Drebrin preserves endothelial integrity by stabilizing nectin at adherens junctions

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Summary
Regulation of cell–cell contacts is essential for integrity of the vascular endothelium. Here, a critical role of the F-actin-binding protein drebrin in maintaining endothelial integrity is revealed under conditions mimicking vascular flow. Drebrin knockdown leads to weakening of cell–cell contacts, characterized by loss of nectin from adherens junctions and its subsequent lysosomal degradation. Immunoprecipitation, FRAP and mitochondrial re-targeting experiments show that nectin stabilization occurs through a chain of interactions: drebrin binding to F-actin, interaction of drebrin and afadin through their polyproline and PR1-2 regions, and recruitment of nectin through the PDZ region of afadin. Key elements are modules in drebrin that confer binding to afadin and F-actin. Evidence for this was obtained using constructs containing the PDZ region of afadin and the F-actin-binding region of drebrin or to lifeact, which restore functional nectin under knockdown of drebrin or both drebrin and afadin. Drebrin, containing binding sites for both afadin and F-actin, is thus uniquely equipped to stabilize nectin at endothelial junctions and to preserve endothelial integrity under vascular flow.

Key words: Actin cytoskeleton, Afadin, Cell–cell junctions, Drebrin, Endothelial cells, Nectin

Article

Introduction
Integrity of the endothelial monolayer is essential for the maintenance of the blood-vessel barrier. Opening and closure of contacts between endothelial cells regulate the eflux of fluid and transmigration of blood cells into surrounding tissues (Ley et al., 2007; Vestweber, 2007; Yuan et al., 2012) and vice versa (van Zijl et al., 2012; Woodfin et al., 2011). The dynamics of cell–cell contacts thus play important roles in physiological vessel function. Consequently, cell–cell contact dysfunctions lead to pathological scenarios including atherosclerosis, chronic inflammation or the formation of edema or hemorrhages (Dejana and Giampietro, 2006; Taguchi et al., 2011). Tight junctions are formed by transmembrane proteins which are intracellularly linked to cytoskeletal complexes (Hartsch and Nelson, 2008; Lampugnani and Dejana, 1997). These connections to the actin cytoskeleton basically enable cell–cell contact dynamics (Duffy et al., 2002; Giepmans, 2004; Kovacs et al., 2002; Prochnow and Dermietzel, 2008; Stehbens et al., 2006; Taguchi et al., 2011). Tight junctions are formed by homophilic interaction between claudins or occludins (Dejana, 2004) and are linked through ZO (zonula occludens)-1 to the actin cytoskeleton (Schneeberger and Lynch, 2004). In gap junctions, connexins assemble into hexameric clusters, which form small pores within the cell membrane and regulate the exchange of ions and metabolites (Bazzoni and Dejana, 2004; Prochnow and Dermietzel, 2008). In endothelial cells, adherens junctions consist of VE (vascular endothelial)-cadherin complexes, which include p120-catenin and β-catenin as intracellular partners (Vestweber et al., 2009). Other adherens junction components include nectins, which directly associate with the scaffolding protein afadin (Reymond et al., 2000; Takahashi et al., 1999). Afadin interacts with nectins and F-actin through independent domains, suggesting that it is involved in linking nectin-based adhesion sites to the actin cytoskeleton (Mandai et al., 1997). Through additional protein interactions (Asada et al., 2003; Mandai et al., 1997; Ooshio et al., 2004) afadin associates directly (Pokutta et al., 2002) and indirectly with α-catenin. PECAM-1 (platelet endothelial cell adhesion molecule-1), another adhesion protein contributing to cell–cell adhesion, although outside of defined junctional structures, can be recruited via β-catenin (Ilan et al., 2000).

The exact progression of cell–cell junction formation is still unresolved. However, one of the earliest events is the recruitment of nectins to adherens junctions (Takai and Nakashima, 2003). Four isoforms of nectin are known (Takai et al., 2008a), of which two, nectin-2 and -3, are expressed in endothelial cells (Lopez et al., 1998). Nectins form trans-dimers between cells, and inhibition of dimer formation by dominant negative nectin-1 inhibits adherens junction formation in MDCK and NIH3T3 cells (Brakeman et al., 2009). The importance of the nectin–afadin system for cell–cell contacts has also been demonstrated in vivo, as afadin-deficient mice show highly impaired adherens and tight junction formation in the ectoderm and in embryoid bodies (Ikeda et al., 1999). Nectins cooperate with cadherin in the regulation of the actin cytoskeleton at sites of cell adhesions. However, the exact role of nectins in mature junctions remains unknown.

In this study, we show that the current model of afadin–nectin regulation is incomplete and reveal the F-actin binder drebrin as an
important regulator of nectin stability in human endothelial cells. Previously, the drebrin isoform E2 has been localized to adherens junctions in human endothelial cells (Peitsch et al., 2005) and to gap junctions in murine astrocytes and Vero cells (Butkevich et al., 2004). Drebrin in vitro shows no actin severing, nucleating or bundling activity (Ishikawa et al., 1994), although binding of drebrin to F-actin alters the helical twist of actin filaments, opposite to that induced by coflin (Sharma et al., 2011). Drebrin binds profilin (Mammoto et al., 1998) and was described to compete with tropomysin and fascin for F-actin binding (Ishikawa et al., 1994), while drebrin overexpression alters actin cytoskeletal organization and cell morphology (Hayashi and Shirao, 1999). Here, we show that drebrinE2 is critical for the integrity of endothelial cell–cell contacts, and that this function is mediated through nectin stabilization at adherens junctions.

Results

Drebrin knockdown leads to functional impairment of endothelial cell–cell junctions

To assess the functional relevance of drebrin in endothelial cell–cell interactions, we performed shRNA-based knockdown of drebrinE2 in HUVEC. Cells were transfected with shRNA and immunostained for drebrin. In untransfected cells, drebrin showed cytosolic punctate staining, as well as enrichment at cell–cell contacts. Cells expressing the drebrin-specific shRNA construct showed a reduction in the drebrin signal but no apparent defects in endothelial integrity. However, preliminary experiments showed that drebrin-depleted cells show a tendency for dissociation under mechanical stress (Fig. 1A–C), pointing to possible weakening of cell–cell contacts (Note: the drebrin shRNA construct enabled bicistronic expression of GFP, thus allowing clear identification of transfected cells. In subsequent experiments, siRNA transfection of HUVEC monolayers was established, reaching >95% of cells; not shown.)

To analyze this phenomenon under more physiological conditions, drebrin-depleted cells were subjected to lateral shear stress by fluid flow. This line of experiments was also performed to unveil potential impairments in cell–cell adhesion that might not have been apparent under static conditions. Treatment of HUVEC with a pool of four siRNAs (or combinations of each time two siRNAs) led to a ~90% reduction of drebrin expression after 3 days (supplementary material Fig. S1A; Fig. S2G–I). HUVEC transfected with a pool of drebrin-specific siRNAs or control siRNA were seeded at

Fig. 1. Knockdown of drebrin leads to functional impairment of endothelial cell–cell junctions.

(A–C) Confocal micrograph of HUVEC treated with drebrin-specific shRNA, bicistronically expressing GFP (B), and stained for drebrin (A); merged image in C. The inset in A shows an enlarged view of drebrin at cell junctions. Note the disassembly of junctions between drebrin-shRNA-expressing cells.

(D–G) Images of HUVEC monolayers, treated with drebrin-specific siRNA pool (D,F) or control siRNA (E,G), and seeded in μ-slides. Cells were submitted to constant fluid shear stress for 1 day (D,E) or 3 days (F,G). The enlarged images (Di–Gi) show the boxed regions of D–G. Arrows indicate the direction of flow.

(H) TER measurement of monolayers treated with the indicated reagents. 3×10 monolayers were analyzed for each treatment and values are means ± s.e.m. of three separate experiments; ****P<0.0001 using one way ANOVA with Bonferroni’s post test. (I–N) Confocal micrographs of monolayers treated with the indicated reagents and stained for VE-cadherin (I–K) and occludin (L–N). Scale bars: 10 μm (A–C, I–N), 100 μm (D–G). For specific values, see supplementary material Table S1. See also supplementary material Fig. S1.
confluence in flow-through chambers and exposed to unidirectional medium flow, with a shear stress of 15 dyn/cm², mimicking flow conditions in medium size vessels (Fig. 1D–G) (dela Paz et al., 2012). At day 1, no differences were detected between drebrin siRNA or control monolayers (Fig. 1D,E). At day 3, cells within both monolayers had developed a spindle-shaped morphology and aligned with the flow (Fig. 1F,G), an organization typifying a potentially athero-protective situation (Dai et al., 2004). Strikingly, drebrin-siRNA-treated monolayers consistently showed numerous ruptures between cells (Fig. 1F), whereas control monolayers remained intact (Fig. 1G). To test basal monolayer integrity, and to detect potential pre-existing defects in cell–cell adhesion under static conditions, transendothelial electrical resistance (TER) measurements were performed (Fig. 1H–N). Cells were transfected with drebrin siRNA or control siRNA and seeded at confluence on Transwell filters. TER measurements were performed after 3 days. Monolayers of drebrin-depleted cells showed a significant (ca. 25%) reduction in TER. As a positive control, addition of 2 mM EGTA, known to disrupt homophilic interactions between cadherins and occludins (Cereijido et al., 1978), led to a further reduction (ca. 50%). These results show that reducing drebrin expression in endothelial cells leads to impairment of cell–cell contact integrity under both static and flow conditions.

**Drebrin stabilizes nectin at adherens junctions**

Decreased monolayer integrity in drebrin knockdown cells pointed to possible effects on the regulation or localization of junctional components. Therefore, we screened drebrin knockdown and control cells for the presence of junctional proteins, including connexin-43, VE-cadherin, nectin-2, occludin and PECAM-1 by immunofluorescence (Fig. 2A–E) and western blotting (Fig. 2F). In both approaches, nectin-2 signals were greatly diminished (Fig. 2C,F), compared to controls (supplementary material Fig. S2). By contrast, connexin-43, VE-cadherin, occludin and PECAM-1 remained localized at cell junctions and their overall expression was unchanged (Occasional decreases of 10–20% in total VE-cadherin and occludin were observed. However, junctional localization of both proteins was preserved in all experiments). Compararable results were achieved when drebrin knockdown was performed with two combinations of siRNAs (supplementary material Fig. S2G–I). In all cases, no alterations in the level or arrangement of cortical actin were discernible.

In addition to nectin-2, also nectin-3 is expressed in endothelial cells (Lopez et al., 1998). Comparable to nectin-2, also nectin-3 signals were greatly diminished at cell–cell junctions (supplementary material Fig. S4), pointing to a general effect of drebrin on endothelial nectin isoforms.

### Fig. 2. Drebrin knockdown leads to loss of nectin-2 from cell–cell junctions which can be rescued through overexpression of drebrin–GFP. (A–E) Confocal micrographs of HUVEC transfected with a pool of four drebrin-specific siRNAs. Monolayers were stained for F-actin (A–E), drebrin (to demonstrate successful knockdown by absence of drebrin staining, not shown) connexin-43 (A), VE-cadherin (B), nectin-2 (C), occludin (D) and PECAM-1 (E). The insets in A–E show enlarged views of boxed areas. Note specific loss of nectin-2 from cell–cell junctions in drebrin knockdown cells. (F) Western blot analysis of lysates from HUVEC transfected with drebrin-specific 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 indicated. (G–K) Junctional localization of nectin-2 is rescued by overexpression of an siRNA-insensitive drebrin-GFP construct (H). Confocal images of HUVEC cultured for 3 days after drebrin siRNA transfection, then co-transfected with siRNA-insensitive drebrin-GFP (G) or GFP (I,J). (K) Intensity of nectin-2 at 100 junctional areas from three experiments was measured between two drebrin–GFP-expressing cells. Values were analyzed (compared to control) using one way ANOVA with Bonferroni’s post test and are depicted as means ± s.e.m. (n=3); ***P<0.001; n.s., not significant. For specific values, see supplementary material Table S1. Scale bars: 10 μm. See also supplementary material Figs S2, S3.
concentrated on nectin-2. As a rescue experiment, we overexpressed an siRNA-insensitive drebrin-GFP construct with multiple silent mutations in the regions complementary to all four sequences present in the siRNA pool (see Materials and Methods) for 16 hours in cells that had been transfected with drebrin siRNA 3 days before. Fluorescence measurements showed that intensities of nectin-2 at cell junctions restored to ca. 80% of normal levels upon re-expression of siRNA-insensitive drebrin–GFP (Fig. 2G–K). Collectively, these results demonstrate that drebrin is required to stabilize nectin at cell–cell junctions.

To address the question whether stabilization of nectin occurs at the mRNA or protein level, we performed RT-PCR from mRNA of cells depleted for drebrin (3 days after transfection with siRNA) or treated with luciferase siRNA as a control. Subsequently, PCR was performed with the obtained cDNA using intron-spanning primers, specific for the nectin-2 sequence, to distinguish the newly synthesized cDNA from contaminations of genomic DNA. We find that nectin-2 mRNA is present in both drebrin knockdown cells and controls (Fig. 3A). These data argue for a stabilizing effect of drebrin on nectin not on the mRNA, but on the protein level.

As drebrin knockdown could lead to increased internalization and degradation of nectin, we next expressed a construct of EEA1 fused to GFP (GFP–EEA1; as a marker for endocytic vesicles) in cells treated for 2 days with drebrin siRNA and stained for endogenous nectin-2. At this time point, nectin-2 was still localized at cell–cell junctions, but also in prominent vesicular accumulations that often co-localized with GFP–EEA1 (Fig. 3B–D). These data point to increased internalization of nectin by endocytosis in the absence of drebrin.

To address a possible degradation of nectin through lysosomes, their activity was blocked using the lysosome inhibitor chloroquine [100 μM (Brown et al., 1984; Wibo and Poole, 1974)] in HUVEC treated for 3 days with drebrin siRNA. Chloroquine treatment resulted in increased formation of large, LAMP-1-positive vesicular structures, comparable to the chloroquine-dilated lysosomes reported earlier in human ARPE-19 epithelial cells (Chen et al., 2011). At time point 0 hours of the treatment, no significant signals of nectin-2 were detected at LAMP-1-positive lysosomes (Fig. 3E–G), but increased accumulation of nectin-2 was observed at time points 5 hours (Fig. 3H–J) and 12 hours (Fig. 3K–M). Collectively, these results point to increased endocytosis and lysosomal degradation of nectin in the absence of drebrin.

**Drebrin does not affect junctional localization, but junctional mobility of afadin**

Afadin is an interaction partner of nectin at adherens junctions (Takai et al., 2008a). Interestingly, afadin levels at cell–cell

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**Fig. 3. Nectin is degraded in lysosomes in the absence of drebrin.**

(A) Agarose gel stained with SYBR Safe, showing PCR products obtained with nectin-2-specific intron-spanning primers, with templates as indicated. Size indicated in base pairs on left. Note comparable amplification of PCR product from cDNA of drebrin knockdown cells [siRNA pool (lane 2) and luciferase siRNA-treated controls (lane 1)].

(B–D) Confocal micrographs of HUVEC treated with drebrin-specific siRNA (pool) for 2 days, overexpressing GFP–EEA1 (B) and stained for endogenous nectin-2 (C); merged image shown in D. (E–M) Confocal micrographs of HUVEC treated with drebrin-specific siRNA (pool) for 3 days, stained for nectin-2 (E,H,K) and lysosomal marker LAMP-1 (F,J,L); merged images shown in G, J and M. Cells were treated for 5 hours and 12 hours with lysosome inhibitor chloroquine, resulting in gradual accumulation of LAMP-1-positive compartments that also acquire nectin-2. Insets show enlarged images of the boxed regions. Outlines of individual cells are indicated by dashed white lines. Scale bars: 10 μm.
junctions (Fig. 4A–F) and in cell lysates were not strongly affected by drebrin depletion (Fig. 4J). This might be a consequence of afadin interacting with other junctional molecules including ZO-1 (Ooshio et al., 2010) or α-catenin (Pokutta et al., 2002), which both colocalize with afadin at endothelial junctions (supplementary material Fig. S5). Notably, drebrin is still recruited to junctions of cells treated with afadin-specific siRNA (Fig. 4G), indicating that afadin is not required to localize drebrin to the cell cortex.

To investigate potentially more subtle effects of drebrin on junctional afadin, we conducted FRAP experiments with drebrin knockdown cells, expressing either afadin–GFP or ZO-1–GFP. Interestingly, the halftime recovery of afadin–GFP, but not of ZO-1–GFP, was decreased in drebrin-depleted cells (Fig. 4K,L). In addition, the mobile fraction of afadin–GFP (Fig. 4M), but not of ZO-1–GFP (Fig. 4N), at junctions was slightly increased upon drebrin knockdown (82% for drebrin knockdown cells, compared to 72% for controls). Still, the presence of afadin was necessary for junctional localization of nectin as the knockdown of afadin resulted in greatly diminished cellular levels of nectin-2, and concomitant loss of nectin from adherens junctions (supplementary material Fig. S5), comparable to previous publications (Kurita et al., 2011; Ooshio et al., 2010; Takai and Nakanishi, 2003).

To determine whether nectin and afadin are involved in the regulation of shear stress resistance by drebrin (Fig. 1D–G), we performed flow chamber experiments with nectin-2, nectin-3 and afadin-siRNA-treated cells. Both knockdown of nectins (Fig. 5A,B) and of afadin (Fig. 5C,D) led to rupturing of monolayers. Measurements of the area of flow-induced holes showed that afadin knockdown led to results comparable to drebrin knockdown, while combined knockdown of nectin-2 and -3 showed an even more pronounced effect (Fig. 5I). Compromised integrity of drebrin-depleted monolayers could be suppressed by overexpression of FLAG–nectin-2 (Fig. 5E,F). Interestingly, quantification of FLAG–nectin-2 at junctions in drebrin-depleted cells (14.24±3.24 a.u.) showed similar values compared to nectin in luciferase controls (12.66±2.05 a.u.). This may reflect a steady resupply of overexpressed FLAG–nectin-2 to the junctional area. Similar results were obtained in TER measurements using cells treated with nectin-2- and -3-specific and afadin-specific siRNA (Fig. 5J). Treatment with either set of siRNAs led to a similar reduction compared to drebrin knockdown cells. Moreover,
expression of FLAG–nectin-2 in drebrin-depleted monolayers led to a partial rescue of TER. Collectively, these results indicate that drebrin, afadin and nectin are all involved in the regulation of endothelial integrity.

The polyproline region of drebrin binds afadin

To address possible interactions between drebrin, nectin and afadin, we next performed co-immunoprecipitations of cells transfected with respective GFP-fused constructs (Fig. 6). (Note 1: levels of overexpressed versus endogenous protein in drebrin–GFP-expressing cells were 12.3, as determined by immunofluorescence, western blotting and ImageJ analysis; note 2: we first probed for direct binding partners using stringent buffer containing 0.1% SDS.) Consistent with their abilities for homo-dimerization (Lopez et al., 1998; Peitsch et al., 2001; Takai et al., 2008b) drebrin–GFP co-precipitated cellular

Fig. 5. Depletion of afadin or nectin leads to rupture of monolayers under flow. (A–H) Images of HUVEC monolayers grown in μ-slides, treated with the indicated siRNAs (siRNA pool in case of drebrin), with simultaneous overexpression of FLAG–nectin-2 (E,F). Cells were submitted to constant fluid shear stress for 1 day (A,C,E,G) or 3 days (B,D,F,H). (Ai–Hi) Enlarged images of the boxed regions in 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). (I) Quantification of area lacking cells, with total area of the picture set to 100% (each value is the mean of 10 images from three experiments). Arrows show direction of flow. (J) TER measurement of monolayers treated with the indicated siRNAs. Additional expression of FLAG–nectin-2 is indicated by ‘+’, 3×10 monolayers was evaluated for each treatment. Values were analyzed using one way ANOVA with Bonferroni’s post test and are depicted as means ± s.e.m. of three experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n.s., not significant. Scale bars: 100 μm (A–H), 10 μm (Ai–Hi). For specific values, see supplementary material Table S1. See also supplementary material Fig. S4.

Fig. 6. Drebrin interacts with afadin. (A,B) Western blots of lysates of HUVEC expressing drebrin–GFP or GFP, immunoprecipitated with anti-GFP antibody. (C) Western blots of lysates of HUVEC immunoprecipitated using afadin-, nectin-2- or drebrin-specific antibodies. Blots were developed using antibodies as indicated. Dashed lines indicate that lanes were not directly adjacent on original blots; all experiments were performed at least three times, yielding comparable results. Note that anti-GFP antibody was covalently linked to beads and IgG bands are thus absent from lanes of anti-GFP immunoprecipitations. Control IgG was not bound to beads and therefore appears as heavy and light chains in the controls.
drebrin (Fig. 6A), and nectin-2–GFP co-precipitated cellular nectin-2 (supplementary material Fig. S6A). However, drebrin–GFP did not co-precipitate nectin-2 (Fig. 6A), and nectin-2–GFP did not co-precipitate drebrin (supplementary material Fig. S6B). By contrast, drebrin–GFP co-precipitated afadin (Fig. 6B), and afadin–GFP co-precipitated both drebrin and nectin-2 (supplementary material Fig. S6B). Using less stringent buffer and probing for interaction of endogenous proteins, immunoprecipitation of afadin led to co-precipitation of both drebrin and nectin-2, while nectin-2 or drebrin co-precipitated afadin, and drebrin co-precipitated both afadin and nectin-2 (Fig. 6C). Collectively, these experiments indicated a close interaction between drebrin and afadin, but not between drebrin and nectin. Still, co-precipitation of drebrin and nectin-2 together with afadin indicated that all three proteins are able to form a complex at the same time.

To map the region(s) of drebrin involved in afadin binding, GFP-fused truncation constructs were generated and immunoprecipitated from HUVEC cell lysates. Consistently, GFP–drebrin co-precipitated afadin, but only constructs containing the drebrin polyproline region (Fig. 7A) were able to co-precipitate afadin (Fig. 7B). To explore the role of this region in cells, a GFP-fusion encoding the drebrin polyproline region fused to a mitochondrial targeting sequence was generated (mito–drebrin–PP–GFP). Anti-GFP immunoprecipitations confirmed that this construct retained the ability to bind afadin, but not nectin-2 (Fig. 7C). A control construct encoding only the mitochondrial targeting signal did not precipitate either protein (Fig. 7C). As a more specific control, we generated a construct encoding a polyproline region of similar size from WASP (mito–WASP–PP–GFP). When transfected in HUVEC, the mito-drebrin–PP–GFP construct showed a mitochondrial localization (Fig. 7D,G,P,Q). Strikingly, cellular afadin was also retargeted to mitochondria in these cells (Fig. 7E), where it colocalized with mito–drebrin–PP–GFP (Fig. 7F,P,Q). By contrast, nectin-2 was only slightly redirected to mitochondria (Fig. 7G–I). Moreover, afadin was not targeted to mitochondria in cells expressing either mito–GFP (Fig. 7J–L) or mito–WASP–PP–GFP (Fig. 7M–O). These experiments show that the drebrin polyproline region is able to bind afadin in cells, and that afadin binding is a specific property of the polyproline region of drebrin.

The PR1-2 region of afadin binds drebrin

Next, we used a series of afadin deletion constructs to identify the region(s) mediating binding to drebrin (Fig. 7R). Immunoprecipitations of drebrin from HUVEC lysates showed that only constructs containing the PR1-2 regions of afadin were co-precipitated, indicating that this region interacts with drebrin (Fig. 7S).

To test whether the drebrin polyproline region and the afadin PR1-2 region interact directly, GST- or MBP-fusion constructs were created (GST-afadin-PR1-2, MBP-drebrin-PP; Fig. 7T) and expressed in E. coli. In pulldown assays using purified proteins, precipitation of MBP–drebrin-PP, but not of MBP, resulted in co-precipitation of GST–afadin-PR1-2 (Fig. 7T). Collectively, these data show that drebrin and afadin interact directly through their polyproline and PR1-2 regions.

Targeting of the afadin PDZ region to F-actin stabilizes nectin at cell–cell junctions

The results so far indicated an interaction between drebrin and afadin, but not between drebrin and nectin. The stabilizing effect of drebrin on nectin is thus likely to occur indirectly through afadin, which binds nectin through its PDZ domain (Takai et al., 2008a). We therefore hypothesized that drebrin, being a prominent F-actin binder (Hayashi et al., 1999; Xu and Stammes, 2006), may function by coupling nectin, through afadin, to the cortical F-actin network. In this context, a construct of the F-actin-binding region of drebrin, linked to the nectin-binding PDZ region of afadin, should stabilize nectin at cell junctions, even under conditions of drebrin knockdown or double knockdown of both drebrin and afadin. We therefore generated a GFP-fusion construct containing the F-actin-binding coiled-coil region of drebrin (Hayashi et al., 1999) and the PDZ region of afadin (drebrinCC–afadinPDZ–GFP) and expressed it in HUVEC depleted for either drebrin (Fig. 8A–C) or drebrin and afadin (Fig. 8D,E). Strikingly, expression of this construct led to complete recovery of nectin at cell–cell junctions after 16 hours (Fig. 8B,E). By contrast, a construct containing only the afadin PDZ region (afadinPDZ–GFP; Fig. 8G–I) or GFP alone (Fig. 8J–L) did not lead to recovery of normal nectin levels at junctions.

In a second set of experiments, we asked whether the F-actin-binding function of drebrin might be replaced by other F-actin binding modules, and exchanged drebrinCC for lifeact (lifeact–afadinPDZ–GFP). Also expression of lifeact–afadinPDZ–GFP led to full recovery of nectin at cell–cell junctions in cells depleted for both drebrin and afadin (Fig. 8M–O). By contrast, a construct containing only lifeact (lifeact–GFP) did not rescue nectin (Fig. 8P–R).

Finally, we tested the specificity of the PDZ region of afadin using a construct with another PDZ region of similar size, from ZO-1 (lifeact–ZO-1PDZ–GFP). Expression of lifeact–ZO-1PDZ–GFP in cells depleted for drebrin and afadin did not lead to recovery of nectin at junctions (Fig. 8S–U). Collectively, only constructs that bind both actin and nectin were able to rescue nectin at junctions in cells silenced for drebrin and afadin (Fig. 8V,W). We conclude that drebrin stabilizes nectin at cell–cell junctions by indirect coupling of nectin, through the PDZ region of afadin, to the cortical actin cytoskeleton. This phenomenon can be mimicked by other F-actin-binding modules such as lifeact, but it requires specifically the PDZ region of afadin.

Discussion

The vascular endothelium forms a monolayer covering the inner surface of vessels and is in direct contact with blood fluid and cells, while being exposed to secondary phenomena such as fluid shear stress [6–40 dyn/cm² for arteries (dela Paz et al., 2012)]. Here, we show that siRNA-induced depletion of the actin-binder drebrin leads to critical weakening of cell–cell junctions in HUVEC monolayers, characterized by reduced transendothelial electrical resistance and rupturing of cell–cell contacts under conditions mimicking vascular flow (15 dyn/cm²). Testing of a variety of junctional proteins revealed that the decrease in junctional integrity is specifically coupled to loss of nectin from adherens junctions.

Drebrin has been shown to stabilize the gap junction protein connexin-43 in murine Vero cells (Butkevich et al., 2004). The fact that we could not detect any influence of drebrin knockdown on connexin-43 localization or stability suggests that drebrin might regulate different junctional systems in various cell types. Still, experiments using canine epithelial MDCK cells (not
shown) support the data gained with HUVEC, and indicate that drebrin-dependent regulation of adherens junctions seems to be a more general mechanism in endothelial and epithelial cells. This is also corroborated by previous findings identifying drebrin as a component of adherens junctions in bovine epithelial cells (Peitsch et al., 1999).

**Fig. 7. The polyproline region of drebrin binds the PRI-2 region of afadin.** (A) Drebrin domains and truncation constructs: ADF homology region (aa 8–134), coiled-coil region involved in homodimerization and F-actin binding (Peitsch et al., 2001) (CC, aa 176–256), minimal actin remodeling region (Hayashi et al., 1999) (MAR, aa 233–317), polyproline region (aa 364–417), showing moderate homology to membrane-binding domains (mem-like, aa 477–571). The polyproline region (aa 349–421) is deleted in drebrinAPP. Co-precipitation of cellular 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). (C) Immunoprecipitation of indicated constructs fused to a mitochondrial targeting sequence, with upper blots showing co-immunoprecipitation of afadin, but not nectin-2. (D–O) Confocal images of HUVEC expressing mito-drebrin-PP–GFP (D–I), mito–GFP (J–L) or mito–WASP–PP–GFP (M–O). Cells were fixed and immunostained for afadin (E,K,N) or nectin-2 (H). (P–Q) Measurements of (P) afadin intensity at cell junctions versus that at mitochondria and (Q) total intensity of afadin in transfected versus untransfected cells, in cells expressing mito–GFP, mito-drebrin-PP–GFP and mito–WASP–PP–GFP. For each value, 90 junctional areas from three experiments were evaluated. Values were analyzed using one way ANOVA with Bonferroni’s post test and are depicted as means ± s.e.m. of three experiments; ****P<0.0001. (R) Afadin domains 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), PRI-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 cellular drebrin with respective constructs is indicated on the right (+, ‘–’). (S) Immunoprecipitations of indicated afadin constructs (GFP-, FLAG- or HA-tagged) expressed in HUVEC. Western blots of immunoprecipitations of cellular drebrin (lower blot) and co-immunoprecipitated afadin domain constructs detected with tag-specific antibodies (upper blot). (T) 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-PRI-2 fused to GST (GST–afadin-PRI-2). Western blots were developed with the indicated antibodies. Dashed lines indicate that lanes were not directly adjacent on original blots. Molecular mass in kDa is indicated. Scale bars: 10 μm. See also supplementary material Fig. S6.
We cannot exclude that, apart from nectin turnover, additional mechanisms are involved in the drebrin-dependent regulation of endothelial junctions. Possible scenarios include: (i) drebrin influencing the twist of actin filaments, which may protect them from severing by cofilin (Sharma et al., 2011); (ii) an influence on actin-associated proteins, as drebrin competes with tropomyosin, α-actinin and fascin for F-actin binding (Ishikawa et al., 1994; Sasaki et al., 1996; Zhao et al., 2006), and may thus keep actin filaments more dynamic; (iii) regulation of the actin-activated ATPase activities of myosin IIB and V (Cheng et al.,...
Drebrin stabilizes nectin at endothelial junctions

2000; Ishikawa et al., 2007; Kubota et al., 2010), which may influence actin-based transport processes at junctions; or (iv) binding of the microtubule plus tip protein EB3 by drebrin (Bazellieres et al., 2012; Geraldo et al., 2008), which may alter targeting of microtubules to endothelial junctions (Stehbens et al., 2006) and influence trafficking of structural or regulatory factors at these sites (Akhanova et al., 2009).

In particular, the importance of the N-terminal region of drebrin, including the actin depolymerizing factor homology region [ADF-H (Poukkula et al., 2011; Xu and Stannes, 2006)], and the actin-binding domain [ABD (Grintsevich et al., 2010; Hayashi et al., 1999)], are well documented. For example, the destabilizing effect of drebrin on dendritic spine morphology in rat hippocampal neurons seems to be based on its ABD (Biou et al., 2008). Drebrin contains also a proline-rich region, which is of functional relevance, evidenced by its direct binding to afadin. This is underlined by the ability of this region to act as a dominant negative construct that can inhibit the drebrin–afadin interaction and thus the junctional localization of nectin (supplementary material Fig. S7).

Proof of direct binding of the drebrin polyproline region comes from anti-GFP co-immunoprecipitation, pulldown assays using purified polypeptides and from mitochondrial retargeting experiments. Interestingly, in the retargeting experiments, the majority of cellular afadin is re-recruited to mitochondria. In contrast, the majority of nectin remains at the junctional area, where it is most probably stabilized by the remaining afadin and endogenous drebrin. The retargeting experiments also demonstrate the specificity of the drebrin polyproline region, as a polypolyline region of similar size, from WASP, failed to recruit afadin. Interestingly, the PRI-2 region of afadin, the binding site for drebrin, is also necessary for binding ZO-1 (Ooshio et al., 2010) and thus seems to act as a hub for junctional components.

Anchorage of afadin by ZO-1 or z-catenin (Pokutta et al., 2002) may also explain why afadin is not lost from junctions upon drebrin knockdown (supplementary material Fig. S5). Moreover, afadin was originally identified as an F-actin binder, with the binding domain comprising amino acid residues 1691–1829 (Mandai et al., 1997), which is not identical with the PRI-2 region that binds drebrin (aa residues 1219–1399). Afadin thus has actin-binding ability independently of drebrin, and all of these interactions may contribute to anchor afadin at the cell cortex. However, (i) our FRAP measurements show that, in the absence of drebrin, afadin shows an increased mobility in the junctional area, and (ii) the presence of afadin in drebrin knockdown cells is not sufficient to stabilize nectin at cell junctions, whereas expression constructs containing F-actin-binding regions (drebrin-CC or lifeact) is sufficient for this. Drebrin thus seems to provide a higher level of junctional anchorage also for afadin and probably acts as the main F-actin binder in this system.

Knockdown of either drebrin or afadin leads to loss of nectin from junctions. Stabilization of nectin thus clearly requires the complete chain of interactions that anchors it to the subcortical actin cytoskeleton. Key elements in this chain are the modules that confer binding to afadin and to F-actin, demonstrated by a rescue construct containing the PDZ region of afadin coupled to the coiled-coil region of drebrin, which restores junctional nectin even under knockdown of both drebrin and afadin. The requirement for the PDZ region of afadin is specific, as a construct containing a PDZ region of similar size from another junctional protein, ZO-1, fails to rescue nectin at junctions. This is in line with earlier findings that the PDZ domain of afadin binds a specific consensus motif in the nectin C-terminus (Reymond et al., 2000; Takahashi et al., 1999). By contrast, the coiled-coil region of drebrin (aa residues 174–258) appears to function as a general F-actin-binding module that can be replaced by another, even shorter, F-actin binding sequence. For this, we chose lifeact (17 aa residues), based on its role as a general F-actin binding module with high specificity and little interference in actin dynamics, and on the comparable F-actin binding kinetics of the drebrin ABD \( K_{d} = 6.6 \pm 0.4 \) \( \mu \)M (Grintsevich et al., 2010) and lifeact \( K_{d} = 2.3 \pm 0.9 \) \( \mu \)M (Riedl et al., 2008). These experiments demonstrate that drebrin stabilizes nectin at cell–cell junctions by indirect coupling of nectin, through the afadin PDZ region, to the cortical actin cytoskeleton. This function can be mimicked by other F-actin-binding modules such as lifeact, but it requires specifically the PDZ region of afadin. Drebrin, as a binder of both F-actin and afadin, fulfills both requirements and is thus able to stabilize nectin at cell–cell junctions.

We propose a scenario where nectin is constantly delivered to the cell surface. If anchoring to the F-actin cytoskeleton is provided, nectin is then stabilized at the cell cortex, which facilitates junction formation. In the absence of cytoskeletal anchoring provided by drebrin, nectin is still delivered to the junctional area, but is unanchored and thus rapidly endocytosed and eventually degraded in lysosomes (Fig. 3).

Considering that (i) drebrin may influence myosin activity (Ishikawa et al., 2007; Kubota et al., 2010) and (ii) actomyosin contractility can be involved in the regulation of cell–cell contacts (Sawyer et al., 2011; Taguchi et al., 2011), we explored the potential role of the actomyosin system in drebrin-dependent regulation of cell junctions, using myosin II-inhibiting drugs such as Y-27632 (inhibiting ROCK) and blebbistatin (not shown). However, using a wide variety of conditions, we did not detect a significant influence on drebrin-dependent stability of nectin at junctions. We conclude that drebrin appears to function mainly as a tether to F-actin in this system, and not as a transducer of contractile forces. However, we can not exclude that drebrin may regulate the accessibility of the afadin PDZ domain by inducing conformational changes in afadin.

Our results add an additional layer of complexity to the current model of nectin regulation by showing that drebrin provides the crucial anchorage to the actin cytoskeleton. We propose the following model for drebrin-dependent regulation of endothelial adherens junctions: drebrin is present at the cell cortex, where it binds actin filaments through its coiled-coil region and also binds afadin through a direct interaction between its polyproline region and the afadin PRI-2 region. Afadin can recruit nectin through its PDZ region, and nectin becomes stabilized at the junctional area, where it trans-dimerizes with nectin from neighboring cells. If the indirect link to the actin cytoskeleton is disrupted, nectin is no longer anchored and can be endocytosed, which may lead to either recycling to the plasma membrane or degradation. This scenario does not imply a specific sequence of inside-out or outside-in signaling, but proposes that stabilization of nectin by F-actin anchorage facilitates junction formation.

The intricate chain of actin–drebrin–afadin–nectin interactions should provide multiple ways for fine-tuning junctional stability and endothelial function. Drebrin-dependent recruitment of nectin to F-actin may thus be used to modulate barrier function and leukocyte extravasation, in both physiological and
pathological settings such as angiogenesis or inflammation. Targeting this molecular chain may also become an attractive option for therapeutic intervention in vascular diseases such as atherosclerosis. Moreover, considering that drebrin, afadin and nectin have all been identified as central regulators of neuronal function such as dendritic spine plasticity or synaptic remodeling (Beaudouin et al., 2012; Harigaya et al., 1996; Lim et al., 2012), as well as cancer cell invasion (Fabre-Layaf et al., 2007; Fournier et al., 2011; Peitsch et al., 2005), modulation of the actin–drebrin–afadin–nectin chain may prove to be important also in neurological disorders and tumor progression.

Material and Methods

Cell culture

HUVEC were obtained and cultured as described previously (Osiak et al., 2005). Cells were passaged every 4–6 days. Assessment of cell morphology was done under phase contrast on an inverted microscope (Nikon TS100, Germany).

Application of fluid shear stress

10^4 HUVEC in 100 μl endothelial cell growth medium (Promocell, Heidelberg, Germany) were seeded at confluence in collagen-G-coated flow-through chambers (Ibidi μ-Slide II®) and cultured for 16 hours and then treated with chloroquine at a final concentration of 6 μM for 0 hours, 5 hours and 12 hours at 37°C. Inhibition of lysosomal protein degradation was maintained by switching of the valves of the fluidic unit, leading to constant perfusion of media through tubes connecting the Ibidi μ-slide to a media reservoir.

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, 0.4 μm pore size Transwell Filters (Costar, New York, USA) coated with collagen G (a mix of calf skin collagens used for preparation of gels; Biochrom, Berlin, Germany) and cultured for 72 hours in 24-well plates (Nunc, Langenselbold, Germany), with media replaced daily. TER was measured using an Millicell®ERS (Millipore, Schwalbach, Germany) ohmmeter according to the manufacturer’s instructions. 2 mM EGTA was added for 1 hour as a positive control leading to junction disruption.

Cell transfection and siRNA experiments

HUVEC were transfected using microporation technique (Peqlab, Erlangen, Germany), according to the manufacturer’s guidelines. For knockdown of drebrin2, shRNA target and control sequences were selected with siRNA Target Designer (Promega Corp., Madison, USA). The shRNA sequence: 5′-ACCCTGCTCCATCCCAACATGTTACAGTGGTAAAGGAAGGCGCTCTTTT-3′ (sense sequence) and scrambled control shRNA sequence: 5′-ACCCGTCTCCCCAAATTTTGAACAGTTCTCTGACTAATGGGTCCTTTTACCTTCTTCTC-3′ (sense sequence) were synthesized by MWG Biotech (Ebersberg, Germany) and inserted into psiSTRIKE vector using the psiSTRIKE U6 hairpin Cloning System iMGFP (Promega Corp., Madison, USA) according to the manufacturer’s guidelines. Alternatively, knockdown of drebrin2 was performed using pSiGENOME SMARTpool DB1 (Dharmacon Inc., Lafayette, CO, USA) where indicated, or by using pairs of single siRNAs (pSiGENOME DBN1 -01, -02, -04 or -17; see supplementary material Fig. S2). Afadin knockdown was performed using pSiGENOME SMARTpool MLLT4 (Dharmacon Inc., Lafayette, CO, USA). siRNAs against nectin-2 and -3 were obtained from Santa Cruz (Heidelberg, Germany). Luciferase control siRNA was obtained from Eurofins MWG Operon (Ebersberg, Germany). For all experiments, unless mentioned otherwise, HUVEC were cultured for 3 days after siRNA transfection, and re-seeded on collagen-G-coated coverslips after knockdown was established.

Inhibition of lysosomal protein degradation

An inhibitor of lysosomal activity (chloroquine) was 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 for 0 hours, 5 hours and 12 hours at 37°C.

Antibodies and immunofluorescence

Cells were stained for immunofluorescence as described (Linder et al., 1999). Guinea pig antisera against drebrin, dreb254.2gp and dreb2E2/A-Z.2gp, the latter specific for drebrin E2 and A (Peitsch et al., 2003; Peitsch et al., 2001) were used. Nectin-2 and nectin-3 antibodies were a kind gift of Yoshimi Takai. Further primary antibodies were raised against VE-Cadherin, AF6 (afadin), paxillin (BD Biosciences, San Jose, USA), connexin-43, fllm M2 (both Sigma, Deisenhofen, Germany), occludin (Invitrogen, Darmstadt, Germany) and nectin-2 (H108), nectin-2 (R2.477.2), nectin-3, PECAM-1, LAMPI (H4A3) (all Santa Cruz; Heidelberg, Germany). The MBP and HA antibodies were purchased from Abcam (Cambridge, UK), the GST antibody from Pharmacia Biotech (now GE Healthcare, Munich, Germany). GFP was detected using the Living color mAb from Clontech (Mountain View, CA, USA). The integrin-α5 antibody was obtained from Millipore (Darmstadt, Germany). F-actin was stained with Alexa-Fluo-568-, Alexa-Fluo-488- or Alexa-Fluo-647–phallolidin (Molecular Probes, Leiden, Netherlands). Secondary antibodies were TRITC, Alexa-Fluo-488-, Alexa-Fluo-648- or Alexa-Fluo-647-labeled anti-mouse, anti-guinea pig or anti-rabbit IgG (Molecular Probes, Eugene, USA). Coverslips were mounted in Mowiol (Calbiochem, Schwalbach, Germany), containing p-phenylendiamine (Sigma).

Microscopy and FRAP experiments

Immunofluorescence images of fixed samples were acquired with a confocal laser-scanning microscope (Leica DM IRE2 with a Leica TCS SP2 AOB5 confocal point scanner) equipped with an oil-immersion HCX PL APO 63× NA 1.42 water objective. Phase contrast images of cells seeded in flow-through chambers were captured using a Zeiss Axioplan upright microscope (Carl Zeiss, Jena, Germany) equipped with a SPOT 1.4MP monochrome CCD camera (Diagnostic Instruments, Sterling Heights, USA). For FRAP experiments, ZO-1-GFP– or afadin-GFP-transfected HUVEC, 3 days previously treated with drebrin siRNA or luciferase siRNA, were seeded on collagen-coated WillCo-dish glass bottom dishes (WillCo Wells BV, Amsterdam, Netherlands) and imaged using an Ultraview spinning disc confocal microscope (PerkinElmer Life Sciences, Boston, USA). Five pre-bleach images were taken every 5 minutes and several recovery images at maximum speed for 20 seconds followed by a lower frequency of 10 pictures/minute, on a Hamamatsu C9100-50 EM-CCD camera (Hamamatsu, Herrsching am Ammersee, Germany). 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 (Velocity 6.0, PerkinElmer Life Sciences, Boston, USA, Microsoft Excel 2003 and GraphPad Prism 5, San Diego, CA). Videos were processed using Ultraview software (PerkinElmer Life Sciences, Boston, USA). Using Excel software (Microsoft, Redmond, WA), data were 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 two sets of experiments. Normalized fluorescence intensities were fitted to a two-phase exponential association using GraphPad Prism 5 Software (San Diego, CA).

Expression analysis by reverse transcription polymerase chain reaction

HUVEC (5 × 10^4 cells) treated with drebrin siRNA (pool) or luciferase siRNA for 4 days were submitted to total RNA extraction using TRIzol reagent following the manufacturer’s instructions (Invitrogen, Darmstadt, Germany). RNA quality was validated by running a reverse transcriptase reaction using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Darmstadt, Germany). With the obtained cDNA, a PCR was performed using n-2 specific intron-spanning primers (F-Primer: 5'- CCTCTGAAGTGGCTACTCT-3', R-Primer: 5'-TGCGAGAGTCTGATTG-3'). A selection of the PCR product sequence (212 bp for cDNA templates and 507 bp for genomic DNA, containing introns. PCR products were analyzed by electrophoresis on a 1% agarose gel containing SYBR Safe DNA Gel Stain (life technologies, Eugene, USA). The size of the PCR product sequence (212 bp for cDNA templates and 507 bp for genomic DNA, containing introns. 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.

Generation of constructs

Human drebrin2-E2-EF1G constructs were kindly gifted of W. Ludwig. EGFP-tagged drebrin2 domain constructs were created by cloning PCR-generated inserts into the HindIII and BamHI sites of pEGFP-N1. To obtain a drebrin2-siRNA pool, 1–3 silent mutations were introduced (QuickChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) in each of four regions complementary to drebrin siRNA sequences -01, -02, -04 and -17 contained in the siRNA pool. Drebrin2 was obtained by generating PCR-based inserts of aa 1–348, introducing Xhol/EcoRI sites (F-Primer: 5′-CCCTCGAGATGGCCTGCGCGCGTCGACCGCC-3′, R-Primer: 5′-ATATATGAAATTCGAGGTCGAC-3′). The XhoI of the PCR products was purified, inserts inserted into XhoI/BamHI, cloning of the pEGFP-N1 using XhoI/BamHI sites. Drebrin-polyproline-GFP, drebrin-e-t erm-GFP and drebrin-C-GFP domain constructs were subcloned into pEGFP-N1 using the HindIII and BamHI sites. The nectin2-GFP construct (Vaid et al., 2007) was a kind gift of Wayne Vogel. The pito-pCMV-Tag2B vector containing a mitochondrial targeting sequence was used to transfect the mitochondrial targeting sequence was used to transform the mitochondrial targeting sequence.
Drebrin stabilizes nectin at endothelial junctions


References

For immunoprecipitation of endogenous drebrin, nectin-2 and afadin, lysates of 5×10⁶ transfected HEK 293 cells were lysed in buffer I (50 mM NaCl, 1 mM EDTA, 1% Triton X-100, phosphatase- and protease inhibitors) or lysis buffer II (wash buffer I from μ-MACS Protein Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris 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°C. Lysates were processed according to the manufacturer's instructions, washed four 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 (lysis buffer II) of 5×10⁴ 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. Note that antigen-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.

Protein expression

Proteins were expressed in E. coli as glutathione S-transferase (GST) or maltose-binding protein (MBP) fusions after induction with 0.4 mM IPTG. Proteins were dialyzed against PBS, shock-frozen, and stored at −80°C. Purity was tested by SDS-PAGE and Coomassie staining.

Pulldown assay

100 μl of wet volume of amylase resin beads (New England Biolabs, Frankfurt am Main, Germany) equilibrated with buffer A (PBS, protease inhibitor cocktail; Roche, Mannheim, Germany) were incubated for 1 hour with 20 μg of MBP-drebrinPP fusion protein or MBP in buffer A. Beads were washed five times in buffer A and incubated with GST-afadin-PR1-2 (18 μg of protein) for 1 hour at 4°C, then washed five times in buffer B (PBS, 150 mM NaCl, 5 mM MgCl₂, protease inhibitor cocktail; Roche, Mannheim, Germany). 100 μl boiling SDS sample buffer was added to the beads, and an aliquot was run on SDS gel of appropriate percentage.

Immunoblotting

Immunoblotting was performed by standard procedure (Kopp et al., 2006), using the above mentioned antibodies and horseradish peroxidase- and anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) as secondary antibody. Secondary HRP-coupled antibodies against guinea-pig and goat were purchased from Abcam (Cambridge, UK). Protein bands were visualized by using SuperSignal West Femto or SuperSignal West Pico kit (Thermo Scientific, Rockford, USA) and X-Omat AR film (Kodak, Stuttgart, Germany).

Quantifications

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), circular ROIs 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%. Statistic analyses were performed in GraphPad Prism 5 using unpaired Student’s t-test or one-way ANOVA with Bonferroni’s post test.

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Author contributions

K.R. designed and performed experiments as part of a doctoral thesis, L.P. and V.v.V. performed experiments, E.G. provided scientific input, and S.L. designed experiments and wrote the manuscript.

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Drebrin stabilizes nectin at endothelial junctions


**Fig. S1** (related to Fig. 1). Western blots of HUVEC treated with various siRNAs. Western blots of lysates of HUVEC treated for 72 h with drebrin-specific siRNA pool (A), afadin- (B), nectin-2- (C) and nectin-3- (D) specific siRNA or luciferase siRNA as a control. Relative protein expression levels (standardized for β-actin) are given beneath each blot and are representative for all knockdown experiments. Molecular masses in kDa are indicated on the right.
Fig. S2 (related to Fig. 2). Drebrin knockdown specifically affects the localization of nectin at cell-cell junctions whereas control knockdown does not have any effect. (A-E) HUVEC transfected with control siRNA. Cells were seeded on coverslips 72 h after siRNA treatment, then fixed and stained after 24 h for F-actin (upper rows), drebrin (middle rows) and various intercellular junction molecules (lower rows), including connexin-43, VE-cadherin, nectin-2, occludin, and PECAM-1. Scale bars: 10 μm. (F) Western blot analysis of HUVEC cell lysates treated with control siRNA, developed with indicated antibodies. (G,H) Confocal micrographs of HUVEC transfected with different combinations of drebrin-specific siRNAs (G: siRNA 01+02; H: siRNA 04+17). (I) Western blot analysis of HUVEC cell lysates treated with different combinations of drebrin-specific siRNAs, as indicated. Blots were developed with antibodies specific for drebrin or β-actin as a loading control. Numbers above blots indicate days after transfection. Relative expression levels (standardized for actin) are indicated. Note the siRNA-induced decrease of drebrin protein levels at day 3 post transfection. Molecular masses in kDa are indicated on the right.
**Fig. S3** (related to Fig. 2). Direct comparison of nectin-2 levels in drebrin knockdown versus control cells. (A-F) Confocal micrographs of HUCEC transfected with control siRNA or drebrin-specific siRNA pool, seeded on two sides of the same coverslip and stained for drebrin (A,D) and nectin-2 (B,E), with a diagram (C) showing the location of respective cell populations. Note decrease of both drebrin and nectin-2 signals in cells treated with drebrin-specific siRNA compared to control cells.
Fig. S4 (related to Fig. 5). SiRNA-induced knockdown of drebrin leads to reduction of nectin-2 and nectin-3 at cell-cell contacts. (A-L) Confocal micrographs of HUVEC transfected with drebrin-specific siRNA pool (A-C, G-I) or control siRNA (D-F, J-L) and stained at day 4 post-transfection for drebrin (A,D,G,J), nectin-2 (B,E) or nectin-3 (H,K), with merged images. Scale bars: 10 μm.
Fig. S5 (related to Fig. 4). Drebrin knockdown does not affect afadin levels or localization at cell-cell contacts whereas knockdown of afadin leads to decreased levels of nectin-2. (A-F) Confocal micrographs of HUVEC stained for afadin (A,D) and ZO-1 (B) or α-catenin (E), with merged images shown in C,F. (G-L) Afadin colocalizes with α-catenin and ZO-1 at endothelial cell-cell junctions. White boxes indicate enlarged areas shown as insets. (G-K) Confocal micrographs of HUVEC transfected with control siRNA or afadin-specific siRNA, seeded to confluence from two sides of the same coverslip and stained for afadin (G) and nectin-2 (J), with diagrams (I,L) showing the location of the respective cell populations. Scale bars: 10 μm. (M) Western blot analysis of HUVEC cell lysates treated with afadin-specific siRNA. Blots were developed with antibodies specific for afadin, nectin-2 and β-actin. Numbers above blots indicate days after transfection. Relative expression levels (standardized for β-actin) are indicated. Molecular masses in kDa are indicated on the right.
Fig. S6 (related to Fig. 6). Drebrin interacts with afadin. Western blots of lysates of HUVEC expressing nectin-GFP (A) or afadin-GFP (B) or GFP, immunoprecipitated with anti-GFP antibody. Blots were developed using antibodies as indicated. Dashed lines indicate that lanes were not directly adjacent on original blots. 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 from lanes of anti-GFP immunoprecipitations. Control IgG was not bound to beads and therefore appears as heavy and light chains in the controls.
**Fig. S7** (related to Fig. 7). Junctional localization of nectin-2 is reduced in HUVEC expressing drebrin-PP-GFP. Fluorescence micrographs of HUVEC expressing drebrin-PP-GFP (B,F) and stained for nectin-2 (A) or afadin (E), with merged images shown in C,G. Fluorescence intensity measurements of nectin-2 (D) or afadin (H) between adjacent cells expressing drebrin-PP-GFP or between control cells. Note that nectin-2-based intensities are significantly reduced compared to control cells, while afadin-based intensities are unaltered. Bars indicate mean values ± s.e.m. For each value, 90 junctional areas of 3 monolayers were evaluated. ****P<0.0001. Scale bars: 10 µm. For specific values, see supplementary material Table S1.
Table S1. Data relating to Figs 1, 2, 4 and 5

**Figure 1H:**
TER measurements (Ω/cm²) (SEM)

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**Figure 2K:**
rescue of nectin in drebrin knockdown cells through insensitive drebrin-GFP (s.e.m.)

<table>
<thead>
<tr>
<th>target of siRNA/ + construct</th>
<th>intensity of nectin (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>drebrin + GFP</td>
<td>2.98 ± 0.05</td>
</tr>
<tr>
<td>drebrin + insensitive drebrin-GFP</td>
<td>10.33 ± 1.09</td>
</tr>
<tr>
<td>control</td>
<td>13.47 ± 1.10</td>
</tr>
</tbody>
</table>

**Figure 4M,N:**
halftime recovery (s) (s.e.m.)

<table>
<thead>
<tr>
<th>target of siRNA</th>
<th>afadin-GFP halftime recovery (s)</th>
<th>ZO-1-GFP halftime recovery (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>13.4s ± 1.2s</td>
<td>13.3s ± 2.5s</td>
</tr>
<tr>
<td>drebrin</td>
<td>10.2s ± 1.3s</td>
<td>14.7s ± 2.1s</td>
</tr>
</tbody>
</table>

**Figure 5I:**
flow-induced cell free area (% of total area) (s.e.m.)

<table>
<thead>
<tr>
<th>target of siRNA</th>
<th>cell free area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.31 ± 0.19</td>
</tr>
<tr>
<td>drebrin</td>
<td>8.63 ± 1.47</td>
</tr>
<tr>
<td>afadin</td>
<td>7.03 ± 1.41</td>
</tr>
<tr>
<td>nectin-2 + nectin-3</td>
<td>14.54 ± 2.72</td>
</tr>
<tr>
<td>drebrin + overexpression nectin-2-flag</td>
<td>0.84 ± 0.14</td>
</tr>
</tbody>
</table>
**Figure 5J:**
TER measurements (SEM)

<table>
<thead>
<tr>
<th>target of siRNA</th>
<th>TER (ohm/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>9.2 ± 1.47</td>
</tr>
<tr>
<td>afadin</td>
<td>5.14 ± 1.27</td>
</tr>
<tr>
<td>nectin-2 + nectin-3</td>
<td>6.18 ± 0.98</td>
</tr>
<tr>
<td>drebrin</td>
<td>6.28 ± 0.75</td>
</tr>
<tr>
<td>drebrin + overexpression nectin-2-flag</td>
<td>7.30 ± 1.9</td>
</tr>
</tbody>
</table>

**Figure 7P:**
ratio of intensity of afadin at junctions/ mitochondria (SEM)

<table>
<thead>
<tr>
<th>transfection with</th>
<th>ratio transfected/ untransfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>mito-GFP</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>mito-drebrin-PP-GFP</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>mito-WASP-PP-GFP</td>
<td>1.08 ± 0.05</td>
</tr>
</tbody>
</table>

**Figure 7Q:**
ratio of afadins overall intensity in transfected/ untransfected cells (SD)

<table>
<thead>
<tr>
<th>transfection with</th>
<th>ratio transfected/ untransfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>mito-GFP</td>
<td>1.06 ± 0.16</td>
</tr>
<tr>
<td>mito-drebrin-PP-GFP</td>
<td>1.01 ± 0.06</td>
</tr>
<tr>
<td>mito-WASP-PP-GFP</td>
<td>0.97 ± 0.10</td>
</tr>
</tbody>
</table>

**Figure 8V:**
rescue of nectin-2 through minimal constructs (SEM)

<table>
<thead>
<tr>
<th>construct</th>
<th>relative intensity of nectin-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>drebrin siRNA:</td>
<td></td>
</tr>
<tr>
<td>drebCC-afadinPDZ-GFP</td>
<td>98.05% ± 1.75%</td>
</tr>
<tr>
<td>drebrin and afadin siRNA:</td>
<td></td>
</tr>
<tr>
<td>drebCC-afadinPDZ-GFP</td>
<td>105.89% ± 5.09%</td>
</tr>
<tr>
<td>afadinPDZ-GFP</td>
<td>17.67% ± 1.13%</td>
</tr>
<tr>
<td>GFP</td>
<td>9.71% ± 1.99%</td>
</tr>
<tr>
<td>lifeact-afadinPDZ-GFP</td>
<td>110.37% ± 2.84%</td>
</tr>
<tr>
<td>lifeact-ZO-1PDZ-GFP</td>
<td>16.19% ± 0.03%</td>
</tr>
<tr>
<td>lifeact-GFP</td>
<td>28.76% ± 1.56%</td>
</tr>
<tr>
<td>stained for</td>
<td>intensity of junctions between transfected cells</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>nectin</td>
<td>6.91 ± 0.32</td>
</tr>
<tr>
<td>afadin</td>
<td>26.25 ± 0.57</td>
</tr>
</tbody>
</table>

Figure S7D,H: intensity of nectin and afadin (a.u.) at junctions after overexpression of drebrin-PP-GFP (SEM)

control | 100% ± 1.77%