiochem), containing p-phenylendiamine (Sigma).

2.2.9 Microscopy and FRAP experiments

Images of fixed samples were acquired with a confocal laser-scanning microscope (Leica DM IRE2 with a Leica TCS SP2 AOBS confocal point scanner) equipped with an oil-immersion HCX PL APO 63× NA 1.4 λblue objective.

FRAP (**F**luorescence **r**ecovery **a**fter **p**hotobleaching) experiments were performed to investigate the mobility of junction-associated proteins in different conditions. Here, a defined area of fluorescent protein is bleached and the recovery of fluorescence in this now darkened zone can be measured. The fluorescence recovery is a result of lateral exchange of bleached proteins against fluorescent protein, which was situated beside the bleached area. Those proteins, which stay in the bleached are – thus preventing a fluorescence recovery of 100 % - are called the immobile fraction, and are one of the valuable information obtained by these experiments. Another important value is the halftime of recovery ($t_{1/2}$), which describes how quickly half of the amount of protein present, when the plateau is reached, is recovered.

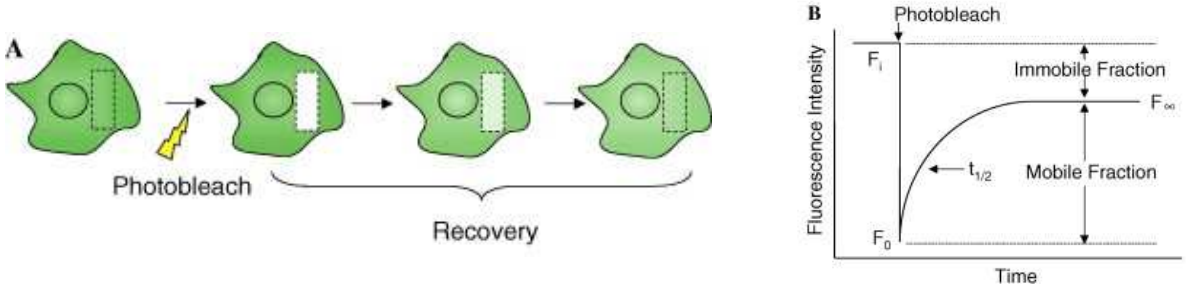


Figure 15 FRAP (Fluorescence Recovery After Photobleaching)
A After bleaching a chosen area, the fluorescence recovery of protein of interest in this zone can be measured.
B From the graphs, characteristics of the mobility of the fluorescent protein can be obtained, for example the halftime of recovery ($t_{1/2}$) or the amount of protein that belongs to the immobile fraction[213].

For FRAP-experiments, ZO-1-GFP or afadin-GFP transfected HUVEC, 3 d previously treated with drebrin siRNA or luciferase siRNA, were seeded on collagen-coated WillCo-dish glass bottom dishes and imaged using an Ultraview spinning disc confocal microscope (PerkinElmer Life Sciences). 5 pre-bleach images were collected every 5 seconds, recovery images at maximum speed for 20 s followed by a lower frequency of 10 pictures /minute, on a Hamamatsu C9100-50 EM-CCD camera (Hamamatsu). A circular ROI (4 µm diameter) of

GFP fluorescence at cell-cell-junctions was bleached using 15 % of a 405 nm laser. Single video frames and FRAP analysis were performed with conventional software (Volocity 6.0, Microsoft Excel 2003 and GraphPad Prism 5). Videos were processed using Ultraview software. Using Excel software, data was corrected for the loss in total fluorescence intensity due to photobleaching. The intensity of the bleached region of interest over time was normalized with the pre-bleach fluorescence intensity. Measurements of fluorescence recovery in the region of interest were quantified, analyzing at least 20 areas from at least 2 sets of experiments. Normalized fluorescence intensities were fitted to a two-phase exponential association using GraphPad Prism 5 Software.

2.3 Biochemical methods

2.3.1 Co-immunoprecipitation

Co-immunoprecipitation of potential, unknown binding partners of a „bait"-protein from whole cell lysates is one of the most common demonstrations of protein-protein interactions, since proteins in the cell extract should be present in their native conformations and complexes[214]
For immunoprecipitation of endogenous drebrin, nectin-2 and afadin, lysates of 5×10^6 confluent HUVEC in lysis buffer I (20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 % Triton X-100, phosphatase- and protease inhibitors) or lysis buffer II (wash buffer 1 from µ-MACS Protein Isolation Kit (Miltenyi Biotec), containing 150 mM NaCl, 1 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris HCl pH 8.0) were incubated with 4 µg of protein-specific antibody and 100 µl of protein A/G coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) overnight at 4℃. Lysates were processed according to the manufacturer´s instructions, washed 4 times with lysis buffer I and eluted with boiling SDS sample buffer. For IgG-controls, species-specific IgG protein (all Abnova, Heidelberg, Germany), was added to lysates and respective A/G beads.
For immunoprecipitation of GFP-fused constructs, lysates of 5×10^6 transfected cells were incubated with 60 µl of beads covalently linked to GFP antibody of µ-MACS GFP Tagged Protein Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and processed according to the manufacturer´s instructions. Lysis buffer II (wash buffer 1 from µ-MACS Protein Isolation Kit (Miltenyi Biotec), containing 150 mM NaCl, 1 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris HCl pH 8.0) was used for all GFP-co-IPs. Note that anti-GFP antibody is covalently linked to beads, while IgG controls are not. IgG heavy and light chains are thus only detected in controls, which is also dependent on cross-reactivity of respective secondary antibodies.

2.3.2 Protein expression

25 ml overnight starter culture of *E. coli* expressing the glutathione S-transferase (GST)- or maltose-binding protein (MBP)- fused proteins of interest were used to inoculate a 1 l main culture that was grown to an OD_{600} of 0.5-0.7. Proteins were expressed after induction with 0.4 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 4 h at 37 ℃ and 180 rpm. Bacterial pellets were collected through centrifugation at 6000 rpm, 15 min, 4 ℃ and resuspended in 5 ml GST-Buffer I or MBP Buffer I, respectively, then kept on ice. After sonification of pellets to disrupt the bacteria, proteins were enriched in the supernatant through centrifugation for 20 min, 15 000 rpm, 4 ℃. 1 ml glutathione- or sepharose resin beads were equilibrated with

GST-buffer I or MBP-buffer I inside polypropylene columns (1 ml), followed by addition of the supernatant containing the recombinant proteins. Proteins were binding to the respective beads while rotating for at least 1 h at 4 ℃. Colu mns were then washed with GST-buffer I / MBP-buffer I for 4 times with subsequent elution of the proteins from the column, using GST elution buffer II, or MBP elution buffer II. Proteins were dialyzed against PBS, shock-frozen, and stored at −80℃. Purity was tested by SDS/PAGE and Coomassie staining.

2.3.3 Determination of protein concentration

The spectroscopic analytical method of Bradford was used to determine concentration of solubilized protein using the BioRad Protein-Assay-Kit. 800 µl of water was mixed with 200 µl of the Bradford reagent and 1 µl of sample added. The absorbance at 595 nm was measured after 5 min against a blank value in a spectrophotometer and analyzed using a protein standard curve based on different concentrations of BSA (1-20 µg/ml).

2.3.4 Pulldown assay

100 µl of wet volume of amylose resin beads equilibrated with GST-buffer I were incubated for 1 h with 20 µg of MBP-drebrinPP fusion protein or MBP in GST-buffer I. Beads were washed 5 times in GST-buffer I and incubated with GST-afadin-PR1-2 (18 µg of protein) for 1 h at 4℃, then washed 5 times in MBP-buffer III. 100 µl boiling SDS sample buffer was added to the beads, and an aliquot was run on SDS-gel of appropriate percentage.

2.3.5 SDS-PAGE

For analysis, protein samples were mixed with 5 x SDS-PAGE loading buffer, boiled for 10 min at 95 ℃ and then subjected to SDS-Page elec trophoresis. Gels were run at 80 V during stacking and 120 V-150 V during separation.

Ingredient	**Stacking gel**	**Resolving gel**		
		7,5%	**10%**	**12%**
ddH_2O	2,6 ml	5 ml	4,2 ml	3,3 ml
Stacking buffer	1,4 ml	--		
Resolving buffer	--	2,5 ml	2,5 ml	2,5 ml
30 % Acrylamide	1 ml	2,5 ml	3,3 ml	4,2 ml
10 % SDS	50 µl	100 µl	100 µl	100 µl
10 % APS	25 µl	50 µl	50 µl	50 µl
TEMED	7 µl	10 µl	10 µl	10 µl

Table 23 SDS-PAGE gel ingredients

2.3.6 Coomassie staining

Proteins present in gels were visualized by staining with Coomassie staining solution for 10 min. Unspecific background staining was removed with destain solution while shaking at least 1 h at RT.

2.3.7 Western Blot

Western blotting was performed using the iblot system (Invitrogen) as described by the manufacturer. Transfer of proteins was performed at 20 V for 6-10 min according to the proteins' size. Subsequently, the membrane was blocked with 5 % fat-free milk powder in TBST for 1 h at RT and incubated with primary antibody in blocking solution overnight at

4 ℃. After washing with TBST, the membrane was incubated with secondary HRP-coupled antibody in TBST for 45 min. After washing, protein bands were visualized by using SuperSignal West Femto or SuperSignal West Pico kit and X-Omat AR film (Kodak).

2.4 Quantifications and statistics

Intensities of junctional proteins were measured using Volocity Version 6.0 software (Perkin Elmer, Massachusetts USA). Circular ROIs of 10 µM diameter were drawn at junctional areas and mean fluorescence intensities were measured. Using Microsoft Excel 2010 (Microsoft Corporation, Redmond USA), intensities were corrected for background fluorescence and statistically analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla USA).

Quantifications of western blots were carried out using ImageJ Version 1.44p software (National Institutes of Health, Maryland USA).

Areas of disrupted monolayers under flow were quantified using Volocity. Complete area of respective images was set as 100 %.

Statistical analyses were performed in GraphPad Prism 5 using unpaired Student's t-test or one-way ANOVA with Bonferroni's post-test.

IV Results

1 The function of drebrin in the endothelium

1.1 Knockdown of drebrin

The F-actin binding protein drebrin is highly expressed in human umbilical vein endothelial cells (HUVEC), where it shows a prominent localization at the cell-cell junctional area. Furthermore, it is present in F-actin rich structures called actin waves, as previously described in our lab, and displays punctuate staining in the cytoplasm, potentially binding to golgi membranes[161,182,215]. In order to explore a functional role of drebrin in the human endothelium, siRNA based gene silencing assays were performed. Therefore, knockdown of drebrin was established using commercially available siRNA directed against sequences that exist in all drebrin isoforms. In humans, drebrin E2 is the isoform found in non-neuronal cells and during embryonic development of the brain (hereafter referred to as drebrin), whereas drebrin A is predominantly expressed in the adult brain[166]. In most cases, three days after siRNA transfection, HUVEC were re-seeded at confluence on collagen-coated glass coverslips for another 24 h, permitting microscopic analyses of the cell monolayers. Using confocal imaging, a loss of drebrin immunofluorescence-based signal could be observed in over 98 % of the cells upon drebrin siRNA treatment (Figure 16A). Concomitantly, western blots of HUVEC cell lysates treated with the same drebrin siRNA showed the disappearance of drebrin signals below detection levels at day three (Figure 16B).
In this study, all drebrin knockdown experiments were performed using a pool of four drebrin-specific siRNAs. Nevertheless, also the single siRNAs were validated to see whether comparable effects can be achieved and it could be shown that a combination of two is sufficient to create a complete knockdown of drebrin.

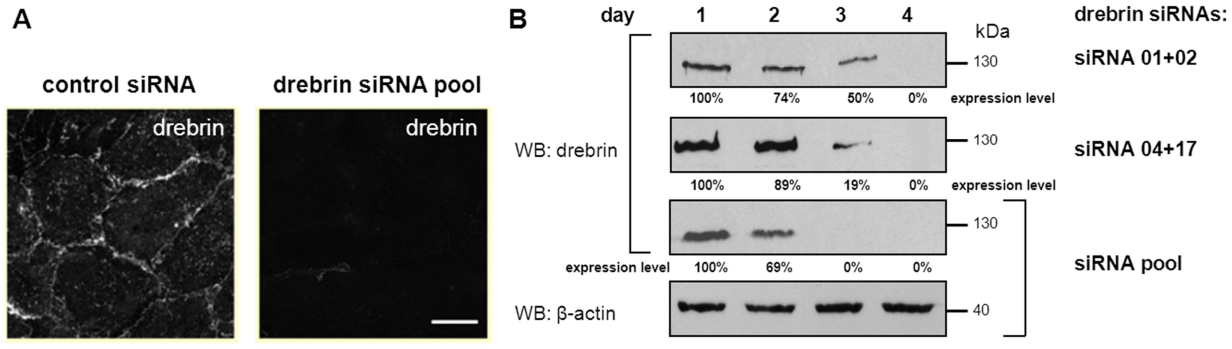


Figure 16 Knockdown of drebrin with different siRNAs
A HUVEC monolayers, treated with control or drebrin siRNA pool for 3 days before seeding them another 24 h on coverslips. Stained with drebrin antibody for confocal imaging. Bar, 10 µm. **B** HUVEC were treated with indicated combinations of drebrin siRNA for 1-4 days and lysed subsequently. Western Blots of lysates treated with drebrin antibody and ß-actin antibody as a loading control. Expression levels were calculated using ImageJ, each drebrin lane referring to actin (loading control for siRNA 01+02 and siRNA 04+17 not shown) with day 1 expression levels set to 100 %.

1.2 TER of drebrin knockdown HUVEC

To investigate a potential functional role of drebrin at endothelial cell-cell junctions, the transendothelial electrical resistance (TER) of control cells and HUVEC depleted of drebrin protein was measured. As the intercellular cleft is sealed through adherens and tight junctions while cells reach confluence, the integrity, and thus the resistance of the monolayer increases. By measuring the transendothelial electrical resistance, slight changes in monolayer integrity of confluent cells grown on transwell filters can be detected. Comparing the resistance of drebrin siRNA treated monolayers and control cells, a pronounced decrease of TER could be observed in knockdown HUVEC, leading to the assumption that drebrin is needed to maintain the functionality of endothelial junctions. As a positive control, monolayers were treated with EGTA that entirely disrupts junction integrity through calcium chelation, which is revealed through a quick decline of TER to approximately 50 % of the control. In this context, it is highly important to verify that the monolayers are intact after performing the experiments, since rupture would lead to pronounced decrease in TER without giving information about the junction functionality. Therefore, HUVEC grown on transwell filters were fixed and stained for immunofluorescence analysis of VE-cadherin and occludin after performing TER measurements (Figure 17).

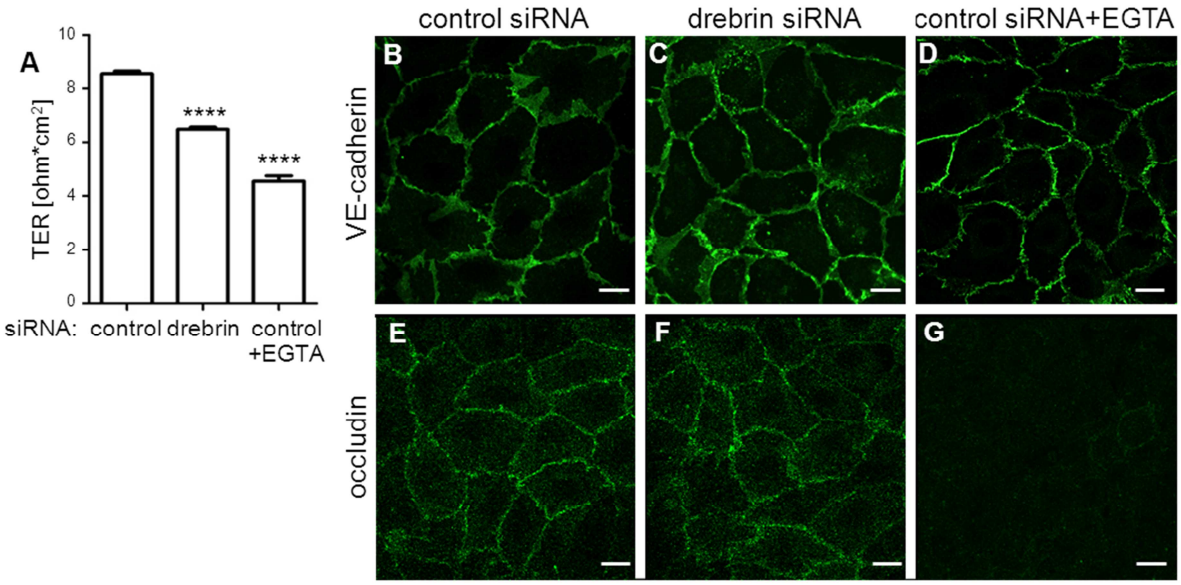


Figure 17 Transendothelial electrical resistance is decreased in drebrin knockdown cells

A TER measurements of monolayers treated with indicated reagents. For each value, 3x10 monolayers were evaluated. Values were analyzed using one way ANOVA with Bonferroni's post-test and are depicted as means ± SEM of n= 3 ****$P < .0001$. **B-G** Confocal images of monolayers grown on transwell filters, treated with indicated reagents and stained for VE-cadherin and occludin to check if monolayers are intact. Note complete loss of occludin signal after EGTA treatment. Bars, 10 µm.

1.3 Drebrin knockdown HUVEC cultured under constant unidirectional flow

It is documented that endothelial cells, such as HUVEC, grown under static *in vitro* conditions are not necessarily representative of *in vivo* endothelial cells aligning blood vessels[216]. In order to investigate endothelial cells under conditions mimicking blood flow in the body,

HUVEC were cultured in microslides connected to a pump system, which creates a constant unidirectional flow. This exposed them to shear stress comparable to the situation in blood vessels, potentially revealing phenotypes of knockdown cells that are not obvious, when cultured under static conditions. Here, HUVEC treated with drebrin- or control siRNA were cultivated under a constant flow of 15 dyne/cm^2 for three days, mimicking average flow conditions in medium sized vessels[217]. The cells developed a spindle-shaped morphology while aligning in the direction of the medium flow, which is an organisation that occurs in potentially athero-protective situations[218]. No differences could be detected between control and drebrin knockdown monolayers at day one, but strikingly, drebrin knockdown monolayers started to display numerous ruptures after three days (Figure 18). Remarkably, this phenotype could only be observed in monolayers cultured under flow conditions, pointing to a role of drebrin in cell-cell adhesion of endothelial cells subjected to shear stress.

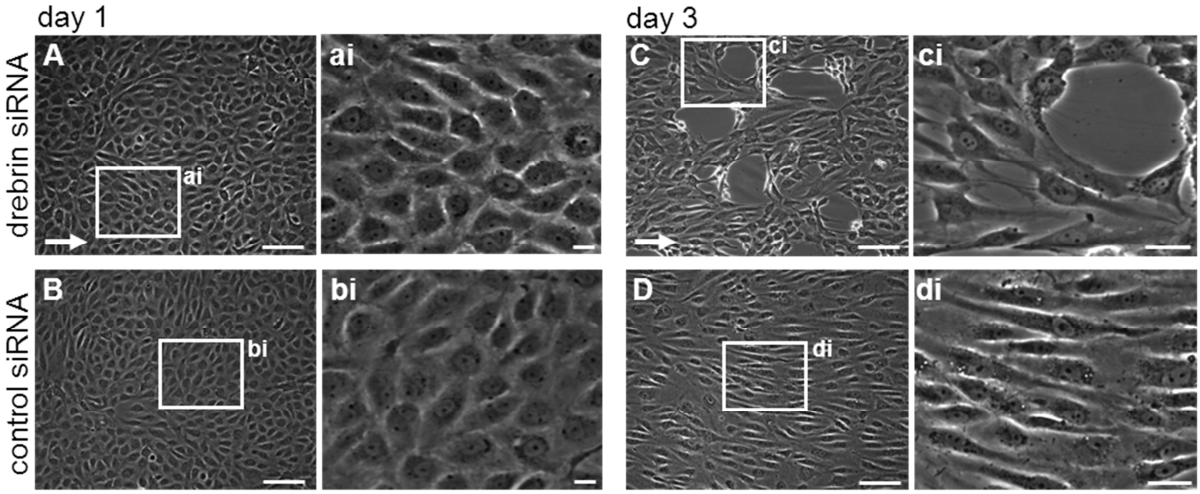


Figure 18 Drebrin knockdown monolayers show ruptures while cultured under constant flow
Images of HUVEC monolayers treated with drebrin-specific (A,C) or control siRNA (B,D) and seeded in microslides. Cells were submitted to constant fluid shear stress for 1 - 3 days. The enlarged images (ai-di) show boxed regions of (A-D). Arrows indicate the direction of flow. Note the rupture of drebrin knockdown monolayers at day 3. Bars, 10 µm in (ai-di), 100 µm in (A-D).

1.4 Cell-matrix adhesion of drebrin knockdown HUVEC

The numerous ruptures observed throughout the monolayers of drebrin knockdown HUVEC brings up the question whether they are due to defects in cell-cell adhesion, or in cell-matrix adhesion. First, cell-matrix adhesion was examined in control versus drebrin knockdown HUVEC. An adhesion assay was performed, where the relative adhesion capabilities of control and knockdown cells to their supporting matrix were compared[209]. Additionally, a typical protein involved in matrix adhesion, integrin-α5, was immunostained in knockdown and control monolayers and alterations in the fluorescence based intensities were evaluated (Figure 19). In both experiments, no differences were observed between control and drebrin knockdown cells, indicating an intact cell-matrix adhesion even without drebrin. Thus, it seemed possible that it is rather the cell-cell adhesion than the cell-matrix adhesion of HUVEC that is altered upon loss of drebrin.

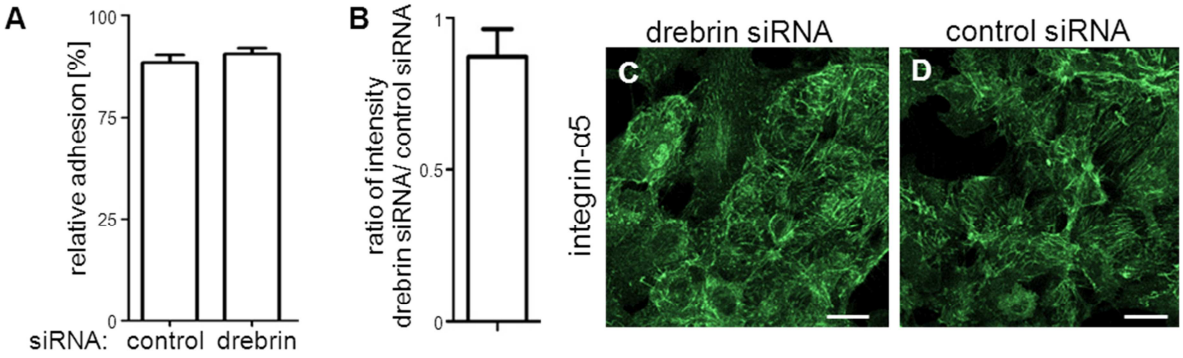


Figure 19 Cell-matrix adhesion is not altered in drebrin knockdown cells
A Relative adhesion of drebrin knockdown cells to collagen coated substratum. 100 % refers to cell-matrix adhesion capabilities of HUVEC before shaking the plate (method: see chapter III.2.2.4) **B** Measurements of antibody-based fluorescence intensities of integrin-α5 in drebrin knockdown vs. control cells (0.87 ± 0.06) with respective immunofluorescence images of drebrin knockdown (C) and control cells (D). Bars, 10 µm.

2 Drebrin knockdown affects the localization of nectin at junctions

2.1 Localization of junctional proteins under drebrin knockdown

To further investigate the idea that alterations in cell-cell adhesion might occur upon drebrin depletion, an immunofluorescence-based screening of the localization of typical endothelial intercellular junction proteins was performed. HUVEC were seeded on coverslips three days after siRNA transfection, when drebrin knockdown was already established. After 24 hours, coverslips were fixed and stained for drebrin, F-actin and different junctional proteins to examine their distribution on a subcellular level using a confocal microscope. Simultaneously, to detect changes in the total protein levels of the respective proteins upon drebrin knockdown, HUVEC were lysed on day one, two, three and four after transfection and western blots were performed. Considering all types of adhesion systems, a protein member of each typical endothelial junctional complex was chosen – connexin-43 as a gap junction protein, VE-cadherin and nectin-2 as members of the adherens junction complex, occludin, a tight junction protein and PECAM-1, since it is a typical endothelial protein involved in adherence without being part of a specific cell-cell junction system.
VE-cadherin, occludin and PECAM-1 showed no significant alterations in their localization to the junctional area, although the overall protein expression levels of VE-cadherin and occludin were slightly diminished at day four of drebrin knockdown (Figure 20 F). Similarly, connexin-43 shows its typical punctuate staining along the junctional area in control and drebrin knockdown cells (Figure 20 A,G). However, contradictory findings were reported by Butkevitch *et al*., claiming that the functional state of connexin-43 based gap junctions is dependent on drebrin and that connexin-43 is degraded upon drebrin knockdown in astrocytes and Vero cells (see V.1)[192].
A very significant difference compared to control cells could be observed regarding the distribution of the adherens junction component nectin-2 – there was no signal detectable at the junctional area after four days of drebrin depletion. Concurrently, a diminished protein

expression level of only 5 %, compared to the nectin-2 expression levels at day zero of drebrin knockdown, was observed (Figure 20 C,F).
After these experiments were performed using a pool of four drebrin siRNAs, we checked whether only two siRNAs are sufficient to result in the same phenotype. Therefore, all possible combinations of each time two of the four siRNAs were transfected into HUVEC and expression of the above mentioned proteins was examined. The results achieved with the pool of four siRNAs were reproducible in all cases, displaying loss of nectin-2 from the junctional area (data not shown).
As a verification of the drebrin knockdown phenotype in direct comparison to HUVEC transfected with control siRNA, cells were seeded from two sides on the same collagen-coated coverslip and thus subjected to exactly identical experimental conditions. A clear distinction of the drebrin knockdown from control cells, with the cells depleted of drebrin showing diminished nectin-2 distribution to junctional areas could be observed (Figure 21).

Taken together, a depletion of drebrin through transfection of HUVEC with siRNA leads to a loss of nectin-2 from the junctional area while the distribution other typical cell-cell junction components shows no alterations. Simultaneously, nectin-2 levels were greatly diminished in cell lysates of drebrin knockdown HUVEC at day four. Since the other junctional proteins are mostly unaffected by drebrin depletion, the functional alterations observed in drebrin knockdown cells are likely to be due to decrease of nectin-2 at endothelial cell-cell junctions.

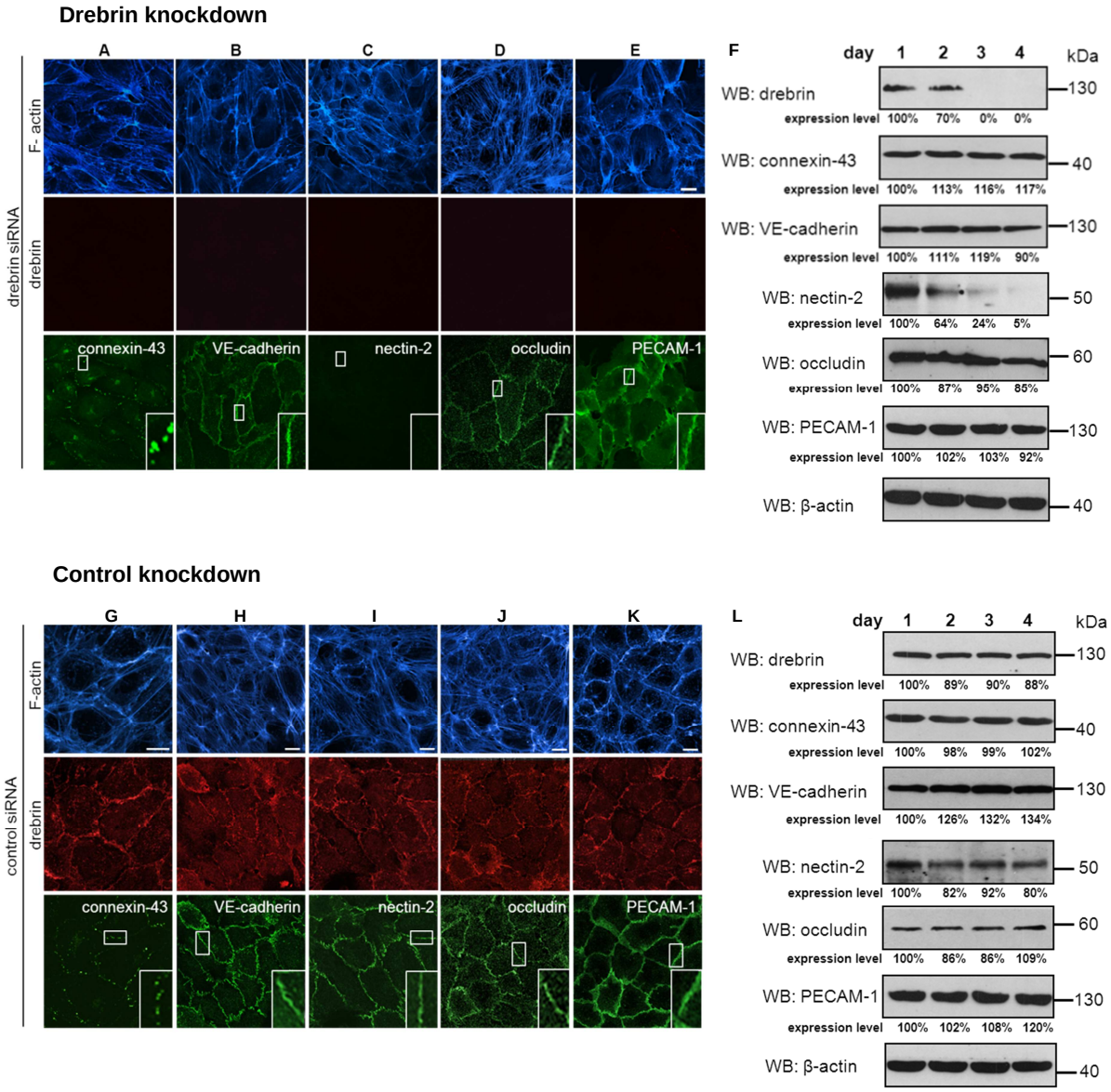


Figure 20 Localization of junctional proteins in drebrin knockdown cells

A-E, G-K Confocal images of HUVEC transfected with a pool of 4 drebrin-specific siRNAs or control siRNA, respectively. Monolayers were stained for F-actin (upper rows), drebrin (middle rows), and different junctional proteins (lower rows). The insets show enlarged views of boxed areas. Note specific loss of nectin-2 from cell-cell junctions in drebrin knockdown cells (C). **F,L** Western blot analysis of lysates from HUVEC transfected with drebrin-specific- or luciferase control siRNA. Proteins were detected with antibodies specific for junctional proteins or β-actin. Numbers above blots indicate days post transfection, relative expression levels (standardized for β-actin) are referring to day 1. Bars, 10 µm.

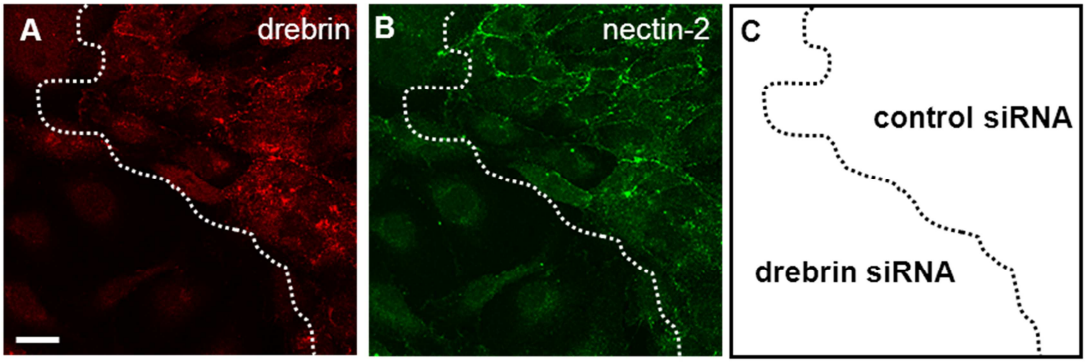


Figure 21 Direct comparison of drebrin knockdown and control cells
Confocal micrographs of HUVEC transfected with a pool of 4 drebrin-specific siRNAs or control siRNA for 3 days, which were seeded simultaneously on coverslips from two different sides. After 24 h, coverslips were fixed and stained for drebrin and nectin-2. The dashed line outlines the border separating the different knockdown pools. Note absence of nectin-2 signal in drebrin knockdown cells. Bar, 10 µm.

In addition to nectin-2, nectin-3 is also known to be expressed in endothelial cells, potentially forming *cis*- or *trans*-hetero-dimers[89]. Comparable to nectin-2, immunofluorescence signals of nectin-3 were also greatly diminished at cell-cell junctions upon drebrin depletion (Figure 22B), pointing to a general effect of drebrin on endothelial nectin isoforms. However, in the following study, we concentrate on nectin-2.

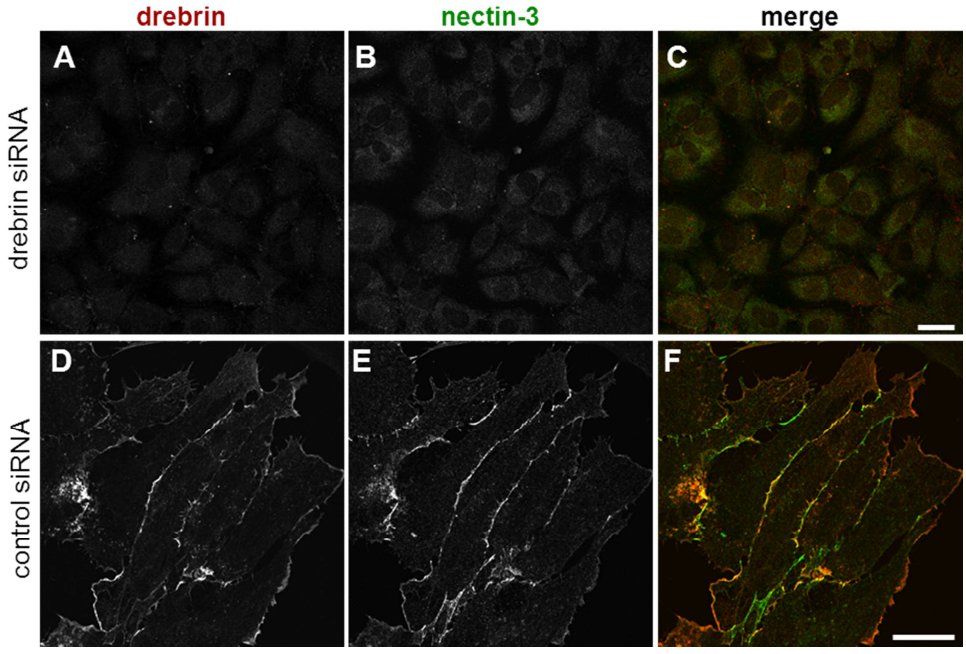


Figure 22 SiRNA-induced knockdown of drebrin leads to reduction of nectin-3 at cell-cell contacts
Confocal micrographs of HUVEC transfected with drebrin-specific siRNA pool (A-C) or control siRNA (D-F) and stained at day four post-transfection for drebrin (A,D) and nectin-3 (B,E), with merged images. Bars, 10 µm.

2.2 Rescue of nectin-2 through overexpression of drebrin

The drebrin depletion experiments, which led to functional impairments of HUVEC monolayers due to loss of nectin-2 at cell-cell junctions, can be further validated by performing “rescue” experiments where drebrin protein is re-expressed from a transiently transfected vector. To prevent the degradation of the vector-encoded mRNA by the drebrin siRNA, silent mutations were introduced into the vector, corresponding to the target regions of each of the four drebrin siRNAs. Consequently, this transcribed mRNA was insensitive to drebrin siRNA silencing[219]. As a validation that loss of nectin-2 really is a specific result of drebrin depletion, the exogenously re-expressed drebrin protein should be able to rescue the knockdown phenotype.

HUVEC were silenced for drebrin via transfection of the siRNA pool for three days. After knockdown was established, cells were transfected with either GFP or siRNA insensitive drebrin-GFP and grown to confluence on coverslips for one day. Cells were stained for nectin-2 and fluorescence intensities of junctional nectin were measured in luciferase siRNA transfected control cells, GFP- and siRNA-insensitive drebrin-GFP-transfected knockdown HUVEC (Figure 23). Since the siRNA insensitive drebrin-GFP was able to rescue nectin-2 protein at junctions, reaching levels comparable to control cells, it can be postulated that drebrin’s absence is indeed causative for nectin’s disappearance at junctions.

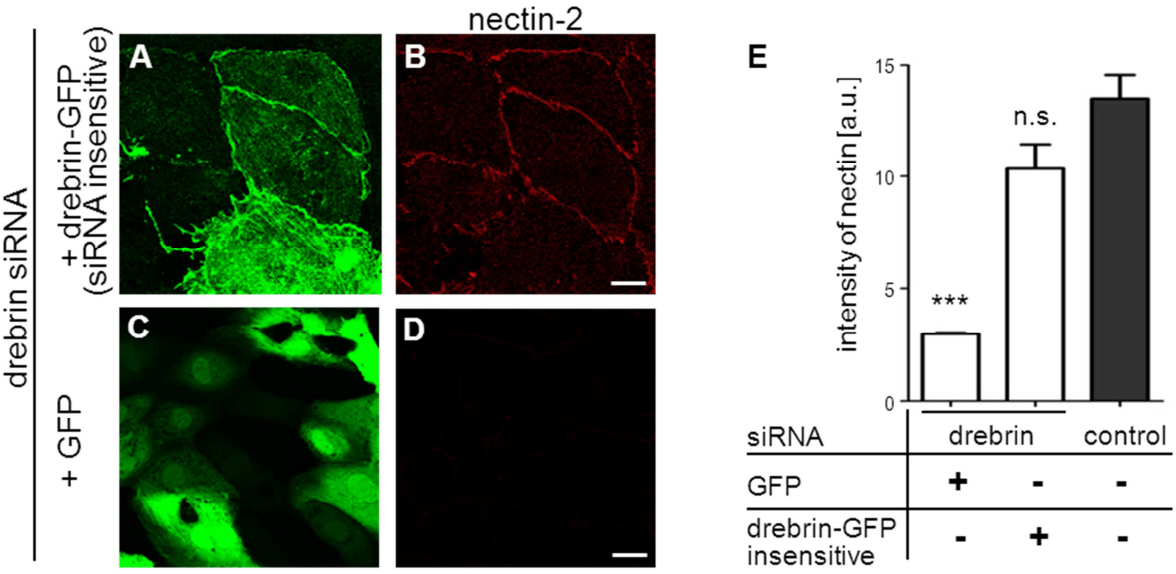


Figure 23 Junctional localization of nectin-2 is rescued by expression of siRNA-insensitive drebrin-GFP
A-D Confocal images of HUVEC cultured for 3 days after drebrin siRNA transfection, then cotransfected with siRNA insensitive drebrin-GFP (A) or GFP (C). Bars, 10 µm. **E** Intensities of nectin-2 (100 junctional areas from 3 experiments) were measured between two drebrin-GFP or GFP expressing cells. Values were analyzed (compared to nectin-2 intensities between luciferase siRNA transfected control cells, picture not shown) using one way ANOVA with Bonferroni’s post-test and are depicted as means ± SEM of n= 3.*** $P < .001$. n.s., not significant.

2.3 Nectin-2 is endocytosed and degraded in lysosomes

The diminished nectin-2 levels upon drebrin knockdown raised the question whether this reflects increased endocytosis and subsequent degradation of the protein or a shutdown in nectin-2 gene expression characterized through reduced mRNA levels.
With the aim to elucidate, if drebrin functions as a regulator of nectin-2 expression, we checked the primary control point for gene expression – the transcription. To find out whether nectin-2 mRNA is produced in the absence of drebrin, total RNA was isolated from drebrin knockdown and control HUVEC and reversely transcribed into cDNA, which will only contain sequences of expressed genes. The obtained cDNA was used as a template for PCR using nectin-2 specific primers situated in two different exons. This was done to make sure that the template is certainly cDNA, lacking introns and thus giving rise to a small PCR product, as opposed to contaminations of genomic DNA, which would result in a larger product. In both cases, even in cells depleted of drebrin protein, a respective PCR product could be visualized on agarose gels leading to the conclusion that nectin-2 mRNA was transcribed (Figure 24A).

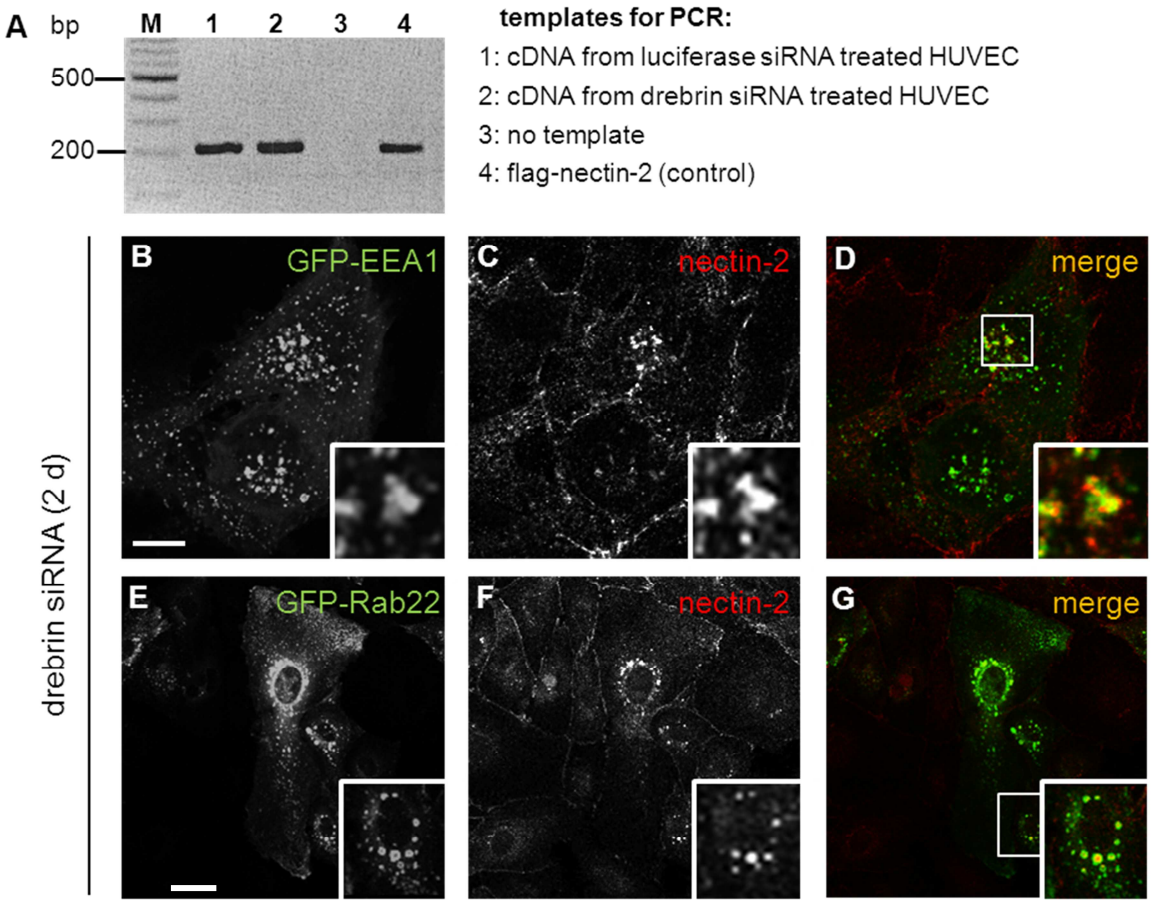


Figure 24 Nectin-2 shows pronounced endocytosis upon drebrin depletion
A Agarose gel stained with SYBR Safe, showing PCR products obtained with nectin-2 specific intron-spanning primers, with templates as indicated. Genomic DNA would give a product of 500 bp. As a positive control, flag-nectin-2 was used as a template, containing no introns. Size indicated in base pairs. Note comparable amplification of PCR product from cDNA of drebrin knockdown cells (siRNA pool) (2) and luciferase siRNA-treated controls (1). **B-G,** Confocal micrographs of HUVEC treated with drebrin-specific siRNA (pool) for 2 days, overexpressing GFP-EEA1 (B) or GFP-Rab22 (E) and stained for endogenous nectin-2 (C,F). Bars, 10 µm.

Many situations are known, where depletion of a junction-related protein (for example catenins, p120-catenin) can lead to an immediate endocytosis of the respective junctional component, in that case VE-cadherin[68]. Since there is no effect of drebrin on nectin-2 gene expression, nectin-2 might be internalized and degraded upon drebrin knockdown. With the aim to examine whether nectin-2 undergoes endocytosis, HUVEC were treated with drebrin siRNA for only two days, in order to have more nectin-2 protein left than after the usual four days of drebrin knockdown. Cells were then co-transfected with GFP-EEA1 and GFP-Rab22, both markers of early endosomes, which are the first compartments proteins enter after being internalized from the cell-surface[220,221]. A clear colocalization of nectin-2 and the early endosome compartments could be observed (Figure 24B-G), emphasizing the idea that nectin-2 is endocytosed in cells depleted of drebrin.

Surface proteins entering the endocytic pathway are first collected in early endosomes. From here, proteins are either recycled back to the membrane or sorted into late endosomes for subsequent degradation. Since there is nothing known about degradation pathways of nectin-2 in general, we wanted to find out whether it undergoes proteasomal or lysosomal degradation. Lysosomes are acidic organelles, where plasma membrane proteins and receptors are typically degraded after they were endocytosed, whereas proteasomal degradation is mostly applied to cytoplasmic proteins[222]. Lysosomal activity in drebrin knockdown cells was inhibited through chloroquine, and cells were fixed after 0 h, 5 h and 12 h and stained for nectin-2 and LAMP-1 (**L**ysosomal-**A**ssociated **M**embrane **P**rotein-1), a marker of lysosomes. Chloroquine treatment led to the formation of large dilated lysosomes as reported earlier in human ARPE-19 epithelial cells[223]. Before the inhibition (0 h) no significant signals of nectin-2 were detected at LAMP-1 positive vesicles, but increased accumulation was observed after 5 h, propagating into a complete colocalization of nectin-2 and LAMP-1 after 12 h (Figure 25). Proteasomal inhibition through lactacystin did not lead to an accumulation of nectin-2 (data not shown).

Collectively, these results demonstrate that nectin-2 gene expression is not altered upon drebrin knockdown – its disappearance is rather due to endocytosis from the cell surface and subsequent lysosomal degradation.

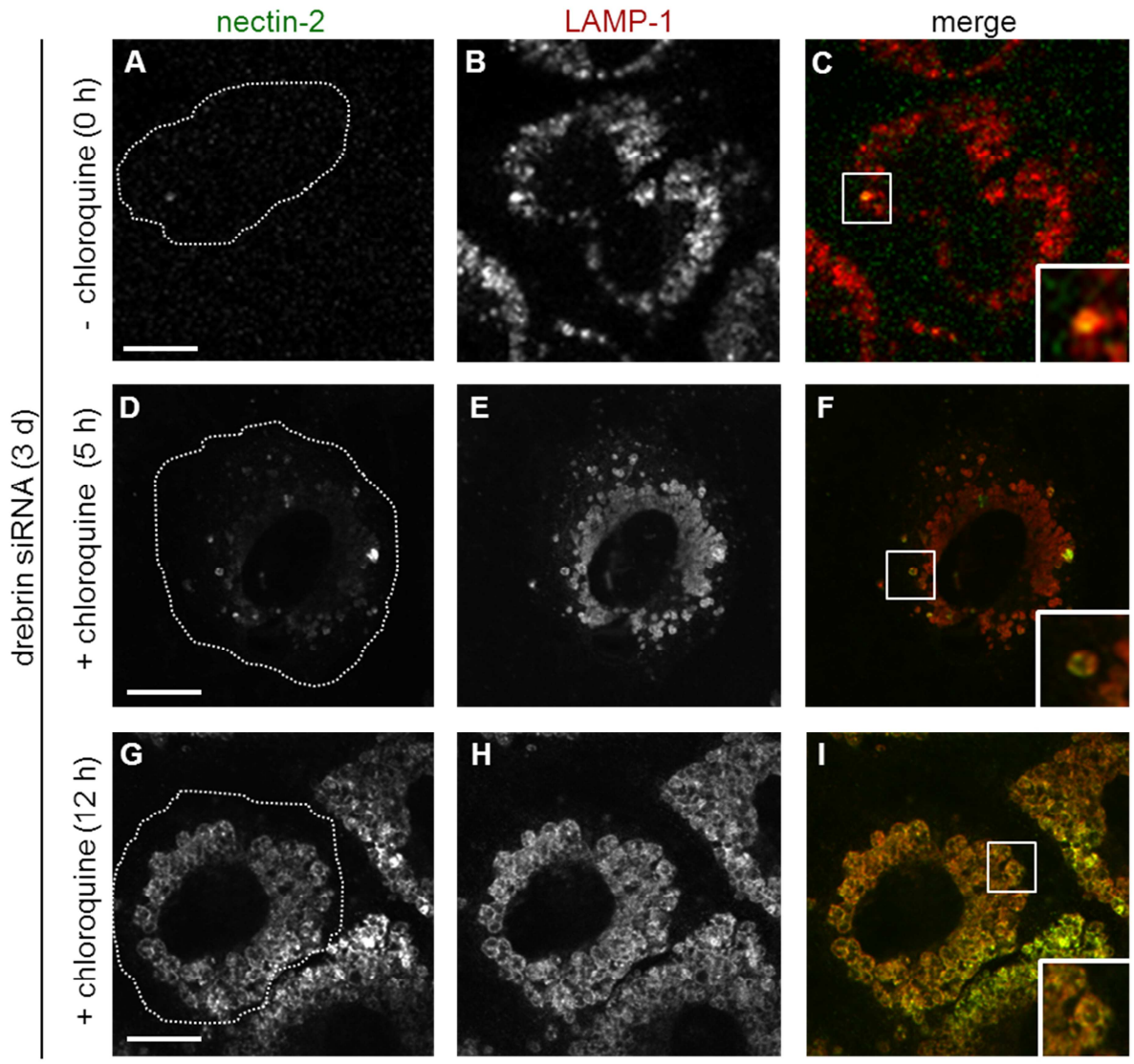


Figure 25 Nectin-2 is degraded in lysosomes
Confocal images of HUVEC treated with drebrin-specific siRNA (pool) for 3 days, stained for nectin-2 and lysosomal marker LAMP-1, with merges. Cells were treated for 5 h and 12 h with 100 µM lysosome inhibitor chloroquine, resulting in dilation of LAMP-1 positive compartments that acquire nectin-2. White boxes indicate areas of detail images shown in insets. Outlines of individual cells indicated by dashed white lines. Bars, 10 µm.

3 Effects of drebrin knockdown on afadin

Nectins have a well described cytosolic binding partner, afadin. They interact through nectin's COOH-terminal motif of four amino acid residues (E/A-X-Y-V) that confer binding to the PDZ domain of afadin[94]. Since nectin-2 disappears upon drebrin depletion, we wondered what happens to its partner afadin under the same conditions.

3.1 Effects on afadin's subcellular localization

To start with, HUVEC depleted for drebrin were examined under static conditions by immunofluorescence staining of drebrin and afadin and subsequent confocal imaging. Surprisingly, while drebrin expression levels were beyond detection, the fluorescence intensity of afadin at the junctional area was only slightly reduced. Concomitantly, expression levels of afadin in cell lysates of drebrin knockdown cells were marginally reduced to 76 % compared to day one (Figure 26). Thus, it seemed apparent that afadin is able to maintain its junctional localization, although slightly reduced, even without its intercellular junctional binding partner nectin.

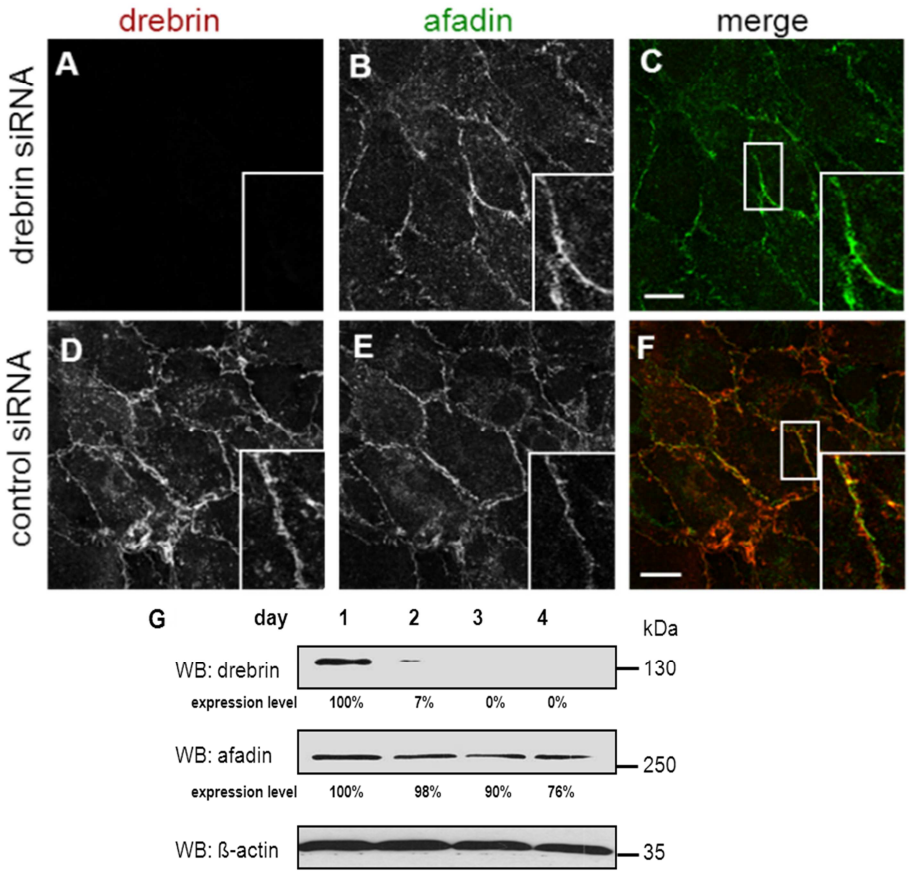


Figure 26 Afadin's subcellular localization is unaltered upon drebrin knockdown
A-F Confocal images of HUVEC treated with drebrin siRNA (A-C), or control siRNA (D-F), stained for drebrin (A,D) and afadin (B,E). Insets show an enlarged view of boxed regions. Bars, 10 µm. **G** Western blots of lysates from HUVEC treated with drebrin-specific siRNA, developed with indicated antibodies. Numbers above blots denote days after transfection. Relative expression levels (standardized for β-actin) are referring to day 1.

3.2 Colocalization of afadin with ZO-1 and α-catenin

Afadin contains many functional domains (Figure 6), allowing its interaction with a variety of proteins. Some of afadin's binding partners are also present at other cell-cell adhesion systems, for example α-catenin that is mainly localized at cadherin based junctions, and ZO-1, a tight junction associated protein. JAM-A, another transmembrane protein at tight junctions is also able to interact with afadin[224]. This fact led to the assumption that even when nectins are not present to serve as afadin's main binding partner, it might still be present at the junctional area through its interaction with α-catenin, ZO-1, or JAM-A. Immunofluorescence-based colocalization studies were performed with a confocal microscope, resulting in the observation that afadin localizes together with ZO-1 and α-catenin at endothelial cell-cell junctions.

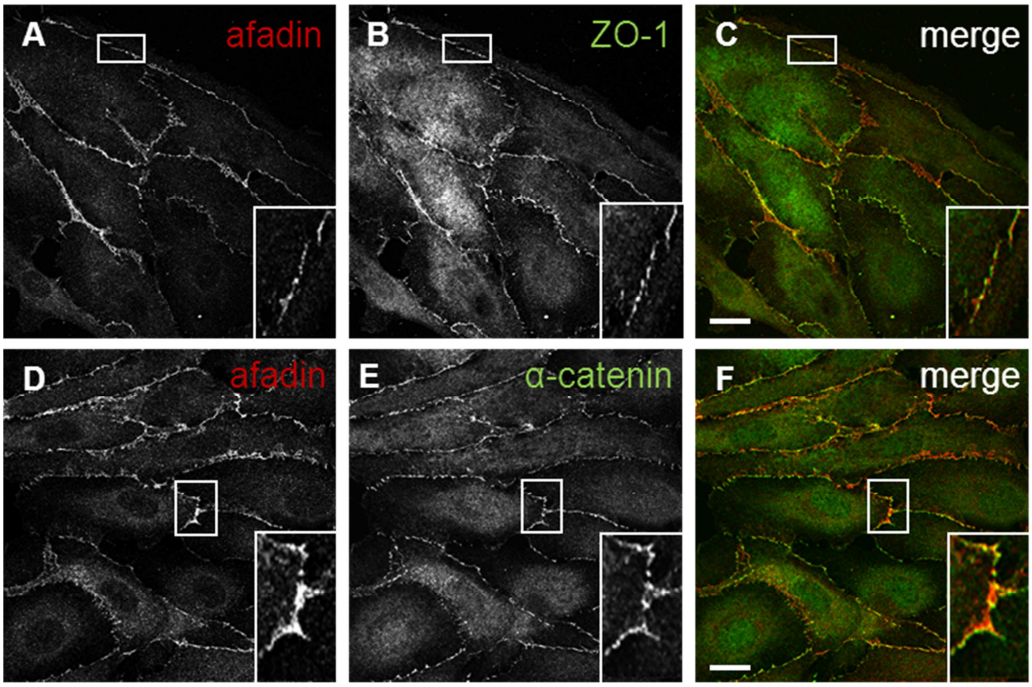


Figure 27 Afadin colocalizes with ZO-1 and α-catenin at endothelial junctions
A-F Confocal micrographs of HUVEC stained for afadin (A,D) and ZO-1 (B) or α-catenin (E), with merged images. Afadin colocalizes with α-catenin and ZO-1 at endothelial cell-cell junctions. White boxes indicate enlarged areas shown as insets. Bars, 10 µm.

3.3 FRAP of afadin-GFP

Even though afadin still localizes to endothelial junctions in drebrin knockdown cells, which lack nectins, probably because it is bound to several junction-associated proteins, other methods might unravel more subtle effects that drebrin knockdown could have on afadin. Taking into account that nectins are directly involved in intercellular adhesion, but other afadin binding partners like ZO-1 and catenins are only junction-associated proteins, afadin might have a different mobility when only bound to junction-related proteins.

To investigate the mobility of proteins, a well-established, microscopy-based method is FRAP, where the lateral diffusion of a fluorescent protein into a previously bleached area is measured (explained in III.2.2.9). Here, drebrin knockdown and control cells were transfected with afadin-GFP and the protein was bleached in defined junctional areas between two transfected cells. As a control, a protein, which was not affected by drebrin depletion (ZO-1-GFP), was bleached in both siRNA treatments, too.
The fluorescent intensities of the bleached areas were measured, corrected for the overall bleaching of the cells and normalized for pre-bleach intensities. The average recovery of afadin-GFP in over 20 measured regions shows that the mobile fraction of protein is elevated, when drebrin is depleted in the cells (Figure 28 A). Concomitantly, afadin's halftime of recovery is reduced in drebrin knockdown cells, whereas ZO-1-GFP shows no difference in drebrin knockdown and control cells (Figure 28B,D). Taken together, this means that drebrin depletion does not only have effects on nectins, but also on afadin, which becomes more mobile at the junctional area.

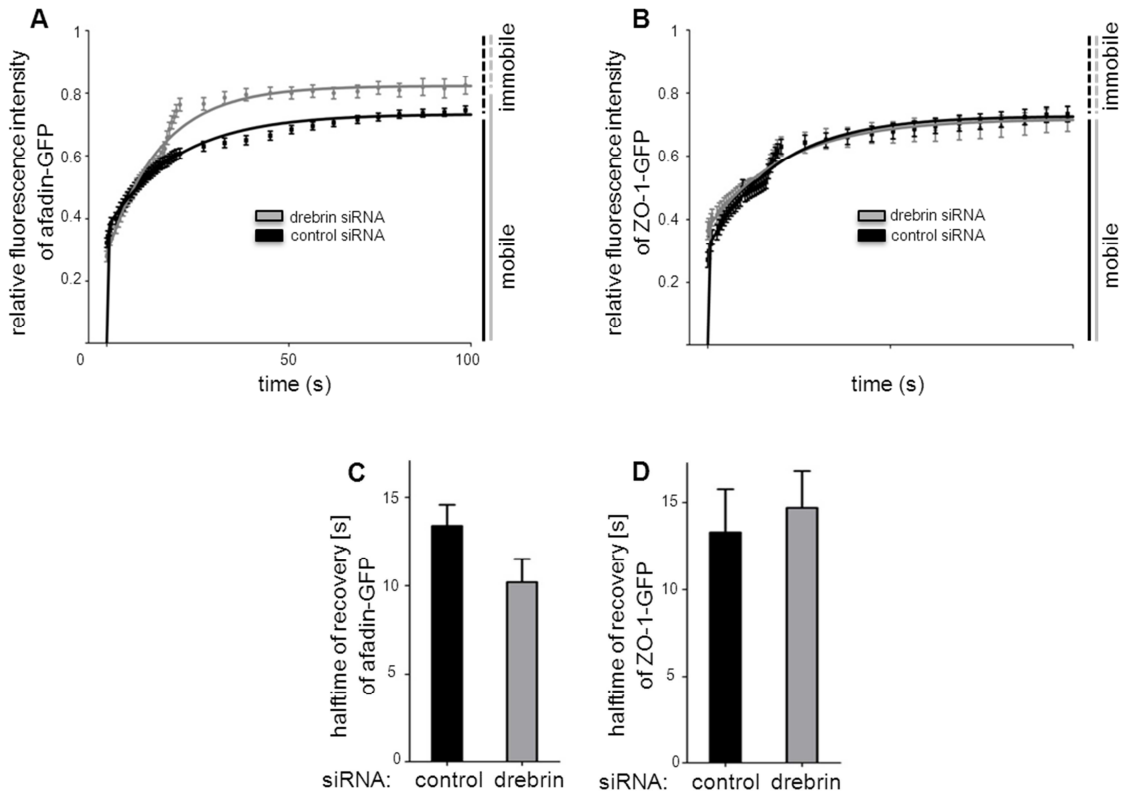


Figure 28 FRAP of afadin under drebrin knockdown
A, B FRAP measurements of afadin-GFP or ZO-1-GFP in drebrin knockdown and control knockdown cells. Means of at least 20 recoveries are shown, with mean of pre-bleach intensities set to 1.0. Experiments were analyzed using two-phase-exponential fitting, with respective graphs showing average curves ± SEM. Note that the mobile fraction (solid line) of afadin-GFP (A), but not of ZO-1-GFP (B), is increased upon drebrin knockdown. **C,D** Halftime recovery of afadin-GFP (C), but not of ZO-1-GFP (D) is decreased upon drebrin knockdown. Graphs show mean halftime ± SEM. Statistical analyses were performed using Student's t-test.

4 Knockdown of nectin and afadin leads to a similar phenotype as drebrin depletion

4.1 TER of nectin and afadin knockdown monolayers

Drebrin knockdown leads to loss of nectins from endothelial junctions, which results in functional impairment of cell-cell adhesion. We wanted to know next whether a direct knockdown of those nectin isoforms, which we found to be expressed in the endothelium (nectin-2 and -3 (Figure 22)), would cause similar limitations in adhesion of HUVEC. To prevent functional redundancy due to the expression of nectin-2 and -3 in the endothelium, an efficient knockdown of > 80 % regarding both nectin isoforms was established (Figure 29 B,C). These nectin knockdown cells were cultivated on transwells and the method of measuring the transendothelial electrical resistance was applied to detect minor changes of monolayer functionality. When nectins were absent, the TER of intact monolayers was decreased about 30 % to levels remarkably similar to drebrin knockdown cells (Figure 29D).

Since afadin's mobility at junctions is affected in HUVEC depleted of drebrin protein, and afadin has been shown to be important for establishing functional monolayers, we were also interested in the effects that afadin knockdown would possibly have on HUVEC monolayer integrity. It has already been published that afadin's binding to nectin is necessary for their clustering at cell–cell adhesion sites[225]. Also, epithelial cells of afadin knockout mice not only show alterations in nectin-based adhesion but even display impaired E-cadherin–based adherens junctions assembly, underlining afadin's key role in the proper organization of cell-cell junctions[106].

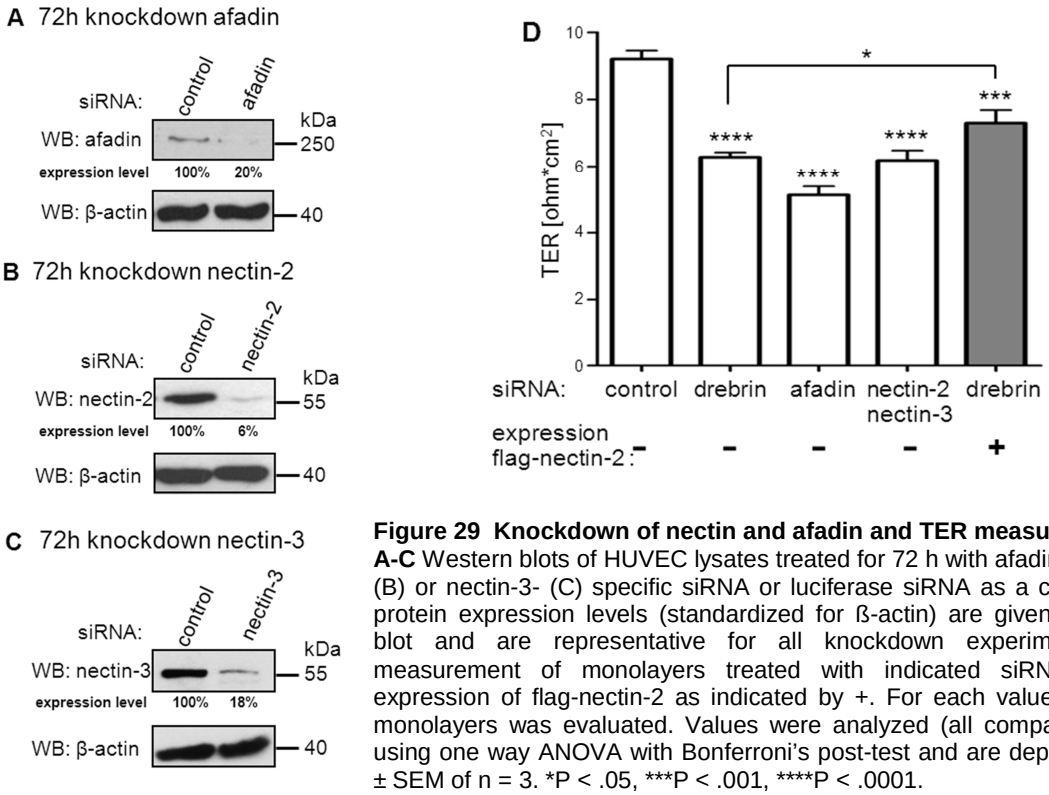


Figure 29 Knockdown of nectin and afadin and TER measurements
A-C Western blots of HUVEC lysates treated for 72 h with afadin- (A), nectin-2- (B) or nectin-3- (C) specific siRNA or luciferase siRNA as a control. Relative protein expression levels (standardized for ß-actin) are given beneath each blot and are representative for all knockdown experiments. **D** TER measurement of monolayers treated with indicated siRNAs. Additional expression of flag-nectin-2 as indicated by +. For each value, TER of 3x10 monolayers was evaluated. Values were analyzed (all compared to control) using one way ANOVA with Bonferroni's post-test and are depicted as means ± SEM of n = 3. *P < .05, ***P < .001, ****P < .0001.

To examine afadin's functions concerning junction functionality in HUVEC, we established an siRNA based knockdown of afadin, reducing its levels to 20 % compared to day one. Subsequently, HUVEC were cultured on transwells and TER measurements were performed in afadin knockdown and control monolayers. A significant decrease of monolayer resistance to about 50 % was observed, comparable to the resistance of HUVEC monolayers depleted for endothelial nectins or drebrin (Figure 29).

4.2 Nectin and afadin knockdown HUVEC cultured under constant unidirectional flow

The drebrin knockdown phenotype became most obvious when HUVEC were cultured under conditions mimicking blood flow in the body. Consequently, this experiment was also performed with afadin or nectin-2/ nectin-3 knockdown cells, respectively.

When afadin was depleted in the cells, a rupture of the monolayers could be observed, resulting in a cell-free area comparable to drebrin knockdown cells. Concomitantly, in cells depleted of both endothelial nectin isoforms, cultured under constant flow conditions, the formation of holes throughout the monolayer could be observed even in greater extent than in drebrin knockdown HUVEC (Figure 30A,B,I).

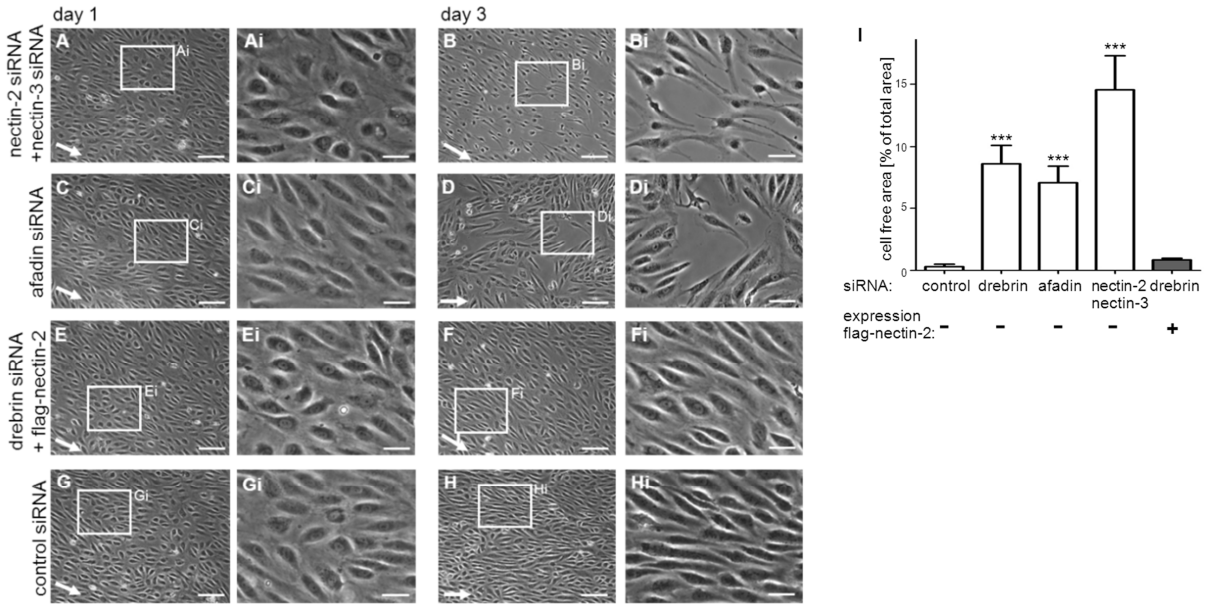


Figure 30 Depletion of afadin or nectin leads to rupture of monolayers under flow
A-H Images of HUVEC monolayers grown in microslides, treated with indicated siRNAs, in E,F with simultaneous overexpression of flag-nectin-2. Cells were submitted to constant fluid shear stress for 1 day (A,C,E,G) or 3 days (B,D,F,H). The enlarged images (Ai-Hi) show boxed regions of (A-H). Note compromised integrity of the monolayer at day 3 in cells treated with afadin- or nectin siRNA (B, D), but not in cells expressing flag-nectin-2 (F) or in controls (H). Arrows show direction of flow. Bars, 100 µm in A-H, 10 µm in Ai-Hi. **I** Quantification of area lacking cells, with total area of the picture set to 100 % (each time 10 images of 3 experiments). Values were analyzed (all compared to control) using one way ANOVA with Bonferroni's post-test and are depicted as means ± SEM of n = 3. ***P < .001.

Together, these data indicate that drebrin and afadin are both required to maintain monolayer integrity and their absence leads to reduced TER and rupturing of monolayers. The extent of functional impairments regarding the cell-cell adhesion is even more pronounced when the endothelial isoforms of the adhesion protein nectin, which is secondarily affected through drebrin and afadin knockdown, are directly depleted in the cells.

We next set out to see whether an overexpression of nectin-2 from a transiently transfected vector could rescue the drebrin knockdown phenotype, regarding TER experiments and monolayer integrity under flow. Indeed, expression of the protein being actually responsible for adhesion, led to intact monolayer integrity in both experiments (Figure 29D, Figure 30E,F,I). Together, these data show that the defects in adhesion observed after drebrin depletion are really due to loss of endothelial nectins at adherens junctions, and that nectin's stabilization at the junctional area is dependent on the presence of drebrin and afadin.

5 Interaction of drebrin with the nectin-afadin system

We found that drebrin knockdown in endothelial cells leads to a disappearance of the adherens junction component nectin, resulting in an impairment of junction functionality. Furthermore, nectin's binding partner afadin is also affected, showing a higher mobility at the junctional area. In order to understand this effect of drebrin depletion on the presence of nectin at the junctional cleft, we next wanted to know whether drebrin influences the nectin-afadin system by binding to one, or both of the proteins.

5.1 Drebrin and nectin-2 do not co-immunoprecipitate

A common scenario, also found in other adhesion systems, implies a cytosolic protein binding to the juxtamembrane region of an intercellular adhesion protein to protect it from degradation. The presence of cadherin at adhesion sites requires its interaction with presenilin-1 and particularly p120-catenin, preventing its endocytosis[226,227].

To investigate whether this model of interactions could be transferred to drebrin and nectin-2, co-immunoprecipitation studies with those proteins were performed. To facilitate the immunoprecipitation of the „bait"-protein, recombinant expression systems were applied, allowing the broad expression of our protein of interest fused to a GFP-tag that can easily be precipitated using commercially available systems. Thus, nectin-2-GFP or drebrin-GFP were transfected into HUVEC for 16 h and the GFP-fused proteins were isolated from the cell lysates utilizing magnetic beads covalently bound to a GFP-antibody. Lysis buffer II, containing SDS, was used for all experiments. Subsequently, the eluate was separated on SDS-PAGE gels and subjected to western blotting, employing antibodies against drebrin and nectin-2. Due to their ability for homo-dimerization, drebrin-GFP co-precipitated endogenous drebrin (Figure 31A) and nectin-2-GFP co-precipitated endogenous nectin-2[89,170,228] (Figure 31B). However, no interaction of nectin-2 with drebrin and vice versa could be observed, which could explain drebrin's effect on nectin-2 stability at endothelial junctions.

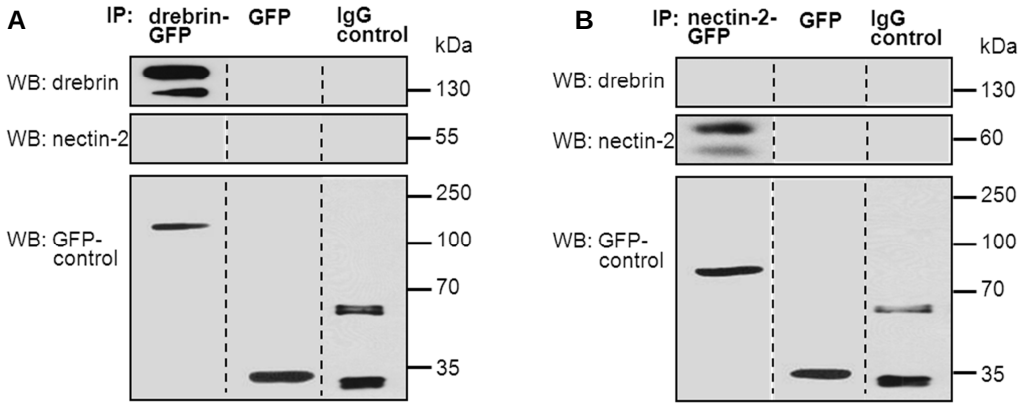


Figure 31 Immunoprecipitation of drebrin and nectin-2
Western blots of whole cell lysates of HUVEC transiently expressing drebrin-GFP (A), nectin-2-GFP (B) or GFP as a control (A,B) subjected to immunoprecipitation using magnetic beads coupled to anti-GFP antibody. Drebrin-, nectin-2- or GFP-specific antibodies were applied to detect the respective proteins. Western blots probed with anti-GFP antibody show the presence of the transfected constructs in the eluates. Note co-immunoprecipitation of cellular drebrin with drebrin-GFP (A), of cellular nectin-2 with nectin-2-GFP (B), but no co-immunoprecipitation of drebrin by nectin and vice versa, in both experiments. All experiments were performed at least 3 times, yielding comparable results. Note that anti-GFP antibody was covalently linked to beads and IgG bands are thus absent in lanes of anti-GFP immunoprecipitations. Control IgG was not covalently bound to beads and therefore appears as heavy and light chains in the controls. Dashed lines indicate that lanes were not directly adjacent on original blots.

5.2 Drebrin and afadin co-immunoprecipitate

Since drebrin knockdown also had an effect on nectin's most prominent binding partner afadin, increasing its mobility at the junctional area, a possible interaction of drebrin with afadin was examined next. Again, GFP-IPs were performed - this time with HUVEC expressing afadin-GFP or drebrin-GFP for 16 h. The GFP-fused proteins were isolated from the cell lysates (lysis performed with lysis buffer II, containing SDS) using magnetic beads bound to a GFP-antibody. After separating the eluate on SDS-PAGE gels, western blotting was performed, employing antibodies against afadin, drebrin and nectin-2 as indicated (Figure 32A,B). Afadin-GFP was able to co-immunoprecipitate nectin-2, verifying their already described interaction in HUVEC (Figure 32B).
Remarkably, drebrin-GFP was able to co-immunoprecipitate cellular afadin and afadin-GFP could co-immunoprecipitate endogenous drebrin, hinting at a so far unknown protein-protein interaction of these two proteins that has never been observed before (Figure 32A,B).

It is of major importance to verify the protein-protein interaction among the endogenous proteins as well, thereby avoiding their overexpression and possible artefacts arising through tag sequences. Accordingly, antibodies against cellular nectin-2, drebrin or afadin were coupled to protein A/G (bound to magnetic beads) via their heavy chains, thus presenting their antigen-binding sites. Thereby, the antibody-coupled "bait"-protein, drebrin, nectin-2 or afadin could be immunoprecipitated from untransfected HUVEC lysates, as verified through western blots employing the respective antibodies. Whether the three proteins were able to co-immunoprecipitate their potential binding partners from cell lysates was tested by western

blotting. The experiments were performed several times, lysing HUVEC in buffers that differed in their stringency.
In addition to the well described connection of nectin and afadin, our newly found interaction of drebrin and afadin could also be confirmed regarding the interaction of the endogenous proteins. Furthermore, we detected also nectin-2 in those eluates, where drebrin was used as “bait”-protein (Figure 32C). This co-immunoprecipitation of nectin-2 by drebrin was achieved through the use of a less stringent non-denaturing lysis buffer (lysis buffer I), which stabilizes also weakly bound protein complexes in the lysates. This result suggested the formation of a tripartite complex between all three proteins at the same time with interactions existing between drebrin and afadin and between afadin and nectin-2.

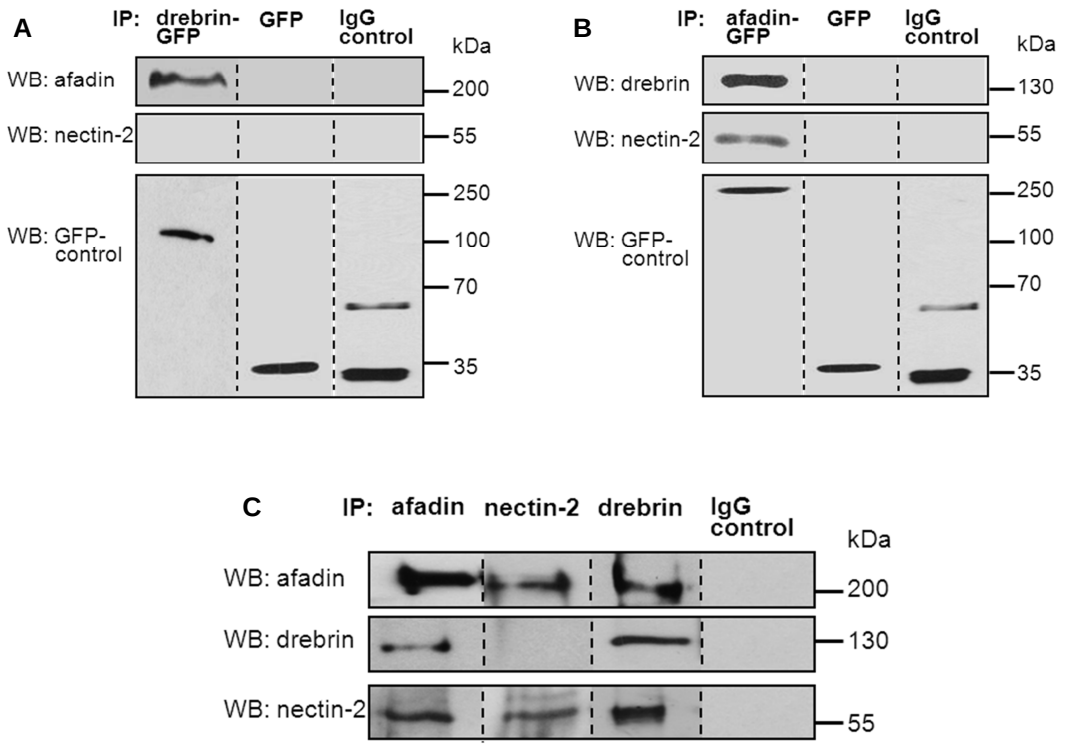


Figure 32 Co-immunoprecipitation of drebrin and afadin
A,B Western blots of whole cell lysates of HUVEC transiently expressing drebrin-GFP (A), afadin-GFP (B) or GFP as a control (A,B) subjected to immunoprecipitation using magnetic beads coupled to anti-GFP antibody. Drebrin-, nectin-2-, afadin- or GFP-specific antibodies were applied to detect the respective proteins. Western blots probed with GFP-antibody show the presence of the transfected constructs in the eluates. Note co-precipitation of cellular afadin with drebrin-GFP (A) and cellular drebrin and nectin-2 with afadin-GFP (B). Experiments were performed 3 times, yielding comparable results. Note that anti-GFP antibody was covalently linked to beads and IgG bands are thus absent in lanes of anti-GFP immunoprecipitations. Control IgG was not covalently bound to beads and appears as heavy and light chains in the controls. **C** Western blots of HUVEC lysates subjected to immunoprecipitation using afadin-, nectin-2- or drebrin-specific antibodies coupled to Protein A/G magnetic beads, using a non-denaturing buffer. Drebrin-, nectin-2-, or afadin- specific antibodies were applied to detect the respective proteins. Dashed lines indicate that lanes were not adjacent on original blots.

5.3 Drebrin's polyproline region binds to afadin

GFP-immunoprecipitation of recombinant expressed proteins also allows binding studies of GFP-tagged protein fragments or particular domains. If the domains maintain their characteristic tertiary structure that permit binding to other proteins, it is possible to co-immunoprecipitate potential interaction partners by only using specific GFP-tagged domains as "bait", elucidating which protein domains confer binding. Five different drebrin domain constructs, mostly covering the largely uncharacterized C-terminus (Figure 33A) were expressed in HUVEC for 16 h and following cell lysis, an anti-GFP-immunoprecipitation was performed. Eluates were subjected to SDS-PAGE and western blotting and probed with specific antibodies against GFP and afadin, to check which of the domains co-precipitates afadin. It could be shown that only those constructs, which contain drebrin's polyproline region, and even just the polyproline region alone, were able to bind afadin. Furthermore, a polyproline deletion construct lacking just the PP-region could not co-immunoprecipitate afadin. Collectively, these results verify that it is indeed drebrin's PP-region, which is responsible for afadin binding.

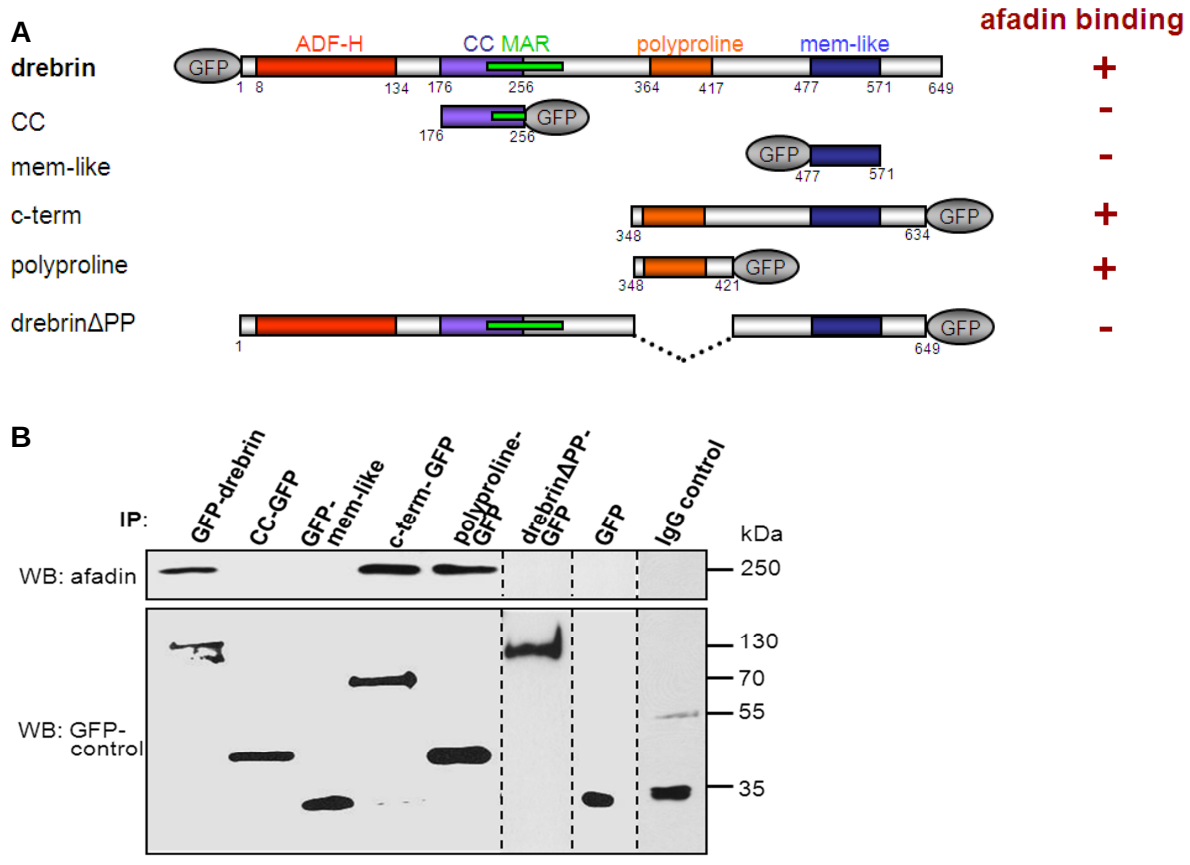


Figure 33 Drebrin's PP region interacts with afadin
A Drebrin domain structure and truncation constructs: ADF-homology region (aa 8-134), coiled coil region involved in homo-dimerization and F-actin binding[170] (CC, aa 176-256), minimal actin remodeling region[181] (MAR, aa 233-317), polyproline region (PP, aa 364-417), a region showing moderate homology to membrane-binding domains ("mem-like", aa 477-571). The polyproline region (aa 349-421) is deleted in drebrinΔPP. Co-precipitation of afadin is indicated by "+". Note: both N- and C-terminally tagged full length drebrin constructs have been used, yielding comparable results. **B** Western blots showing co-immunoprecipitated afadin (upper blot) and presence of the GFP-tagged domain constructs (lower blot). Dashed lines indicate that lanes were not adjacent on original blots.

5.4 Afadin's PR1-2 region binds to drebrin

After successfully identifying drebrin's afadin binding region we investigated, which of afadin's domains is involved in drebrin binding. Here, different truncation constructs of afadin, either GFP-, flag- or HA-tagged were transfected into HUVEC and endogenous drebrin was immunoprecipitated from the lysates. The eluates were then subjected to western blotting and were probed using specific antibodies against the respective tags of the corresponding constructs to examine, which of the domain-constructs could be co-immunoprecipitated by drebrin. In this case, only constructs containing the PR1-2 region of afadin, and even just the PR1-2 region alone, were able to bind drebrin. Furthermore, a PR1-2 deletion construct could not be co-immunoprecipitated by endogenous drebrin, confirming that it is indeed the PR1-2 region, which is responsible for drebrin binding (Figure 34).

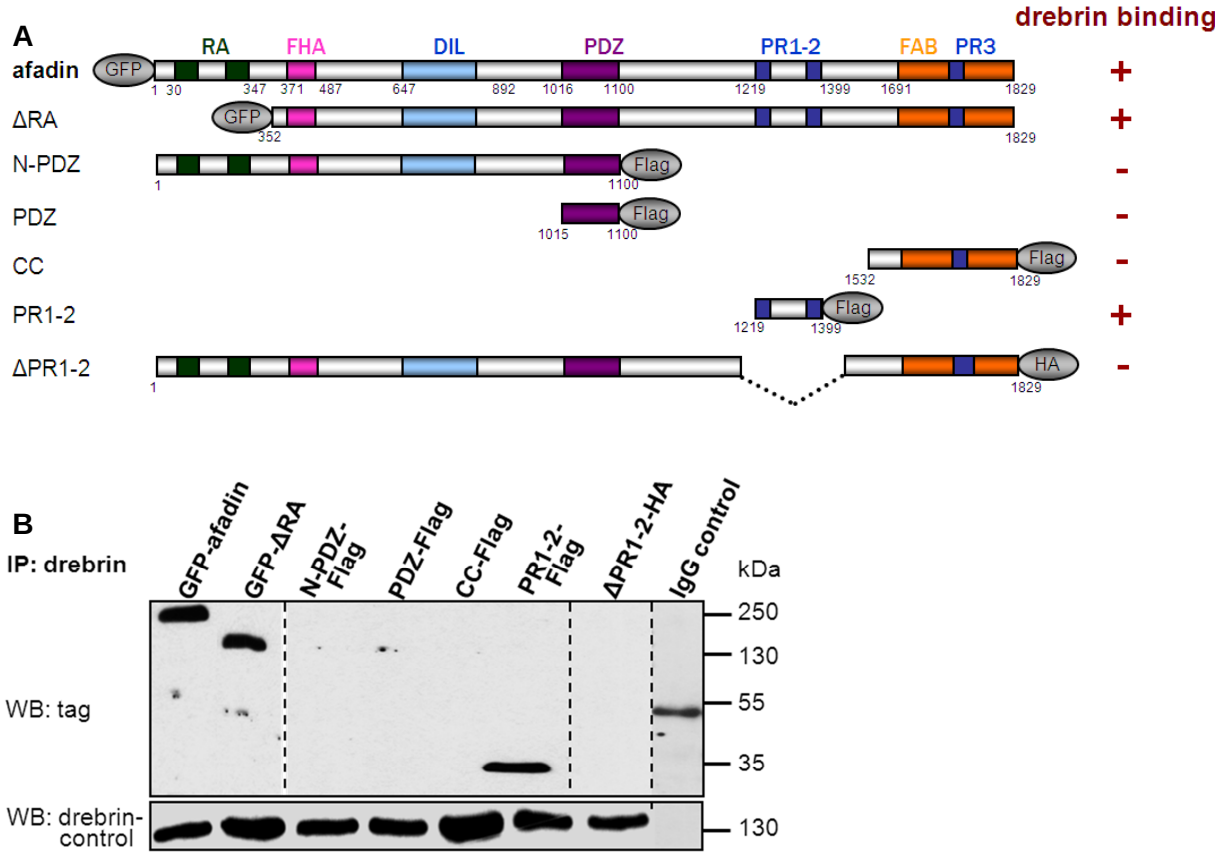


Figure 34 Afadin's PR1-2 region interacts with drebrin

A Afadin domain structure and truncation constructs: RA regions involved in Rap1 binding (aa 30-347), FHA region (aa 371-487), DIL region (aa 647-892), PDZ region involved in nectin binding (aa 1016-1100), PR1-2 region containing two polyproline stretches (aa 1219-1399), FAB region involved in F-actin binding (aa 1691-1829), containing a third polyproline stretch (PR3). Co-precipitation of respective constructs by cellular drebrin is indicated on the right ("+", "-"). **B** Western blots of immunoprecipitations of cellular drebrin, (lower blot) and co-immunoprecipitated afadin domain constructs (GFP-, Flag- or HA-tagged) detected with tag-specific antibodies (upper blot). Dashed lines indicate that lanes were not adjacent on original blots.

5.5 Drebrin's polyproline and afadin's PR1-2 region interact directly

An obvious limitation of the widely used method of co-immunoprecipitating protein binding partners from whole cell lysates is the fact that the identified interactions might not be direct, but rather mediated through other proteins present in the lysates. One way to prove if an interaction is direct is to perform pulldowns using purified bacterially expressed proteins or protein domains tagged with GST (**G**lutathione **S**-**T**ransferase) or MBP (**M**altose-**B**inding **P**rotein). These tag sequences enable binding of the recombinant protein to glutathione sepharose or amylose resin, respectively, and thus allow their purification. After dialysis of both purified proteins, they can be used for direct interaction studies: following coupling one of the potential binding partners to a column, the other purified protein can be applied to it and subsequently, the eluate can be examined for their interaction.

Here, the „bait"-proteins, either recombinant expressed drebrin-PP fused to MBP (MBP-drebrin-PP) or solely MBP (as a control), were immobilized on amylose resin beads. Then, GST-afadin-PR1-2 was incubated with either of the MBP proteins and the eluates were subjected to SDS-PAGE and western blotting with specific antibodies against the tags (Figure 35)

MBP-drebrin-PP clearly binds GST-afadin-PR1-2, which is not the case in the MBP control, thus proving the direct interaction between drebrin's PP and afadin's PR1-2 region in a cell free system.

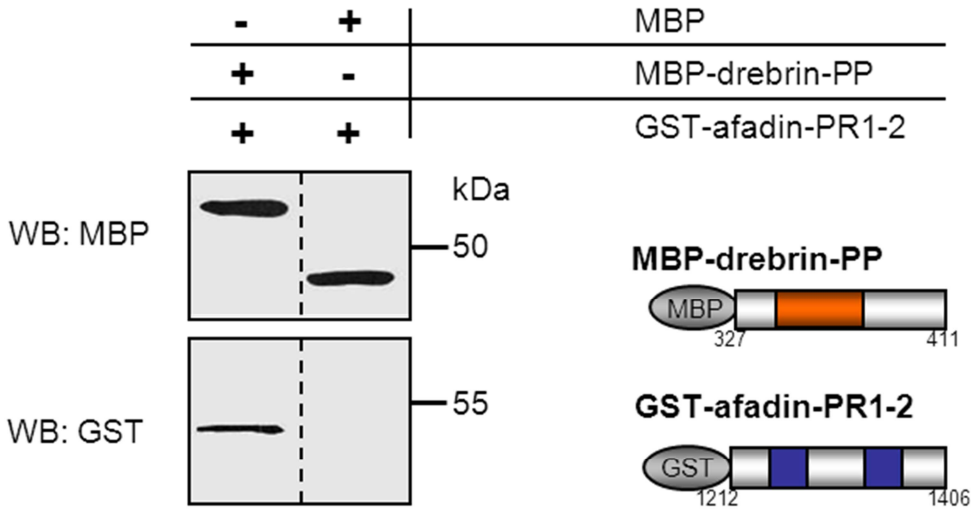


Figure 35 Direct interaction of drebrin's PP and afadin's PR1-2 domain

Western blots of pulldown assay using drebrin-PP fused to MBP (MBP-drebrin-PP) or MBP as control immobilized on amylose resin beads, incubated with afadin-PR1-2 fused to GST (GST-afadin-PR1-2). +/- indicates, which components were present in the respective experiment. Western blots developed with indicated antibodes. Dashed lines indicate that lanes were not directly adjacent on original blots. Molecular weight in kDa indicated. Domain constructs with positions of aminoacids are shown on the right.

5.6 Mitochondrial retargeting shows close interaction of drebrin and afadin

So far, the newly found interaction between afadin and drebrin, mediated via their PR1-2 and PP-domains, respectively, was proven on protein levels. At cellular levels, we could show a colocalization of these two proteins at the junctional area using confocal microscopy. However, since the area of cell-cell junctions is very narrow in HUVEC, it is hard to define a "real" colocalization of two junctional proteins, which is not just due to their usual presence at junctions. Furthermore, a colocalization observed with microscopic methods does not necessarily imply that proteins really bind to each other - they could just be situated in the same region. A way to check whether two proteins actually interact in cells is to re-direct one of the binding partners to a cellular region, where it is usually not present. If the potential interactor is co-recruited to that structure, the re-distribution verifies an actual interaction[198].

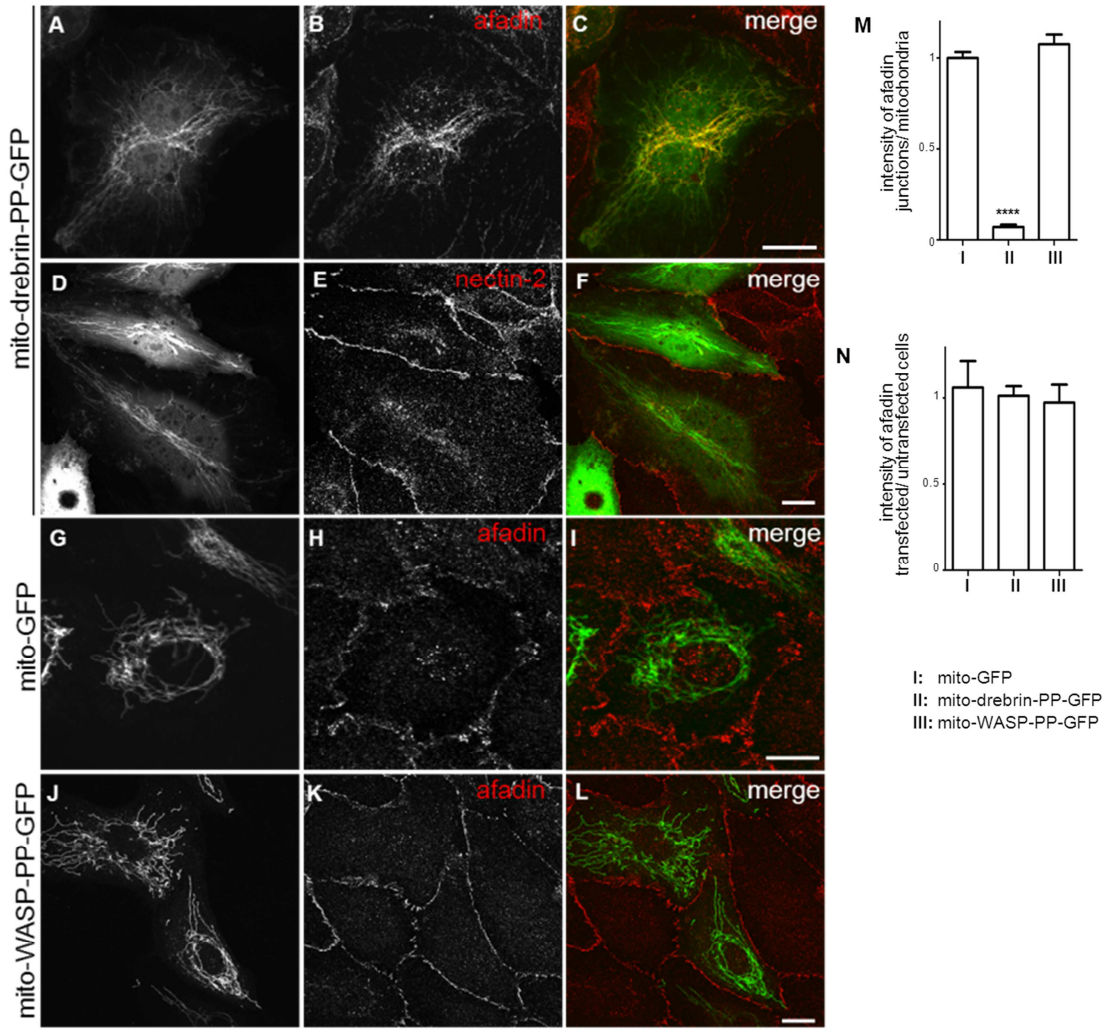


Figure 36 Mitochondrial retargeting shows close interaction of drebrin's PP and afadin on a cellular level
A-L Confocal micrographs of HUVEC expressing mito-drebrin-PP-GFP (A-F), mito-GFP (G-I) or mito-WASP-PP-GFP (J-L). Cells were fixed and immunostained for afadin (B,H,K) or nectin-2 (E). **M,N** Measurements of (M) afadin intensity at cell junctions vs. mitochondria and (N) total intensity of afadin in transfected vs. untransfected cells, in cells expressing mito-GFP, mito-drebrin-PP-GFP and mito-WASP-PP-GFP. For each value, 90 junctional areas of three experiments were evaluated. Values were analyzed (all compared to I) using one way ANOVA with Bonferroni's post-test and are depicted as means ± SEM of n = 3 ****P < .0001. Bars, 10 µm.

To visualize the interaction of afadin and drebrin on a cellular level, we next checked, whether drebrin's PP-domain would be able to recruit cellular afadin to intracellular membranes, where it is usually not situated. Therefore, a mitochondrial targeting sequence was fused to drebrin's PP and GFP (mito-drebrin-PP-GFP), or just to GFP (mito-GFP) as a control, which led to efficient targeting of the fusion construct to the outer mitochondrial membranes of HUVEC (Figure 36A,D,G). When transfected cells were co-stained for afadin, it showed a clear and unusual localization to mitochondria, overlapping exactly with the targeted drebrin-PP fusion construct (Figure 36B). This relocalization of cellular afadin did neither happen in the mito-GFP control transfections nor in another control, where a different PP-domain of similar size (from WASP (**W**iskott–**A**ldrich **S**yndrome **P**rotein)) was targeted to mitochondria (mito-WASP-PP-GFP) (Figure 36H,K). Consistent with the protein-protein interaction studies, these findings demonstrate that specifically the PP-region of drebrin is necessary and sufficient for strongly binding to afadin.
Staining the mito-drebrinPP-GFP transfected cells for cellular nectin emphasized the idea that drebrin can interact indirectly with nectin, through afadin: nectin-2 showed a slight relocalization to mitochondria, indicating that some of the endogenous nectin is retargeted together with afadin to the mitochondria (Figure 36E). Most of the nectin-2 protein is still visible at the junctional area, where it is probably stabilized through endogenous drebrin and the adequate amount of afadin that is still left at the junctional area, not being retargeted towards mitochondria.

6 Coupling nectin-2 to F-actin leads to its stabilization at junctions

The results that we gained so far, point to a close interaction of drebrin and afadin, but not between drebrin and nectin. Since drebrin depletion nevertheless led to disappearance of nectin at junctions, drebrin's effect on nectin is likely to be indirect, potentially through afadin. Drebrin's most pronounced characteristic is its ability to strongly bind to F-actin, which is its main function also in other cell types[181]. In endothelial cells, it has already been included in the list of proteins involved in attaching cortical F-actin to junctional plaques – without elucidating its exact function in this environment[191]. Given the fact that afadin binds strongly to nectin through its PDZ domain, we postulated that drebrin might be responsible for linking the nectin-afadin system to the cortical F-actin, thus stabilizing this complex at adherens junctions.

6.1 Afadin's PDZ fused to drebrin's F-actin binding region can rescue nectin-2 at the junctional area

To elucidate the question whether drebrin might be responsible for stabilizing nectin through afadin, we generated a GFP construct, containing just the PDZ-region of afadin responsible for binding to nectin fused to drebrin's coiled coil region, which enables F-actin binding (drebrinCC-afadinPDZ-GFP)[181]. Upon expression of this minimal construct in HUVEC depleted for drebrin, or drebrin and afadin, we could indeed observe a rescue of nectin-2 at the junctional area (Figure 37A-F). Constructs that contained just the afadin-PDZ region,

without linkage to F-actin, or only GFP, were not able to restore nectin-2 at junctions (Figure 37G-L). Also the quantification of nectin's fluorescence intensity at junctions revealed that the fluorescence recovery at the junctional area between drebrinCC-afadinPDZ-GFP transfected cells reached the amount of nectin-2 levels in control cells (Figure 38).
In order to check whether afadin's PDZ region can be replaced through a different PDZ region of similar size, we generated a construct including the ZO-1 PDZ region and expressed it in drebrin and afadin knockdown HUVEC. Immunofluorescence microscopy and quantifications of nectin-2 signals at junctions revealed that a construct linking the ZO-1 PDZ to F-actin is not able to stabilize junctional nectin-2, due to its inability of binding nectin (Figure 37S-U, Figure 38B).
These results strongly indicate that nectin-2 needs to be linked to the F-actin cytoskeleton for its stabilization at the junctional area, and this linkage could be provided through afadin and drebrin. Therefore, specifically afadin's PDZ region, which allows binding to nectin's C-terminus is required.

6.2 Drebrin's F-actin binding region can be replaced by lifeact

The observation that only a construct containing drebrin's CC region, was able to rescue nectin-2 at junctions, led to the question whether drebrin's CC region might exert additional unknown functions or only plays a role as an F-actin linking unit in this context. If this was the case, it could potentially be replaced by a different F-actin binding unit, which also provides linkage of nectin-2 to F-actin. A peptide currently used for labelling F-actin in living cells, lifeact, was chosen to substitute drebrin's F-actin binding region in the newly generated construct (lifeact-afadinPDZ-GFP). Strikingly, expression of this linker construct in drebrin and afadin knockdown cells also led to a recovery of nectin at junctions, whereas the control (lifeact-GFP), missing the nectin-binding unit, did not lead to a rescue of junctional nectin-2 (Figure 37M-R). This experiment confirmed that the indirect stabilization of nectin through drebrin is indeed due to drebrin's F-actin binding capabilities, thus serving as a linker of the nectin/afadin system to the cortical F-actin.
In addition to the results obtained by immunofluorescence stainings and microscopic analysis, GFP-immunoprecipitations of the different domain constructs were performed to reveal their binding capacities to nectin-2 and actin. Concomitantly with the former results, only those constructs that were able to rescue nectin-2 at junctions could co-immunoprecipitate nectin-2 and actin at the same time (Figure 38B).

Collectively, these results elucidate drebrin's function at endothelial junctions: it is indispensable for linking the nectin-afadin complex to the cortical F-actin, thus maintaining endothelial integrity under flow through the stabilization of nectins at the junctional area. Of major importance are drebrin's functional domains that allow its simultaneous binding to F-actin and afadin, serving as the essential linker of the two systems.

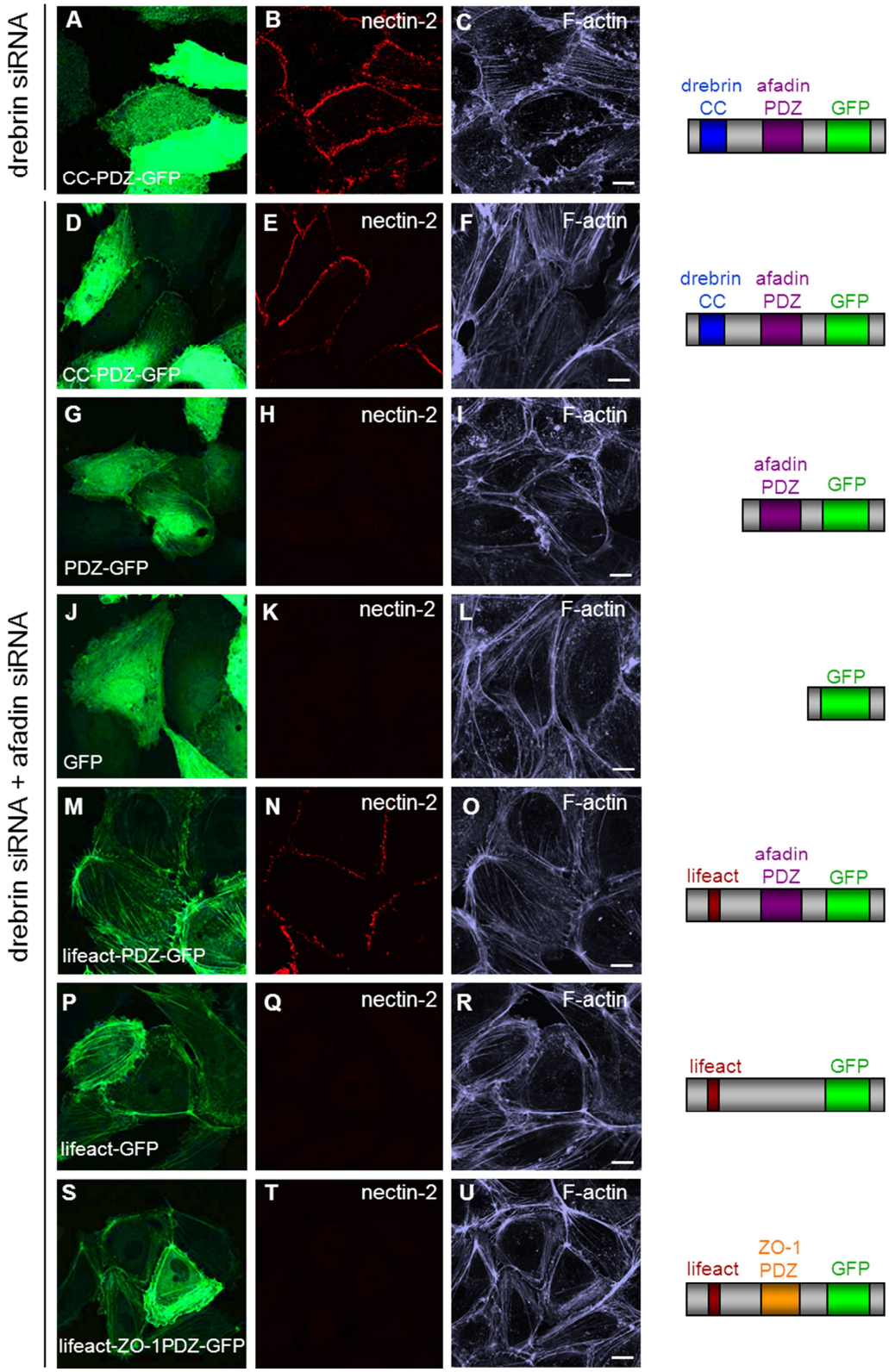


Figure 37 Recovery of nectin at cell-cell junctions by minimal rescue constructs
A-C HUVEC treated with drebrin siRNA pool and co-expressing the drebrinCC-afadinPDZ-GFP construct binding to nectin-2 and F-actin. **D-U**, Confocal micrographs of HUVEC monolayers treated with drebrin- and afadin-specific siRNAs for 3 days and stained for nectin-2. Cells express minimal constructs schematically depicted on the right side. Note recovery of junctional nectin-2 only in cells with transfected constructs that confer binding to F-actin and nectin-2 (E,N). Bars, 10 µm.

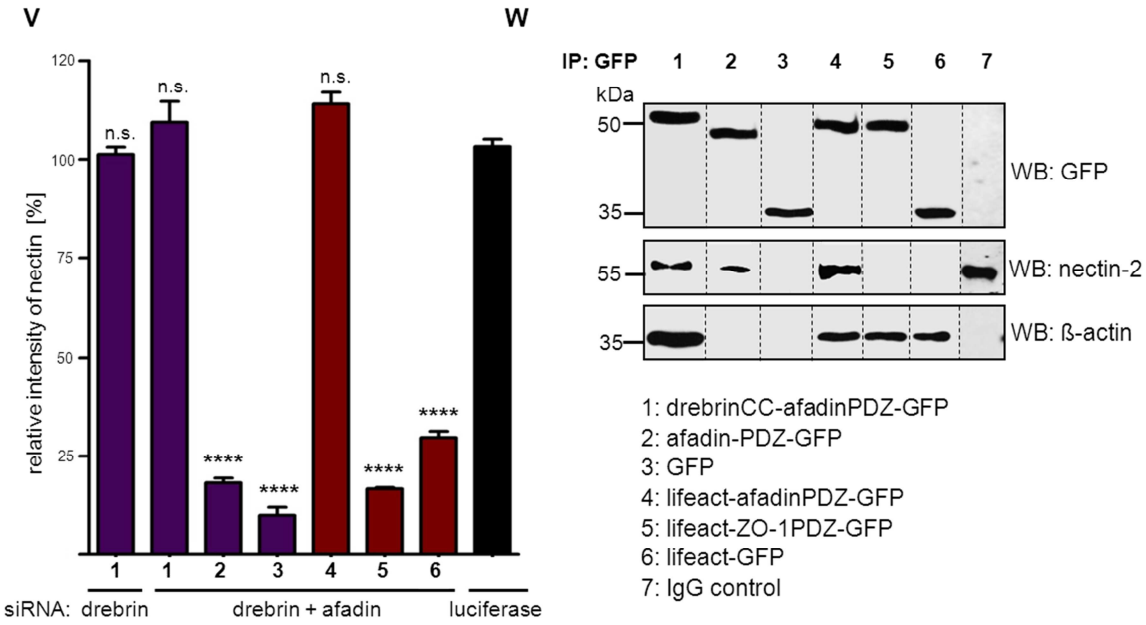


Figure 38 Quantification of nectin-2 recovery through rescue constructs that bind nectin-2 and F-actin
A Quantification of fluorescence-based intensities of nectin-2 at cell junctions upon overexpression of different fusion constructs in HUVEC treated with siRNA specific for drebrin and afadin. For each value, 200 junctional areas of 10 µm x 10 µm sizes from at least 3 different monolayers were measured. Bar diagram shows intensities/area after background correction, with mean value for nectin-2 intensity of luciferase control siRNA treated cells set to 100 %. Values were analyzed (all compared to luciferase transfected control, black bar) using one way ANOVA with Bonferroni's post-test and are depicted as means ± SEM of n = 3 ****P < .0001. n.s., not significant **B** GFP-immunoprecipitations of indicated constructs expressed in HUVEC. Small lines indicate that lanes were not directly adjacent on original blots. Western blots developed with indicated antibodies. Note that the signal in lane 7 is due to detection of free IgG in control.

V Discussion

The major topic of this dissertation has been to elucidate the role of drebrin E2 (hereafter referred to as drebrin) in the human endothelium and to understand its function at endothelial cell-cell junctions at a molecular level. At the time this work was initiated, merely drebrin's localization to the junctional area in endothelial cells had been described by Peitsch *et al.,* without an explicit function defined so far[191]. In this study, we found that drebrin is a critical regulator of monolayer integrity in primary human umbilical vein endothelial cells (HUVEC) and its presence at junctions is crucial to maintain their functionality. On the molecular level, drebrin is needed to stabilize the nectin/afadin system at adherens junctions by linking them to the underlying F-actin cytoskeleton. This prevents nectin's endocytosis and can thus maintain endothelial integrity under conditions mimicking vascular flow.

1 Stabilization of junctions through interaction with cortical F-actin

Coordination of cell-cell adhesion through regulating the availability of intercellular adhesion proteins at the cell surface, explicitly cadherins, nectins and tight junction proteins determines the strength of intercellular adhesion. A newly formed adhesion site, with just initial contacts made through few proteins is less strong than a cluster of many recruited adhesion proteins[229]. Accordingly, adhesion sites are weaker, when a large pool of intercellular proteins is endocytosed and rather present in vesicles than at junctions. However, regulating the presence or absence of junctional proteins is not the only way how strength of intercellular adhesion is influenced – also the linkage of these proteins to the underlying F-actin cytoskeleton is of major importance for regulating the adhesion capabilities[230]. It has been shown that the assembly of first contact sites is not dependent on the F-actin cytoskeleton, but once this contact is made, the development of mature junctions relies on an intact cortical F-actin distribution, concomitant with the activation of small Rho-GTPases Cdc42 and Rac[229,231]. F-actin occurs in different structures, highly depending on associated proteins that bundle, crosslink or form networks of F-actin, depending on the cells requirements. First assembly of cell-cell junctions is usually made between migrating cells, which form characteristic lamellipodia with unevenly distributed adhesion molecules at their leading edge[70,232]. Lamellipodia are shaped through assembly of F-actin into branched networks, as accomplished by the ARP2/3 complex, which is active at this stage of junction formation[233]. There are different opinions whether first junction assembly relies on the interaction of nectins, which subsequently recruit cadherins, or if nectins are dispensable for this process[102,232]. Nevertheless, different researchers agree on the fact that quickly after initial adhesion site formation at the lamellipodia, ARP2/3 is deactivated, or potentially repressed by an enrichment of α-catenin[73,234]. Consequently, other actin binding proteins, among them VASP and Mena, are subsequently recruited through α-catenin, leading to the formation of thicker F-actin cables needed to stabilize the developing junctions (Figure 42)[73,235,236]. The presence of this circumferential cortical F-actin ring has been shown to be essential for adhesion in general - if disrupted by latrunculin A or cytochalasin D, junction integrity is lost[75,237-239].

However, it quickly became clear that not just the presence of a stable cortical F-actin ring is essential for proper junction stabilization, but especially the presence of linker proteins connecting the adhesion proteins to F-actin. It has been shown that knockdown of either α-catenin, VASP, EPLIN or afadin, which are all involved in anchoring adhesion systems to F-actin, leads to severe problems in junction formation and maintenance[240-243]. This failure to form junctions has different reasons, depending on the function of the protein. One of them is that the cells are not able to seal their membranes due to defects in F-actin re-organization, as in knockdown of VASP or α-catenin[236]. In afadin depleted epithelial cells, adherens junctions do not form, since afadin is important for the recruitment of cadherins to initial adherens junction sites[244]. Showing similarities to our findings, knockdown of EPLIN leads to misorganization of the cortical F-actin, due to the missing linkage of cadherin/catenin to F-actin[141]. We added another protein, drebrin, to the list of junction-associated proteins that link adhesion molecules to F-actin. Drebrin is important for stabilizing the nectin/afadin system at adherens junctions by linking them to F-actin, and loss of drebrin leads to impaired monolayer integrity due to degradation of nectin (see V.2 and V.3).

2 Drebrin's function at junctions

Drebrin, a protein of major importance for organizing F-actin in dendritic spines of neurons, was first thought to be brain specific[245]. However, since 1999, its shorter splice variant drebrin E2 has been described also in tissues of non-neural origin, for example smooth muscle, epithelia and endothelia[191]. In those tissues, cells characteristically form compact monolayers with close interactions between individual cells, which are enabled through adhesive transmembrane proteins assembling into adherens- and tight junctions. While cells are still migrating, drebrin is found at protrusions, but once the monolayers have formed it localizes along cell-cell boundaries[170]. This presence of drebrin at junctional areas has been clearly observed in vein and artery endothelial cells, as well as in many other cell types[191]. Among them are kidney and bronchial epithelial cells, different kinds of carcinoma and some specialized cells, such as sertoli cells of the human testis[160,162,246]. At the junctional area, drebrin was described to be especially concentrated at F-actin-rich plaques of adherens junctions[191]. Some attempts have been made before to elucidate the actual molecular function of drebrin at the junctional area: Butkevich *et al.* found that drebrin binds to the gap junction protein connexin43 and is needed to maintain gap junction functionality in astrocytes and Vero cells[192]. Keon *et al.* described drebrin being enriched at the apical part of certain acid-secreting cells of stomach and kidney, the same subcellular region, where Bazellières *et al.* found it to be enriched in columnar epithelial cells while they acquire their elongated shape[162,163]. Drebrin's localization at apical junctions in stomach and kidney cells could not be explained so far. Bazellières *et al.* succeeded to describe a function of drebrin in their cell system: at apical junctions of intestinal epithelial Caco2 cells, drebrin is needed to maintain the F-actin network, and thus enables cell elongation and microvilli distribution[163]. Knockdown of drebrin leads to disruption of the proper F-actin-myosin IIB-spectrin network in Caco2 cells, a function that rather belongs to drebrin's effects on F-actin than on junctional components[163]. Nevertheless, in endothelial cells, knowledge about the reason for drebrin's localization to the junctional area is very limited, giving rise to the main question of this thesis, what drebrin's function at endothelial cell-cell junctions might be.

To receive a first impression about drebrin's role in the endothelium, we established an siRNA-based knockdown, which lead to almost complete disappearance of drebrin from junctions. Concomitantly, drebrin protein was not detectable in whole cell lysates any more, allowing studies of its function in HUVEC (Figure 16).
To start with, we wanted to investigate whether loss of drebrin has effects on the cells' adhesion to their substratum. Therefore, we performed an adhesion assay with drebrin knockdown and control cells, as described by Humphries *et al.* (Figure 19)[209]. However, we could not detect any alterations in cell-matrix adhesion of HUVEC upon drebrin depletion leading to the assumption that drebrin might rather be involved in cell-cell junction dynamics.
Indeed, we could show that siRNA induced knockdown of drebrin leads to functional impairments of HUVEC monolayers. A very pronounced effect could be observed when HUVEC were cultured under constant unidirectional flow of 15 dyne/cm^2 mimicking flow conditions in medium-sized blood vessels[217]. Monolayers depleted for drebrin showed numerous ruptures under flow, revealing the cells' difficulties in cell-cell adhesion (Figure 18). Also under static conditions, a decrease of monolayer integrity could be detected using a more sensitive method - measuring the transendothelial resistance (TER) of drebrin knockdown and control monolayers grown on transwells filters. Even though this method is not frequently used on HUVEC, due to their very low basal resistance arising from their flat appearance and lack of numerous tight junctions, a significant decline of 25 % regarding TER was observed (Figure 17). Following these observations, we set out to find the source of the monolayer alterations visible upon drebrin depletion.

We could reveal that drebrin knockdown leads to a complete loss of nectins from endothelial adherens junctions, and from HUVEC cell lysates (Figure 20, 21). Nectins are transmembrane proteins that form adherens junctions in conjunction with cadherins, thus enabling cell-cell adhesion between endo- or epithelial cells (Figure 3)[107]. Four members of the nectin family have been identified (nectin-1,2,3,4), showing diverse expression patterns in different tissues[44,63]. Nectin-1 and -3 are abundantly expressed in epithelial and neuronal tissue, whereas nectin-2 is ubiquitously expressed, including cells that do not express cadherins, such as blood and sertoli cells[247-249]. Nectin-4 is the most recently discovered member of the nectin family, with an expression mainly restricted to placental tissue[85].
In addition to nectin-2, nectin-3 is expressed in the endothelium as well. We could demonstrate a loss of nectin-3 at junctions upon drebrin depletion, leading to the assumption that drebrin might have effects on all nectin isoforms (Figure 22). However, in the following experiments, we concentrated on nectin-2.
After realizing that drebrin knockdown lead to loss of nectins from adherens junctions, we wanted to confirm that this effect is really due to drebrin depletion. The expression of an siRNA-insensitive drebrin-GFP construct in drebrin knockdown cells was sufficient to stabilize nectin-2 at the junctional area, which enabled us to attribute the functional impairments and the concomitant loss of nectin-2 and nectin-3 to the lack of drebrin (Figure 22,23).

Starting with co-immunoprecipitation experiments, we wanted to find out whether drebrin interacts with nectin-2, thereby displaying an effect on its junctional localization. We could not co-immunoprecipitate the two proteins together, but we found that drebrin can bind to nectin's cytosolic binding partner, afadin (Figure 31, 32). Through co-immunoprecipitations

using domain constructs of both proteins, we could attribute their binding to drebrin's polyproline (PP) and afadin's PR1-2 domain (Figure 33, 34). This interaction was confirmed by pulldown studies using the bacterially expressed protein domains, revealing that it is indeed a direct interaction (Figure 35).
The binding of drebrin's polyproline region to afadin was strong enough that a re-distribution of drebrin's PP to mitochondria through a mitochondrial targeting construct (mito-drebrinPP) led to a simultaneous re-localization of the majority of afadin to the mitochondrial outer membrane (Figure 36). Interestingly, also a slight relocalization of nectin-2 to mitochondria could be observed, suggesting that some nectin-2 protein was still bound to the re-distributed afadin. Nevertheless, most of the nectin-2 protein remained at the junctional area, where it was probably stabilized through endogenous drebrin and the residual afadin. Concomitantly, immunoprecipitations of endogenous drebrin led to co-immunoprecipitation of afadin and additionally nectin-2, when a less stringent lysis buffer was used, which stabilizes also weakly bound protein complexes in the lysates. This probably allowed the formation of unstable tripartite associations between all three proteins, with interactions existing between drebrin and afadin and between afadin and nectin-2.

We also elucidated that drebrin acts on nectin by stabilizing the nectin/afadin system through linking it to the cortical F-actin network (Figure 39). Drebrin contains a coiled-coil region (CC), which serves as an F-actin binding domain[179]. Additionally, we could demonstrate its polyproline region, situated in the not well described C-terminus, to be necessary and sufficient for binding afadin (Figure 32, 33). Being equipped with both of these modules, conferring binding to F-actin and afadin, drebrin can serve as a strong linker between the afadin/nectin complex and the cortical F-actin cytoskeleton. Even with a minimal construct that only contained drebrin's CC domain fused to afadin's nectin-binding PDZ region, a rescue of nectin-2 at the junctional area could be achieved in drebrin knockdown HUVEC (Figure 37, 46).
Altogether, we could show a new function of drebrin in endothelial cells, which is to stabilize the nectin/afadin system at adherens junctions by linking them to the cortical F-actin. Being not well characterized in the endothelium in general, nectins are usually only mentioned in the context of junction formation – if acknowledged at all. Here, we could also demonstrate for the first time that they do also fulfil functions at established endothelial junctions, where their linkage through afadin and drebrin to F-actin is of major importance for the upkeep of adherens junctions.

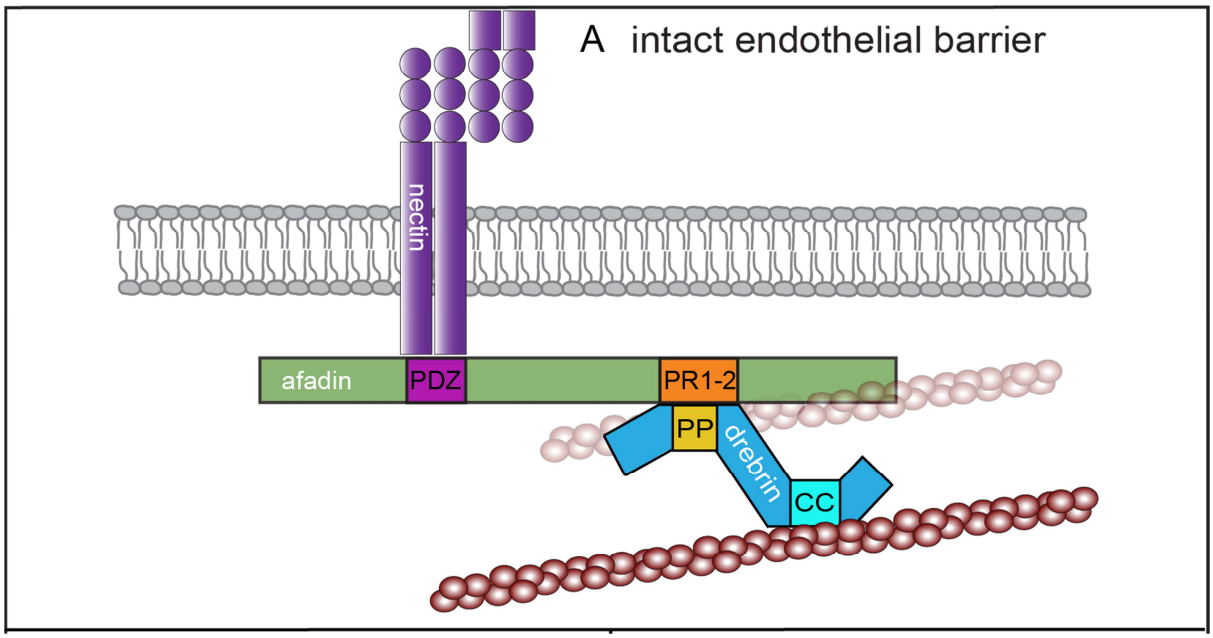


Figure 39 Drebrin's function in endothelial cells
Adherens junction protein nectin binds to afadin's PDZ domain. Afadin can simultaneously interact with drebrin's PP region through its PR1-2 domain. Thereby, drebrin can link the nectin/afadin system to F-actin via its F-actin binding CC-domain. If this linkage is established, nectin is stabilized at the junctional area and endothelial barrier function is intact.

Interestingly, an overexpression of flag-nectin was able to rescue the drebrin knockdown phenotype, regarding rupture of monolayers under flow and TER experiments, even while drebrin was depleted in the cells and thus not available for linking flag-nectin to F-actin (Figure 29, 30). As the word overexpression already indicates, the transfection of an artificial construct into HUVEC results in an oversupply of the protein encoded by the vector. Nevertheless, quantifying the amount of flag-nectin-2 at the junctional area based on immunofluorescence staining of the flag tag revealed only slight increase (ca. 10 %) of flag-nectin-2 at the surface, compared to endogenous nectin-2 in control cells. However, a high number of vesicles containing flag-nectin-2 could be visualized in the cells, which might lead to a constant delivery of flag-nectin-2 to the cell surface (data not shown). This could give rise to more frequent events of spontaneous nectin-cis and trans-dimerization. Even if these nectin-dimers might be unstable and quickly endocytosed (see V.3), since they are not anchored to F-actin via drebrin, the multitude of the transient interactions could be sufficient to restore the properties of the HUVEC monolayers.

3 Degradation and turnover of nectins

After observing a disappearance of nectin-2 from the junctional area upon knockdown of drebrin we wanted to know what its disappearance implies. First, we excluded that drebrin has an effect on nectin-2 gene expression characterized through reduced nectin-2 mRNA levels, by performing an expression analysis using RT-PCR. We could not detect any effect of drebrin depletion on nectin-2 expression levels with this method (Figure 24).
Since we could not see any accumulation of nectin-2 in the cytoplasm and its protein level was also depleted in whole cell lysates, we examined whether it is degraded upon drebrin knockdown. By performing immunofluorescence staining and confocal microscopic analysis, we revealed that nectin-2 is present in early endosomes upon drebrin knockdown, which were visualized by expression of EEA1-GFP (Figure 24). The usual degradative route, which follows endocytosis, is the degradation of internalized proteins in lysosomal compartments. We immunostained drebrin knockdown HUVEC for LAMP-1, a lysosomal marker, but could not detect endogenous nectin-2 in these vesicles. However, after lysosomal function was blocked using chloroquine, nectin-2 started to accumulate in the compartments in a time-dependent manner, verifying that it undergoes quick degradation in lysosomes (Figure 25). This is a new finding about how nectin-2 is generally degraded. In fact, degradation of nectins has only been -to some extent- examined regarding nectin-1. This nectin isoform is by far the best studied one, since it serves as an entry receptor for herpes simplex viruses into epithelial and neuronal cells, where nectin-1 is mainly expressed[250].
In agreement with our findings that nectin-2 degradation takes place through an endocytic/lysosomal pathway, Stiles *et al.* could show that nectin-1 is also degraded in lysosomes following endocytosis induced by herpes viruses (Figure 40). Interestingly, an accumulation of nectin-1 could only be observed, when they inhibited lysosomal function through bafilomycin A1, which prevents activation of lysosomal proteases[251,252]. This goes in line with our experiments, where accumulation of nectin-2 in lysosomes upon drebrin depletion was only visible, when their function was inhibited by chloroquine. Together, these data suggest that nectins are generally degraded by a lysosomal pathway – at least when their internalization is triggered either by interaction with herpes simplex viruses in case of nectin-1, or by drebrin depletion as we have shown for nectin-2.

A very interesting question, which has hardly been addressed so far, is how -or rather if- nectin trafficking is achieved in untreated cell monolayers. Concerning cadherins, extensive studies have revealed that a certain pool is constantly endocytosed and recycled back to the cell surface, thus regulating its availability for junction arrangement[153,253]. After endocytosis, the internalized cadherins are usually visible in early endosomes. Subsequently, they are either delivered to late endosomes and lysosomes for degradation, or to recycling endosomes for transferring the proteins back to the surface (Figure 40)[254]. This constant trafficking is a possibility to regulate adhesion properties of the cell and the amount of cadherin present at junctions or in vesicles is regulated through a multitude of proteins, executing the transport between all endocytic compartments[153]. Included are members of G-protein families, adaptors, kinases, motor proteins and many other yet unknown candidates, since many aspects of cadherin turnover still have to be elucidated[158,255].

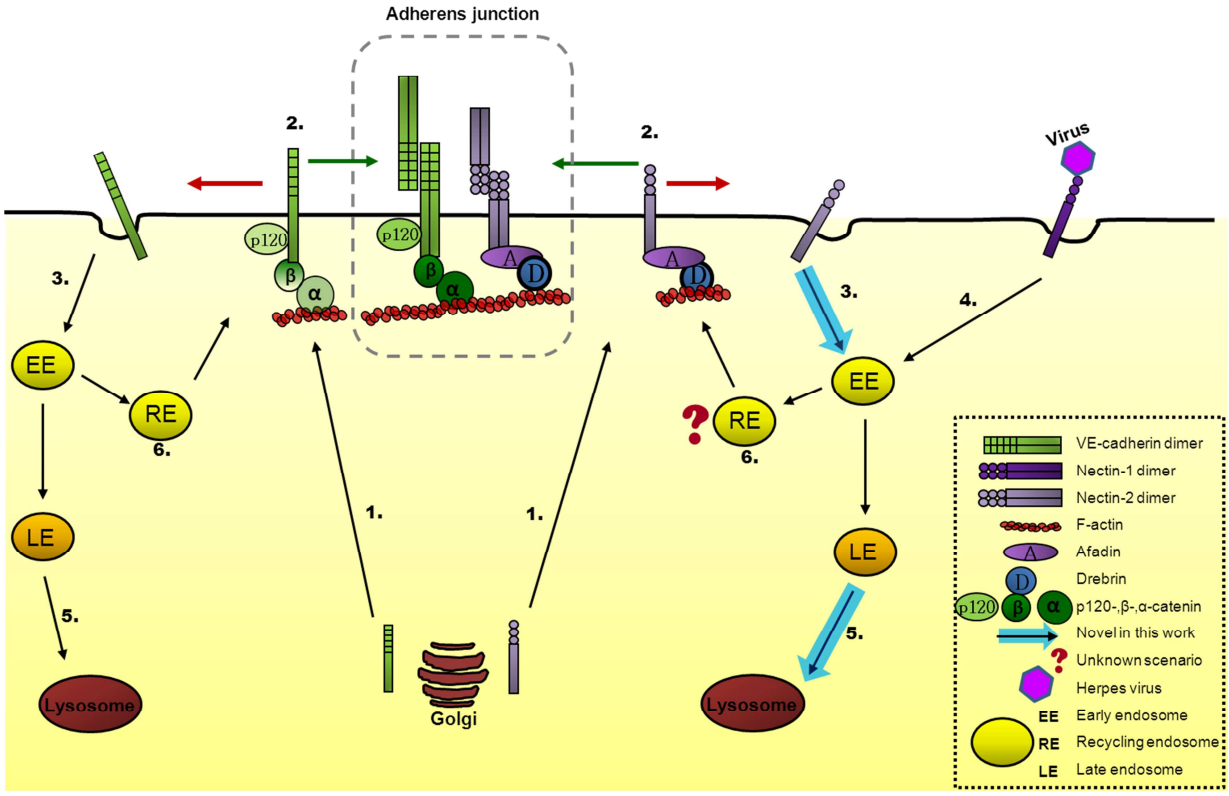


Figure 40 Cadherin and nectin trafficking
Schematic mechanism of nectin and cadherin trafficking events known so far. New results described in this work are marked by
(1.) Cadherins and nectins are initially delivered from the golgi complex to the cell surface as monomers. (2.) Here, they either form a complex with their adaptor proteins and assemble into mature adherens junctions (green arrow), or they are directly endocytosed again (red arrow). This either happens during natural turnover, or upon knockdown of important proteins needed for their stabilization, such as drebrin or p120-catenin. (3.) The destabilized adhesion proteins are then endocytosed and present in early endosomes. (4.) Additionally, nectin-1 has been described to undergo endocytosis after infection with herpes viruses. From early endosomes, proteins are either transported to late endosomes, leading to subsequent lysosomal degradation (5.), or they might be recycled back to the surface (6.). The cells can influence their adhesion capabilities by determining, which route is preferred and how much protein is present at the surface or in transporting vesicles. Whether these recycling routes (6.) are also common for nectin trafficking is not known so far. Cadherin trafficking based on[254]

It is rather astonishing that no such trafficking events have ever been described for nectins, which makes this question an interesting open field for research. It was only once mentioned by Stiles *et al.* that natural nectin-1 turnover at junctions is usually not taking place in quiescent monolayers - nectin-1 is stable at the junctional area for at least three hours[252]. Accordingly, we could also demonstrate a high stability of nectin-2 (data not shown) by inhibiting protein synthesis using cycloheximide.
Drebrin might play a role in natural turnover of nectins at adherens junctions - when drebrin is present at junctions in a state where it can bind afadin and thus stabilize nectins, their endocytosis is prevented. We propose a scenario, where nectin-2 might be constantly delivered to junctions. When drebrin and afadin are present to anchor nectin to the F-actin cytoskeleton, it is stabilized through the nectin-afadin-drebrin-F-actin chain, incorporated into adherens junctions and shows almost no natural turnover (Figure 40,(2.) green arrow). If not stabilized, due to unavailability of drebrin, it is endocytosed and delivered to lysosomes.

Speculating along these lines, one could propose that through the organization of drebrin's presence at the junctional area, nectin's incorporation into adherens junctions could be influenced as a regulation of its natural turnover. Regarding the question if nectin undergoes recycling in wildtype cells, it is interesting to mention that we could observe nectin-2 being present in endocytic vesicles - not only in drebrin knockdown, but also in control cells (data not shown). Whether all of the endocytosed nectin-2 is actually degraded, or if some amount is recycled back to the plasma membrane is currently unknown and would be an interesting point for further investigations. A transfection of HUVEC with a marker of recycling endosomes, could already give some hints whether nectin-2 is present in this subtype of endosomes[256].

Concomitantly, afadin has previously been shown to be essential for nectin's presence at adherens junctions – upon depletion of afadin, nectin is lost from junctional areas[106]. We confirmed this data by knocking down afadin and observing the same functional impairments of HUVEC monolayers as in drebrin knockdown cells (Figure 30). Also immunofluorescence staining of nectin-2 revealed its loss from the junctions in HUVEC upon afadin knockdown, indicating that there are at least two proteins directly involved in regulating nectin stabilization (data not shown).
Again, a similar scenario has been described for cadherins - their stabilization occurs through a central player in the field of cadherin turnover, p120-catenin[257,258]. This protein binds the juxtamembrane region of cadherins and exerts functions in regulating their surface exposure through a complex interplay with a variety of proteins and signaling cascades[257]. Knockdown studies revealed that absence of p120-catenin does not alter cadherin mRNA levels and their delivery to the cell surface, but their stabilization at the membrane once they reach the periphery[68]. If p120-catenin is not available to bind cadherin at the membrane, it will immediately enter a degradative pathway[77]. This well-studied mechanism is remarkably similar to the effect that drebrin has on nectin, although stabilization occurs not through direct binding, but indirectly, through afadin and F-actin.

4 Adaptor, linker, or rather scaffolding proteins?

It is important to mention that the textbook models of how adhesion proteins are simply linked to F-actin through one or two linker proteins (VE-cadherin/ β-catenin/ α-catenin/ F-actin, occludin/ ZO-1/ F-actin, nectin/afadin/F-actin) are currently questioned by a few groups, after Drees *et al.* showed that α-catenin is not able to bind β-catenin and F-actin at the same time[72,73]. Emerging concepts of how interactions between junction proteins, their adaptor proteins and F-actin might take place rather consider the adaptor proteins as "scaffolding" proteins than as mere linker units of just one junctional protein. Evidence for this idea might be that in several knockdown experiments of one linker protein (for example ZO-1), junction properties are rather altered or their assembly is slowed down than being completely abolished[259]. This could be due to the fact that most of the typical adaptor proteins can bind to several other junction-related proteins and especially interact among each other (Figure 41)[260]. An example is afadin, which typically interacts with nectin but also exerts binding to JAM, ZO-1 and α-catenin, interconnecting all these proteins[93,244]. A similar scenario accounts for ZO-1 – it can bind all typical tight junction adhesion proteins, occludin,

claudin and JAM, in addition to its scaffolding interactions with ZO-2 and afadin[55,199]. These interconnecting effects between adherens and tight junction proteins are especially important during junction formation: cadherin assembly strongly relies on the preceding establishment of nectin-based adhesion sites where they are recruited to through afadin[107]. Furthermore, the existence of adherens junctions is mandatory for the establishment of tight junctions, with JAM proteins and claudins being recruited through the interaction of ZO-1 and afadin[104,199].
Seeing the linker proteins as scaffolding proteins forming a complex network, the interconnection of the intercellular adhesion proteins and F-actin can be envisioned as a sum of many chains of potentially weak or transient interactions, adding to proper adhesion of the whole system. A scenario like this is especially interesting concerning endothelial cells, with the systems of adherens and tight junctions being quite intermingled along the contact zone, enabling a quick remodelling of junctions upon for example extravasation of leukocytes[39].

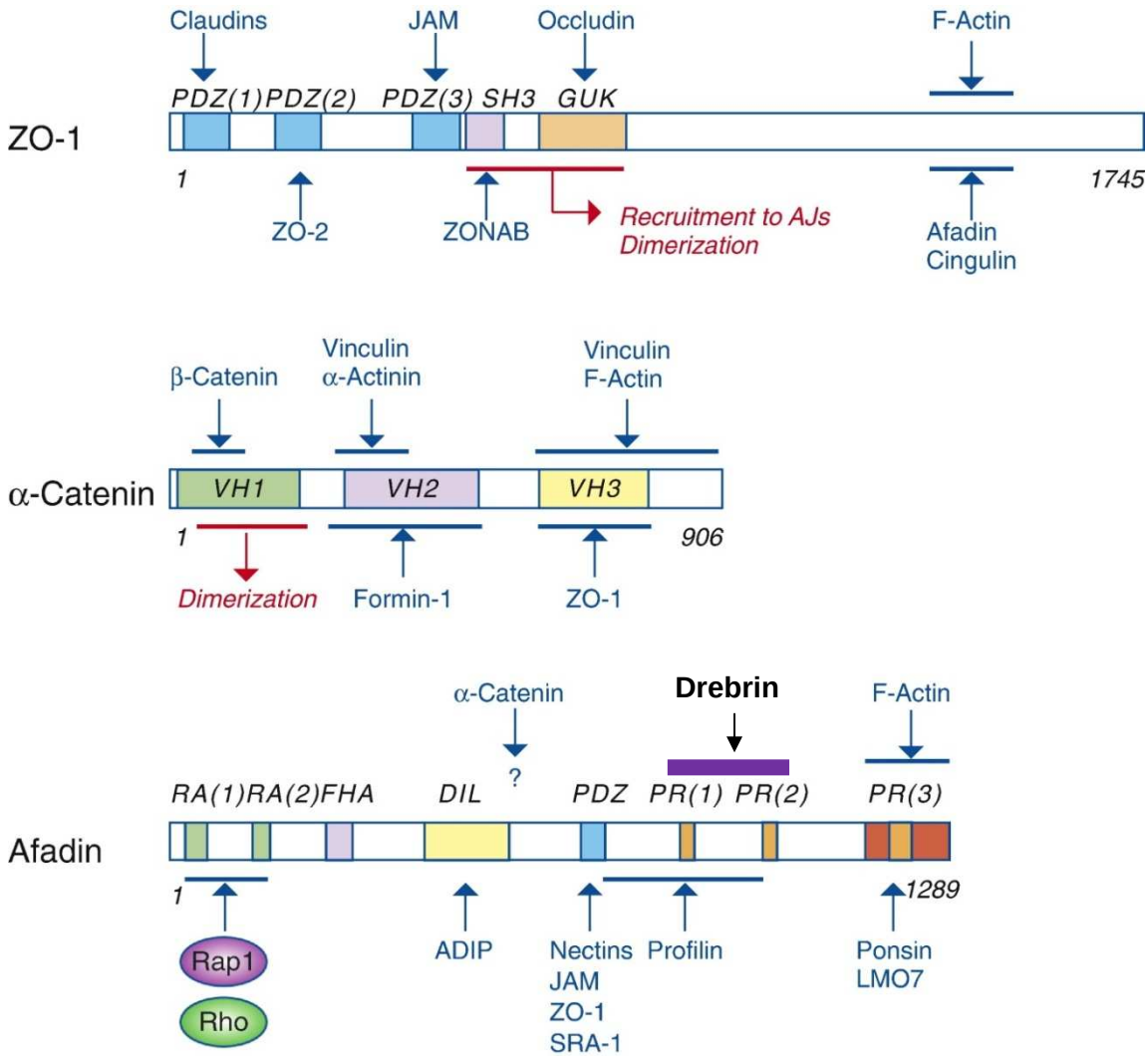


Figure 41 Adaptor proteins at cell-cell junctions
ZO-1, α-catenin and afadin have a C-terminal actin-binding domain and a variety of other functional domains, enabling their interactions with different proteins and each other. It is not clear whether their primary function is to link adhesion proteins to F-actin or interacting with other proteins as scaffolds.
SH3= Src-homology 3 domain; GUK= guanylate kinase; VH1–3= vinculin-homology 1–3; RA= Ras-associated domain; FHA= forkhead associated; DIL= dilute domain; PR= proline-rich domain.[260], with modifications.

Here, we identified drebrin as a new scaffolding protein present at endothelial junctions. Comparable to other adaptor proteins, drebrin has different functional domains, allowing the interaction with many other proteins. We could identify drebrin's polyproline region as the module responsible for the interaction with afadin's PR1-2 domain (Figure 35). Afadin's PR1-2 region is also responsible for ZO-1 binding, indirectly linking the nectin system to tight junction proteins. Upon drebrin knockdown and the concomitant loss of nectin-2 from junctions, afadin was still clearly visible at cell-cell boundaries. Potentially, binding of afadin to ZO-1 is enhanced, when drebrin is not present to compete with ZO-1 for binding to afadin's PR1-2 region, resulting in elevated localization of afadin to tight junctions.

The close interconnection of the adherens- and tight junction proteins through their adaptors could explain why we could also observe a slight decrease of VE-cadherin (90 %) and occludin (85 %) protein levels upon drebrin depletion (Figure 20). Interestingly, the other examined components, PECAM-1 and the gap junction protein connexin43, which do not belong to the closely connected system of adherens and tight junctions but form separate adhesion structures, were not affected by loss of drebrin. It cannot be excluded that additional typical junctional components of the endothelium, such as JAM-A, JAM-B, JAM-C, claudin, or N-cadherin might also be affected - especially those known to bind also to afadin, for example JAM-A[224,261-263].

The hypothesis that it is not simply linkage of adhesion proteins to F-actin, which is important for junction stabilization, moreover reinforces why afadin is not able to stabilize nectin proteins by itself – it does indeed have an F-actin binding region, which was only once described in 1997[83] (Figure 6). This region comprises amino acid residues 1691-1829 and can thus bind to F-actin independently of drebrin. Nevertheless, drebrin knockdown leads to disappearance of nectins from junctions, even though they could in theory be still anchored to F-actin via afadin. Also a minimal construct, containing afadin's F-actin binding region coupled to afadin's PDZ region was not sufficient to prevent nectin's endocytosis (data not shown) while a construct containing drebrin´s F-actin binding region coupled to afadin´s PDZ region was able to do so. Either, drebrin simply binds to F-actin with higher affinity than afadin, or it has additional functions, potentially influencing the activity of afadin, which should be further investigated (see also V.5).

5 Mechanotransduction at cell-cell junctions

We cannot exclude that drebrin is involved in additional mechanisms of endothelial junction regulation, apart from linking nectin to actin filaments with high affinity or serving as a scaffolding protein. A concept of how protein interactions work includes the idea that one protein induces conformational changes of another protein, which is only then able to fulfil its function[264]. This could generally occur through binding of the proteins, leading to different orientations of amino acid side chains that may influence the tertiary structure of the whole protein[265].
A special kind of conformational change implies that proteins can be stretched via the actomyosin system of the cell. Since drebrin is able to interact with myosin II and attenuates

actin-myosin V binding, an influence of drebrin on actomyosin properties of junctional F-actin can be considered[266-268]. Recently, there has been rising acknowledge of mechanotransduction being an important regulatory mechanism at cell-cell junctions, although not much is known about how cells are able to transform a force stimulus into chemical responses. Major structures of eukaryotic cells, indispensable for mechanotransduction events are stress fibers, which consist of crosslinked F-actin filaments and bipolar bundles of the motor-protein myosin II. Once activated through phosphorylation of its light chain, myosin moves along F-actin filaments, leading to the contraction of the stress fiber[269]. Additionally, myosin II has been shown to localize to the junctional area and contributes to expansion of membranes, leading to junction formation by increasing the cell's contact zones[270-272]. Afterwards, myosin II activity drives the development of initial cell-cell contacts into more linear adherens junctions[273]. Furthermore, it was verified that its inhibition leads to instability of cortical F-actin bundles, thereby leading to junction weakening[274].

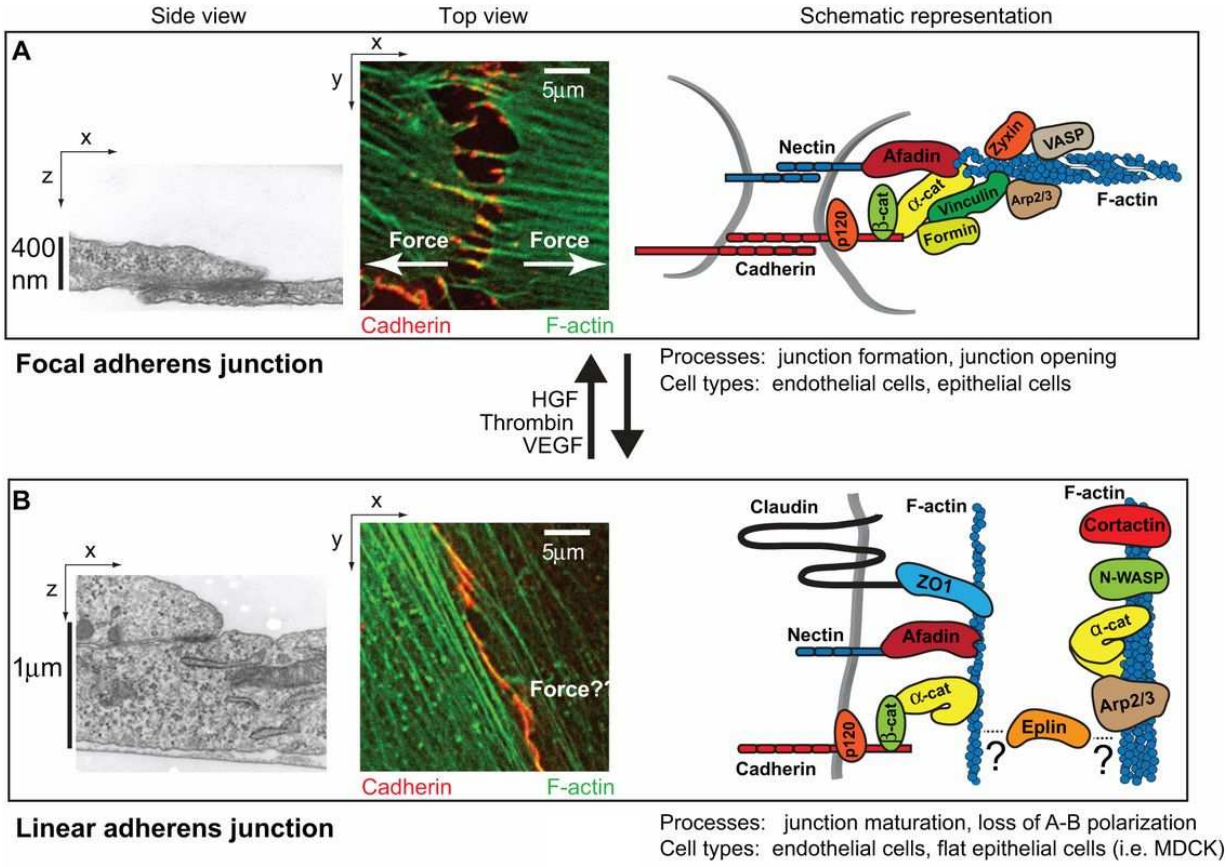


Figure 42 Different types of adherens junctions and their associated actomyosin systems
A During initial junction formation or remodelling of junctions, highly mobile, punctuate junctions are formed through assemblies of nectin and cadherin dimers. They are contacted by radial actomyosin fibers, leading to mechanical stretching of α-catenin, which enables binding of vinculin and thus enhancement of F-actin linkage. Various proteins are recruited to the initial junctions, such as zyxin, VASP, formins and the ARP2/3 complex, which bind to adaptor proteins or are associated with the actin cytoskeleton. **B** After myosin-dependent expansion of membranes and initial junctions into linear junctions, F-actin bundles run in parallel to the cell surface. At this stage, tension might not be of major importance, but there is need for investigations. Inflammatory hormones lead to reorganization of linear to focal adherens junctions, enabling passage of blood cells.
[151], with alterations

An especially interesting mechanism of actomyosin dependent mechanotransduction occurs during junction formation and remodelling in endothelial cells. While initial junctions (also referred to as focal adherens junctions) are formed through primary contacts made by nectins and cadherins present at lamellipodia of migrating cells, the adhesion proteins are contacted by perpendicular actomyosin bundles (Figure 42)[151]. These are connected to nectin and cadherin through their adaptor proteins, thus exerting a pulling force upon the entire junctional complex. Effects of these forces have recently been closer examined concerning the cadherin-catenin system: they bring α-catenin under tension, which leads to stretching of the molecule and reveals a cryptic binding site for vinculin[275,276] (Figure 42, 43). Thereby, vinculin can bind to α-catenin and connect it more strongly to the F-actin cytoskeleton, thus enhancing junction formation[303,304].

The idea of tension-induced unfolding of proteins is not new - stretching talin, a focal adhesion protein interacting with integrins, through the stress fibers attached to the focal adhesion sites has also been shown to reveal cryptic vinculin binding sites in the talin protein[277]. Recruitment and binding of vinculin to talin then enhances the maturation of focal adhesions via their additional linkage to F-actin[277].
Another example are spectrins and other major scaffolding proteins of red blood cells, which expose sterically shielded cysteine residues upon shear stress induced changes of the proteins[278]. These cysteines can be approached by in situ fluorescence labelling with dyes recognizing the cryptic cysteines after unfolding of the protein, which is a way to investigate the conformational changes of the protein[278].

A crucial point of interest referring to our findings on the nectin/afadin/drebrin system in endothelial cells is whether afadin/drebrin could have a function similar to that of α-catenin/vinculin. It would be certainly interesting to check whether the model of tension revealing cryptic binding sites of α-catenin, thus enabling binding of vinculin could be transferred to drebrin and afadin. Eventually, nectin-bound afadin binds with low affinity to F-actin, is stretched via the actomyosin system, and thus reveals its drebrin binding site. Drebrin could then bind simultaneously to actomyosin and afadin, leading to proper linkage and enhanced junction formation (Figure 43).
A possibility to check whether actomyosin-dependent tension is important for the formation of a functional drebrin/afadin/nectin complex is to inhibit myosin II contractility using blebbistatin[279]. It would be interesting to investigate, if the distribution of drebrin, afadin or nectin at cell-cell junctions is altered in blebbistatin-treated HUVEC at different timepoints of junction formation, hinting at an effect of myosin II-dependent contractility.

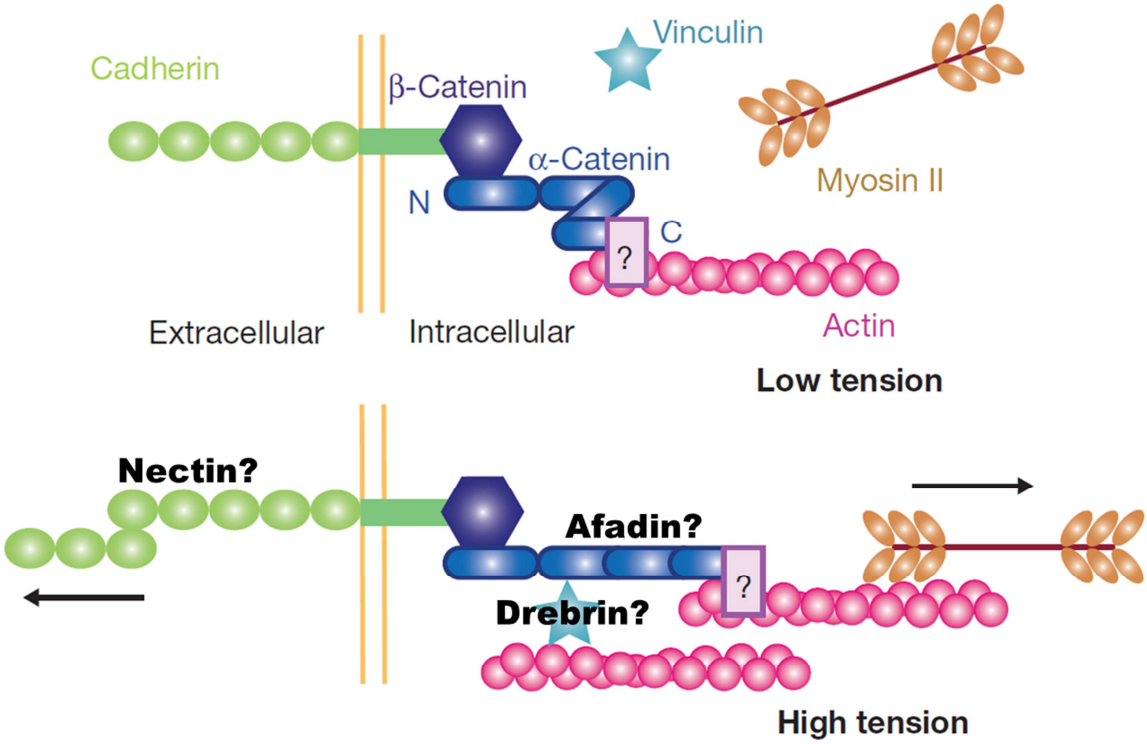


Figure 43 Tension-induced unfolding of proteins
Model of tension-induced vinculin binding to α-catenin. Under low tension conditions, the conformation of α-catenin prevents vinculin binding. Upon myosin II dependent stretching of α-catenin, the cryptic vinculin binding site is exposed, leading to enhanced anchorage of α-catenin to F-actin, which is required for junction formation. Putative similarities to drebrin/afadin/nectin are indicated and need to be investigated. N,C = N and C termini of α-catenin. A possible linker protein is indicated with a question mark. [275], with modifications

To further investigate the idea that drebrin might lead to (tension-induced) conformational changes of afadin, FRET (**F**luorescence **R**esonance **E**nergy **T**ransfer) experiments could be performed. Here, a protein (for example afadin) is labelled with two fluorescent tags, one of them serving as an acceptor (YFP, **Y**ellow **F**luorescent **P**rotein) and one as a donor (CFP, **C**yan **F**luorescent **P**rotein). Upon excitation, the donor fluorochrome releases energy of a certain wavelength. If the acceptor fluorochrome is located closer than 10 nm apart, this energy automatically excites the acceptor, leading to release of energy of a different wavelength than the donor[280]. Thus, depending on the wavelength detected, it can be concluded whether donor and acceptor were in close physical distance, or not.

It will be interesting to approach the subject of potential conformational changes of drebrin or afadin in general, and the potential influence of mechanotransduction events concerning nectin/afadin/drebrin in further experiments. Especially in endothelial cells that are situated in an environment where constant shear stress is created upon them through the bloodstream, the translation of mechanical forces into biochemical signals is of major importance for cellular processes. Preserving monolayer integrity is crucial for endothelial function, and since we showed drebrin to be important for maintaining this integrity, a role of drebrin in mechanotransduction events at cell-cell junctions could be worth to further investigate.

6 Drebrin's effects on microtubules

Beside F-actin, also microtubules are getting rising acknowledge of being involved in adherens junction maintenance. Comparable to F-actin, microtubules are dynamic structures that constantly assemble and disassemble at their plus end[25]. The minus end is typically anchored at a centrosome, which is mostly situated beside the nucleus[117]. Since the plus ends are usually oriented towards the cell periphery, they have been considered to influence cellular processes, mostly through delivering required proteins to certain peripheral structures, for example focal adhesions or adherens junctions[281]. At these structures, microtubules are often connected to the F-actin cytoskeleton[282]. This cytoskeletal crosstalk occurs in various processes and relies on proteins that can simultaneously bind to F-actin and microtubules, or their associated proteins[283,284].

Recently, drebrin has been shown to bind the microtubule plus end binding protein EB3 (**E**nd-**B**inding 3) in neuronal filopodia, connecting F-actin and microtubules in growth cones[176]. Disruption of this interaction leads to impaired extension of neurites, underlining the importance of their connection[176]. Furthermore, drebrin is specifically localized to the transitional zone of axonal growth cones, an area where networks of F-actin and microtubules overlap[285]. A similar function has been observed in columnar human intestinal Caco2 cells, where drebrin's interaction with EB3 is needed for cell elongation by linking the two cytoskeletal systems to each other[163]. These studies suggest that drebrin might have general functions in connecting microtubules to F-actin, and it would be interesting to see whether an analogous function could be observed in HUVEC.

Concerning functions of microtubules at adherens junctions, Stehbens *et al.* observed a reduction of E-cadherin accumulation at the junctional area after blocking microtubule extension[286]. The cadherin binding protein p120-catenin has been shown to directly interact with microtubule plus ends, tethering them to cadherin based adherens junctions, which seems to be a generally important mechanism for maintaining junction integrity[287,288]. With its ability to bind EB3 and afadin, drebrin could be involved in tethering microtubules to nectin-based adherens junctions, thereby influencing trafficking of structural or regulatory factors at these sites[289]. Alternatively, microtubules could have functions in positioning drebrin at the cell periphery. Disrupting microtubules with nocodazol could give a first idea whether they might play a role in locating drebrin to the junctional area.

7 Drebrin's function in other cell types

Drebrin has frequently been described as an F-actin interacting protein, which mostly exerts its functions through competing with other F-actin binding proteins. Since drebrin's effects on regulation of actin filament assembly were shown to be quite similar in entirely different cell types, ranging from neurons to intestinal epithelial cells, it would be interesting to find out whether also drebrin's effect of stabilizing nectin by linking it to F-actin could be a widespread phenomenon[168,170,194].

We started to test this hypothesis by using MDCK cells, a cell type that is known to establish well-defined adherens and tight junctions and is therefore an interesting candidate for studying junction properties[107,199]. We found that drebrin is expressed in those epithelial cells and established a knockdown of drebrin using the same siRNAs as in HUVEC. Since MDCK

cells are kidney epithelial cells derived from *Canis lupus familiaris*, a cocker spaniel, only one of the four drebrin siRNA sequences was complementary to the canine sequence[290]. Nevertheless, this siRNA was sufficient to create a knockdown of drebrin. We could observe a disappearance of nectin-1 and nectin-3 from the junctional area upon drebrin depletion and could detect similar functional impairments as in HUVEC via TER measurements (data not shown). This already serves as a hint that stabilization of nectin through drebrin is a widespread phenomenon not just limited to endothelial-, but at least also observed in epithelial cells.

An interesting cell type, explicitly mentioned as being remarkably rich in drebrin at the junctional area, are sertoli cells, which cultivate and embrace spermatids during their differentiation in the testis (Figure 44)[191]. These cells are known to exclusively express one of the four existing nectin isoforms, nectin-2, being the one we mainly described as being influenced by drebrin in this study[291]. In 2002, Ozaki-Kuroda *et al.* showed that loss of nectin-2 leads to defective sperm morphogenesis, due to problems in the connection between the cell-cell adhesion system and the cortical F-actin[91]. Since we found drebrin to be responsible for linking these two systems in HUVEC, it might be also an interesting candidate for linkage of nectin-2 to F-actin in sertoli cells. In addition, already existing studies on drebrin in rat sertoli cells revealed that it is needed for the proper distribution of cortical F-actin bundles[292,293].

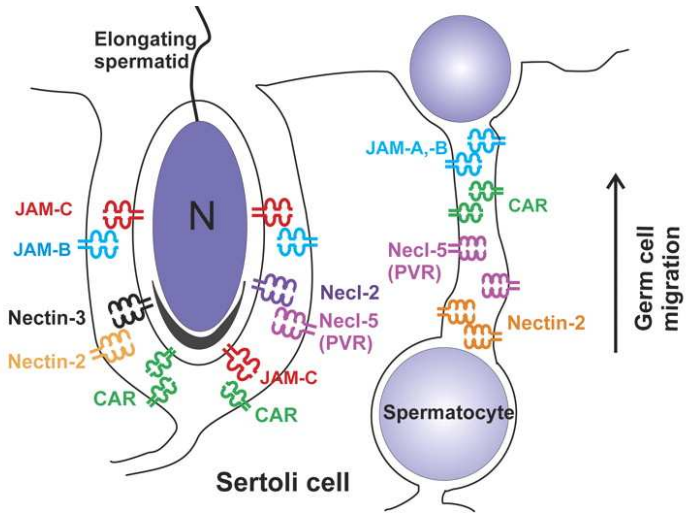


Figure 44 Sertoli-spermatocyte junctions
Germ cells develop in the seminiferous epithelium of the adult testis while they migrate through sertoli cells. Heterophilic junctions between germ cells and the surrounding sertoli cells are comprised of nectin-2 and nectin-3, as well as JAM and CAR proteins, all belonging to the immunoglobulin-like protein family.
Necl= Nectin-like, CAR= Coxsackie and Adenovirus Receptor, JAM= Junctional Adhesion Molecule
Modified from [294]

Our data showing an interaction between drebrin and the nectin/afadin system could also become important in other contexts such as maintaining the functionality of synapses.
Drebrin was a long time thought to be brain specific, being expressed in neurons where it localizes to dendritic spines, which are filopodia-like protrusions serving as the receptive region of synapses[295 296]. Drebrin is crucial for their maintenance by keeping the dendritic F-actin cytoskeleton dynamic and organized at the same time[245]. Consequently, loss of drebrin leads to disorganization of F-actin, and thus to reduced dendritic spine stability, which has been shown to decrease synaptic function[297-299]. Loss of dendritic spine plasticity due to diminished drebrin levels has attracted special attention regarding the hippocampus - the

part of the brain, which is important for learning and memory - since drebrin's loss from hippocampal neurons has been connected to Alzheimer's disease[183-185].

Synapses can be considered as a special kind of junction where the axon of one pre-synaptic neuron comes into functional contact with the dendrites of the post-synaptic neuron where the signal is transmitted to[300]. Two kinds of specialized junctions have been described at synapses: the first one is the synaptic junction, which is important for signal transmission through neurotransmitters[301] (Figure 45). The other type is morphologically similar to the non-neuronal adherens junctions, contains no synaptic vesicles, but intercellular adhesion proteins and is termed "puncta adherentia junction"[300].
Interestingly, nectin-1 and nectin-3 have been found to establish these neuron-specific junctions together with afadin, especially in the hippocampus – the same region of the brain where drebrin is also of significant importance[302]. When nectins, or respectively afadin, are inhibited, the formation of synapses is altered[302-304]. Recently, knockdown of nectin-3 has been shown to reduce dendritic spine density going along with learning deficiencies in mice, as well as their loss of memory[305].

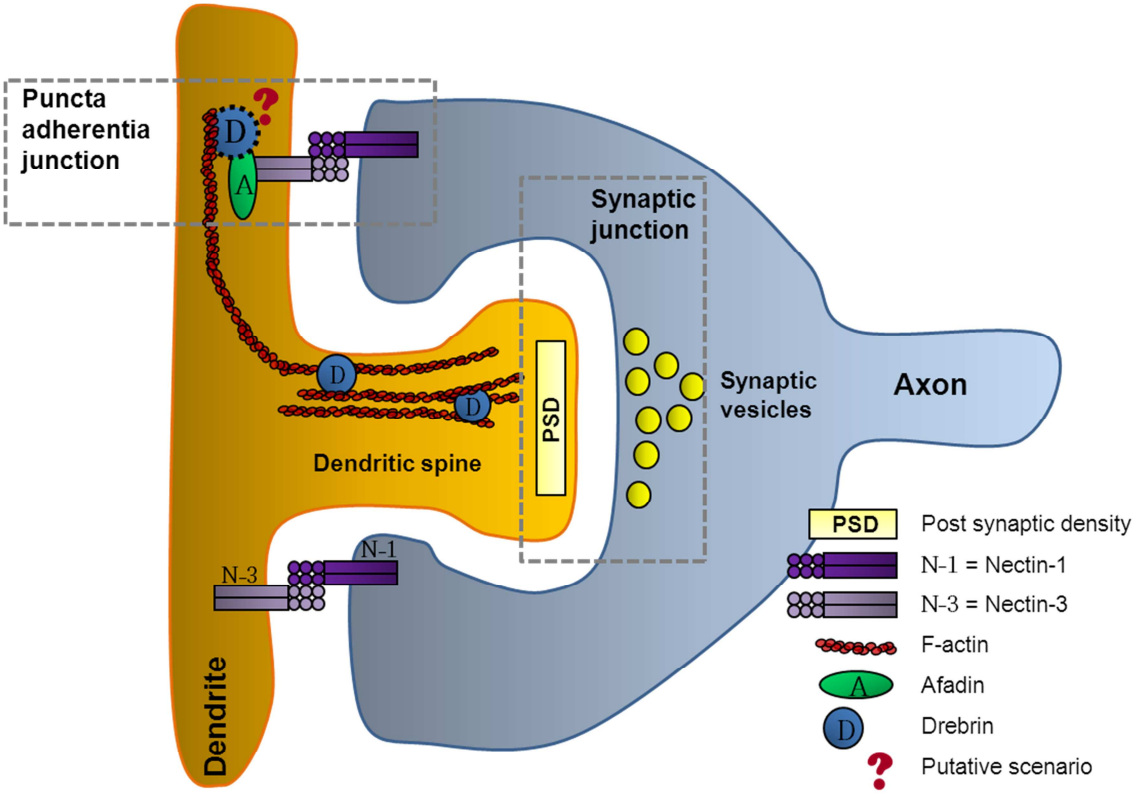


Figure 45 Schematic synapse with drebrin and nectin
Synapses connect pre-synaptic axons to post-synaptic dendrites, which receive the transmitted signal. Synaptic vesicles release neurotransmitters at the pre-synaptic side through Ca^{2+}-channels. These chemical compounds diffuse through the synaptic cleft and contact multiple receptors positioned at the dendritic side, forming the post synaptic density. Together, this functional apparatus is termed synaptic junction. Dendritic spines are rich in drebrin that keeps F-actin dynamic and maintains dendritic spine plasticity (schematic). Next to the dendritic spines, puncta adherentia junctions allow adhesion of the two neurons through interactions of nectin/afadin and cadherins (not shown). Potentially, nectins could also be stabilized at F-actin through drebrin and afadin.

Notwithstanding of the knowledge that dendritic spine maintenance is important for their function, the molecular mechanism of keeping them dynamic and functional is still poorly understood. The striking observation, that nectins, afadin, F-actin and drebrin have each individually been shown to be important for the function of synapses leads to the idea that an interaction between them, as we described it in HUVEC, could also be important in neurons. Taking into account that loss of each of these proteins has been connected to Alzheimer or learning deficiencies underlines this idea of them forming a functional network. To prevent diseases linked to synaptic dysfunction, both types of neuronal junctions need to work accurately and drebrin could be important for each of them. Potentially, drebrin plays a dual role in dendrites: it interacts with F-actin at the center of dendritic spines to maintain synaptic junction plasticity as thoroughly described before - and additionally, drebrin could link nectins to F-actin at puncta adherentia junctions, thus preserving anchorage of neurons to each other in a fashion similar to what we described in endothelial cells (Figure 45).

VI Summary

The human endothelium forms a permeable barrier between the blood stream and surrounding tissue, strictly governing the passage of immune cells and metabolites. Regulation of cell-cell contact dynamics between endothelial cells is essential for the maintenance of its function and the vascular integrity. Besides other junctional systems, the accurate adhesion of cells is mainly dependent on the adherens junctions system, which is predominantly composed of intercellular adhesion proteins such as VE-cadherin and nectin, as well as their associated proteins. Drebrin, a protein mainly expressed in neurons, has been included in the growing list of “junction associated” proteins, but its exact role in adherens junction dynamics has so far been unclear.

In this study, we show that knockdown of drebrin leads to functional impairments of endothelial monolayers, as demonstrated by a decrease of transendothelial electrical resistance (TER) and rupturing of HUVEC monolayers cultured under constant unidirectional flow conditions. The observed weakening of cell-cell contacts upon drebrin depletion is characterized by a specific and complete loss of nectin from adherens junctions, due to its endocytosis and subsequent degradation in lysosomes. The importance of drebrin for nectin’s presence at junctions is underlined by rescue experiments, where transient re-expression of siRNA-insensitive drebrin stabilizes nectin at the junctional area. To support the fact that the phenotype is indeed due to loss of nectin, we established a knockdown of all endothelial nectin isoforms, which resulted in an even more pronounced phenotype than drebrin knockdown.

Conducting co-immunoprecipitation experiments, we could show that drebrin does not interact with nectin directly but with its most prominent intracellular binding partner, afadin. Direct binding of drebrin and afadin is mediated through their polyproline and PR1-2 regions as shown by GST-pulldown experiments using bacterially expressed domain constructs of both proteins. Concomitantly, confocal microscopy studies revealed strong binding between the two proteins on a subcellular level: drebrin’s polyproline region fused to a mitochondrial targeting signal is sufficient to relocalize afadin towards the outer membrane of mitochondria. Due to its association with nectin, afadin is also affected by drebrin knockdown – its mobility at the junctional area is enhanced, as shown by FRAP experiments, even though it still localizes to junctions, probably through binding other proteins, such as ZO-1 or α-catenin.

Furthermore, we could demonstrate that drebrin maintains junctional integrity through its ability to link the nectin/afadin system to the cortical F-actin network. Being equipped with an F-actin binding module (CC-region) and the afadin-binding polyproline region, it anchors afadin to F-actin. Simultaneously, afadin binds to nectin through its PDZ region, resulting in a chain of protein interactions: F-actin-drebrin-afadin-nectin, which indirectly stabilizes nectin at the F-actin network (Figure 46A). Evidence, verifying that linkage of nectin to F-actin is essential for monolayer integrity is provided by rescue of junctional nectin, under knockdown of both drebrin and afadin, through overexpression of minimal constructs containing exclusively afadin´s PDZ region coupled to drebrin´s F-actin binding region, or lifeact (Figure 46C). Drebrin, containing binding sites for both afadin and F-actin, is thus uniquely equipped to stabilize nectin at endothelial junctions, thereby preserving endothelial integrity.

Altogether, these results contribute to the current understanding of how junctions are regulated in the endothelium under vascular flow, especially elucidating the significance of nectins. In particular, the newly identified interaction between drebrin and afadin, which establishes the necessary linkage of nectins to cortical F-actin, is shown to be crucial for junctional integrity.

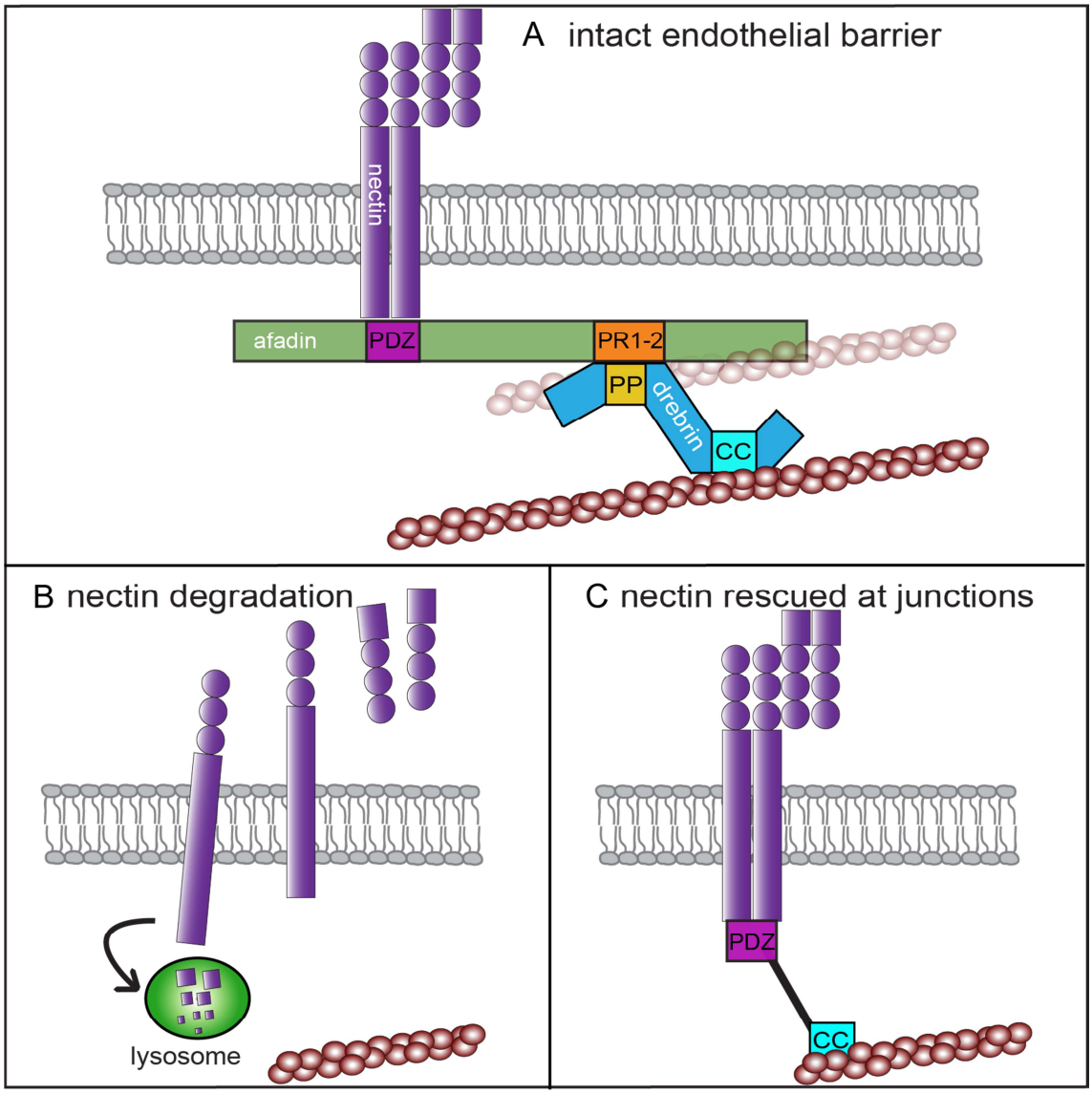


Figure 46 Summary and model
A Drebrin binds to F-actin with high affinity through its coiled coil region (CC) and via its polyproline region (PP) to afadin's PR1-2 region. Afadin's PDZ domain simultaneously binds nectin. Nectin is thus stabilized at the junctional region and can form cis- and trans-oligomers, leading to endothelial integrity. **B** Absence of drebrin and/or afadin leads to loss of nectin´s indirect anchorage to the actin cytoskeleton. Nectins are internalized and degraded by the lysosome, resulting in impaired endothelial integrity. **C** Nectin can be stabilized at junctions even in the absence of both afadin and drebrin upon overexpression of constructs containing the afadin PDZ region and drebrin´s CC region, which re-establish proper anchorage to the actin cytoskeleton.

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VIII List of tables

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X List of Abbreviations

<	smaller as
>	larger as
℃	degree celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
A	Adenine
AA	Aminoacid
ad	add
ADF-H	Actin Depolymerizing Factor-Homology
AF6	ALL-1 fusion partner from chromosome 6
AJ	Adherens Junction
Amp	Ampicillin
ANOVA	Analysis of variance
APS	Ammonium persulfate
Arp2/3	Actin-related protein 2/3
ARPE19	Arising retinal pigment epithelia (RPE) cell line
ATP	Adenosinetriphosphate
bp	Basenpaar
BSA	Bovine Serum Albumin
C	Cytosine
CaCl2	Calcium cloride
Caco2	Human colon epithelial cancer cell line
ca.	circa
cAMP	cyclic Adenosine Mono Phosphate
CapZ	F-actin plus end capping protein
CC	Coiled-coil
Cdc42	Cell division cycle 42 GTP binding protein
cDNA	copy DNA
cm	centimeter
Cx43	Connexin43
CXCR4	C-X-C chemokine receptor type 4
DBN1	gene coding for drebrin protein
d	day
dd	double distilled
dH_2O	distilled water
DIL	Dilute region
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
Drebrin	Developmentally Regulated Brain Protein
Drebrin A	Drebrin, adult
Drebrin E	Drebrin, embryonic
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EB3	End Binding 3
EC	Endothelial cell
E-cadherin	Epithelial-cadherin

ECGM	Endothelial Cell Growth Medium
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EEA1	Early endosome antigen 1
eGFP	enhanced GFP
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N, N, N', N'*-tetraacetic acid
EPLIN	Epithelial protein lost in neoplasm
et al.	and others (lat.: „et alteri“)
FA	Formaldehyde; focal adhesion
FAB	F-actin binding
F-actin	Filamentous-actin
FD	fast digest
FHA	Forkhead associated domain
FCS	Fetal calf serum
Flag	peptide sequence DYKDDDDK
FRAP	Fluorescence Recovery After Photobleaching
FRET	Fluorescence Resonance Energy Transfer
G	Guanine, Germany
g	gram
g	relative centrifugal force
G-actin	Globular-actin
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine 5'-Triphosphate
GUK	Guanylate kinase
h	hour
HA	Hemagglutinin, peptide sequence YPYDVPDYA
HRP	Horseradish peroxidase
HSC-39	Human signet ring cell gastric cancer cell line
HUVEC	Human umbilical vein endothelial cell
HVEM	Herpesvirus Entry Mediator
ICAM-1	Intercellular Adhesion Molecule-1
IF	Immunofluorescence
Ig	Immunoglobulin
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JAM	Junctional adhesion molecule
Kan	Kanamycin
kbp	kilo base pairs
kDa	kilo Dalton
l	liter
l-afadin	large-afadin
LAMP1	Lysosomal-associated membrane protein 1
LB	Lysogeny broth (Luria-Bertani)
M	molar
MBP	Maltose binding protein
mAbp1	Mammalian actin-binding protein 1
mA	Milli Ampere
MACS	Magnetic Cell Sorting System
MAR	Minimal Actin Remodeling
MCS	Multiple Cloning Site

MDCK	Madin-Darby Canine Kidney
mg	milligram
min	minute
mito	mitochondrial
ml	millilitre
MLC	Myosin-Light-Chain
MLCK	Myosin-Light-Chain Kinase
MLLT4	myeloid/lymphoid or mixed-lineage leukemia translocated to 4
mM	millimolar
mRNA	messenger-RNA
MTOC	Microtubule-Organizing Center
MW	Molecular Weight
ms	millisecond
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
Necl5	Nectin-like
ng	nanogram
NIH3T3	fibroblast cell line
nM	Nanomolar
NO	Nitric oxide.
nt	non-targeting, neurotransmitter
OD	Optical density
PAJ	Puncta adherentia junction
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS with Tween-20
PCR	Polymerase chain reaction
PDZ	Post synaptic density protein Drosophila disc large tumor suppressor Zonula occludens-1 protein
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PGI_2	Prostacyclin, effective vasodilator
pM	Pikomolar
pmol	Pikomol
PP	Polyproline
PR1-2	Proline-Rich region 1-2
PR3	Proline-Rich region 3
PVRL	Poliovirus receptor related
RA	Ras associated domain
Rab22	Ras-associated binding 22
Rac	Ras-related C3 botulinum toxin substrate
Rap1	Ras-related protein 1
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
s	second
s-afadin	small-afadin
SD	Standard deviation
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
SH3	Src-homology 3 domain

siRNA	small interfering RNA
SJ	Synaptic Junction
SV 80	Simian virus 80
t1/2	half time (of recovery)
T	Thymine
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TBST	TBS with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
TER	Transendothelial Electrical Resistance
TfB	Transformation buffer
TGF-β	Transforming growth factor beta
TJ	Tight Junction
tPA	Tissue plasminogen activator
Tris	Tris-(hydroxymethyl)-aminomethane
TRITC	Thiol-reactive tetramethylrhodamine-5-(and-6)-isothiocyanate
U	Uracil
UV	Ultraviolet
V	Volt
VASP	Vasodilator-stimulated phospho-protein
VE-cadherin	Vascular Endothelial-cadherin
VEGF	Vascular Endothelial Growth Factor
VH1–3	Vinculin Homology 1–3
WASP	Wiskott–Aldrich syndrome protein
YFP	Yellow Fluorescent Protein
ZO-1	Zonula Occludens-1

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XII Publications

Parts of the content presented here have been published:

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Posters with parts of the content have been presented at:

The French society for cell biology: Invadopodia, Podosomes and Focal Adhesions in Tissue Invasion, Hyeres, France (26.-30.9.2009)

33rd Annual Meeting of the German Society for Cell Biology (DGZ), Regensburg, Germany (10.-13.3.2010)

The invadosome consortium: Podosomes, Invadopodia and Focal Adhesions in Physiology and Pathology, Madrid, Spain (18.-21.9. 2011)

International Meeting of the German Society for Cell Biology: Molecular concepts in epithelial differentiation, pathogenesis and repair, Leipzig, Germany (7.-10.11. 2012)

ASCB - Annual Meeting of the American Society for Cell Biology, San Francisco, USA (15.-19.12. 2012)

1st UKE Light Microscopy Symposium, umif, Hamburg, Germany (5.-7.2.2013)
Acknowledged with 1st poster price

XIII Declaration on oath

I hereby declare, on oath, that I have written the present dissertation on my own and have not used other than the acknowledged resources and aids.

Hamburg, August 2013

Kerstin Rehm