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# Cortactin regulates the activity of small GTPases and ICAM-1 clustering in endothelium

## Implications for the formation of docking structures

Dietmar Vestweber,[1,*] Dagmar Zeuschner,[1] Klemens Rottner[2,3] and Michael Schnoor[4,*]

[1]Department for Vascular Cell Biology; Max-Planck-Institute for Molecular Biomedicine; Münster, Germany; [2]Actin Dynamics and Motility Unit; Institute of Genetics; University of Bonn; Bonn, Germany; [3]Helmholtz Center for Infection Research; Braunschweig, Germany; [4]Department for Molecular Biomedicine; CINVESTAV; Mexico City, Mexico

**Cortactin is an actin-binding molecule that regulates various cellular processes requiring actin dynamics. We recently described cortactin-deficient mice and despite its pivotal role for actin remodeling in vitro, these mice are surprisingly healthy. Analyzing cortactin functions in endothelium under inflammatory conditions, we found that cortactin is required for endothelial barrier functions and leukocyte extravasation in vivo. Importantly, these effects were not regulated by defective actin dynamics but instead by a failure to activate the small GTPases Rap1 and RhoG in endothelial cells. Defective RhoG signaling led to reduced ICAM-1 clustering that supported the interaction with leukocytes. These clusters originally seen as rings surrounding adherent leukocytes actually represented in many cases ICAM-1 containing protrusions as they were described before as docking structures. Thus, cortactin is essential for the formation of endothelial docking structures as well as for leukocyte adhesion and extravasation.**

**Keywords:** docking structure, transmigratory cup, Rap1, RhoG, vascular permeability, leukocyte extravasation, actin, ICAM-1

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*Correspondence to: Dietmar Vestweber and Michael Schnoor;
Email: vestweb@mpi-muenster.mpg.de and mschnoor@cinvestav.mx

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We recently published that cortactin is in vivo required for the maintenance of vascular barrier function and for efficient neutrophil extravasation under inflammatory conditions.[1] Strikingly, the mechanisms underlying the observed effects are primarily related to defective activation of the small GTPases Rap1 and RhoG rather than regulation of actin nucleation, a function that has been originally ascribed to cortactin in vitro.

Cortactin was identified as a major src-substrate about 20 years ago[2,3] and its importance for the actin cytoskeleton became clear around 10 years later when cortactin was shown to bind both F-actin and the actin-related protein (Arp)2/3 complex and to promote the formation of branched actin networks.[4-6] Consistent with this, cortactin primarily localizes to sites of actin dynamics such as lamellipodia and filopodia and is thought to stabilize these structures.[4,7,8] Furthermore, cortactin can regulate actin branching by activating Arp2/3 directly or via Wiskott-Aldrich-Syndrome protein (WASP). However, when analyzing cortactin-deficient murine embryonic fibroblasts in vitro, it became evident that morphology and lamellipodial architecture of these cells was unchanged, suggesting that cortactin is not necessary for Arp2/3-dependent actin network formation and lamellipodia protrusion.[9] These data suggest that cortactin contributes more indirectly to promoting dynamic actin reorganizations, potentially through temporal and spatial regulation of Rho GTPase activation.

Cortactin translocation to sites of active actin assembly and its tyrosine phosphorylation have both been shown to be dependent on Rac1 activation making cortactin a downstream target of this GTPase.[10,11] Again, results from cortactin-deficient murine embryonic fibroblasts implied that cortactin is also required upstream of Rac1 to drive platelet-derived growth factor (PDGF)-mediated actin remodelling.[9]

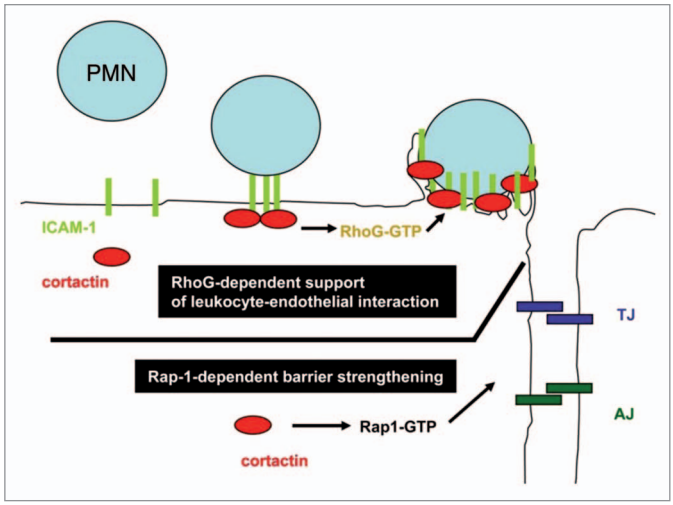


**Figure 1.** Model for cortactin functions in leukocyte extravasation and vascular permeability. Cortactin affects leukocyte extravasation and endothelial cell contact integrity by controlling the activity of two different GTPases in two independent mechanisms: Cortactin is required for activation of RhoG, clustering of ICAM-1 around adhering leukocytes and subsequent transmigration. On the other hand, cortactin regulates endothelial cell contact integrity via controling the activation of Rap-1.

Furthermore, the absence of cortactin in these cells caused a significant reduction of steady-state levels of active Cdc42. Thus, cortactin seems to be a general mediator of smallGTPase signaling both upstream and downstream of GTPase activation. Our recent data from cortactin-deficient primary microvascular lung endothelial cells (MLEC) corroborate this conclusion. We found that steady-state levels of active Rap1 are reduced in the absence of cortactin, which accounts for defective endothelial barrier function (**Fig. 1**). Additionally, we showed that RhoG cannot be activated upon ICAM-1 stimulation without cortactin leading to defective ICAM-1 clustering, reduced leukocyte adhesion to the apical endothelial surface and subsequently to reduced leukocyte transendothelial migration (**Fig. 1**).[1] In vivo, the reduced levels of activated Rap1 in endothelial cells manifested in increased vascular permeability. In addition, the defects in ICAM-1-triggered RhoG activation and ICAM-1 clustering corresponded with a reduced number of firmly adherent and transmigrated neutrophils as demonstrated by intravital microscopy of the TNF-inflamed cremaster. Otherwise, our cortactin-deficient mice did not show any obvious phenotype, which is surprising given the important cellular functions that have been published to be regulated by cortactin.[12,13] This is in contrast to other cortactin-deficient mouse models that have been described leading to embryonic lethality in one case[14] and no obvious effects during embryonic development, at least until E15, in the other.[15] The reason for these discrepancies is currently unknown but may result from the different genetic approaches exploited to generate these mice.[1,14,15]

Neutrophil transendothelial migration has previously been shown in vitro to be regulated by cortactin in an ICAM-1-dependent fashion. We corroborated the findings of the Luscinskas group by also showing reduced formation of ICAM-1- and cortactin-dependent ring-like structures surrounding adherent neutrophils on the endothelial surface.[1,16,17] Moreover, leukocyte transmigration through endothelial cell layers has been shown to be accompanied by the formation of so called endothelial "docking structures" or "transmigratory cups" that form under static as well as under flow conditions and that strengthen cell adhesion and thereby facilitate the transmigration process.[18-21] ICAM-1 is instrumental for the formation of these docking structures. Our recent results revealed that cortactin is indeed needed in vivo for firm adhesion of leukocytes in inflamed venules and for ICAM-1-clustering around adhering leukocytes. Other cytoskeletal adaptors that are important for the formation of docking structures are the ERM proteins[18] and filamin B the latter of which was found to be involved in ICAM-1 recruitment to docking structures and in leukocyte adhesion.[22] Moreover, the small GTPase RhoG has been described to regulate docking structure formation.[23] Importantly, we found that RhoG activation upon ICAM-1 engagement requires cortactin.[1] Thus, we extended our previous studies on ring-structure formation to a search for such docking structures in wild-type and cortactin-depleted endothelium. Using confocal and scanning electron microscopy, both HUVEC transfected with scrambled siRNA and primary MLEC derived from WT mice were observed to form docking structures comprised of ICAM-1-enriched microvilli-like protrusions that engulfed adherent neutrophils (**Figs. 2A and C**). Importantly, cortactin-depleted HUVEC and cortactin-deficient MLEC showed only very few of these docking structures. We quantified the cortactin-dependent effect on the organization of ICAM-1-containing docking structures clustered at sites of neutrophil contact by classifying these structures into the following categories: (1) ICAM-1-positive protrusions engulfing the neutrophil, (2) ICAM-1-positive closed ring structures, (3) ICAM-1-positive discontinuous ring structures and (4) no ICAM-1 clustering (**Fig. 2B**). More than 53% of PMNs on cortactin-positive cells showed ICAM-1 containing protrusions and an additional 31% showed closed ICAM-1-positive ring structures. Only 11% of adherent cells on cortactin-positive HUVEC were surrounded by a structure discontinuously labeled for ICAM-1, whereas merely 4.5% of PMNs showed no ICAM-1 clustering. The situation was completely different for PMNs in contact with cortactin-depleted HUVEC. Here, only 4.3% showed ICAM-1-positive protrusions and less than 10% were surrounded by closed, ICAM-1-positive ring structures. By contrast, more than 44% of total PMNs were surrounded by a discontinuous ICAM-1-labeling and 42% of these cells showed no ICAM-1 clustering. We found

similar ratios for cortactin-deficient and control MLEC (data not shown). In addition, we performed scanning electron microscopy analysis of primary bone-marrow-derived neutrophils adhering to the apical surface of cortactin-deficient or control MLEC, respectively. As shown in **Figure 2C**, neutrophils adhering to control MLEC (WT) were actively engulfed by endothelial membrane protrusions while such structures were absent with cortactin-deficient MLEC (KO). These data corroborate previous reports of other groups describing the existence of endothelial docking structures for adhering neutrophils. Importantly, for the first time, we provide experimental evidence that cortactin is required for the proper formation of ICAM-1-enriched endothelial docking structures for adhering PMNs.

It is presently debated whether docking structures are indeed necessary to prepare leukocytes for diapedesis and whether they really form in vivo.[24] It was raised that leukocytes had been additionally activated by platelet activating factor (PAF) or chemokines on the apical surface of endothelial cells, and under these conditions integrin activation would be enhanced leading to the strong recruitment of ICAM-1-containing endothelial protrusions.[19,21] We have tested this treatment and indeed found that the formation of docking structures was enhanced. However, we also found that this treatment is not necessary for the formation of these structures. Even under conditions where we added the chemokine only from the basal side of the endothelial cells and where PAF was omitted, these structures still formed for the majority of the neutrophils and were easily detectable by electron and confocal microscopy. In line with this, also other studies omitted strong activation conditions and detected docking structures frequently.[18,20] Another critical issue raised dealt with the question whether leukocytes surrounded by docking structures are really able to transmigrate or whether only those leukocytes that for some reason would not migrate would have the chance and time to recruit ICAM-1/VCAM-1-expressing protrusions. The work by Carman and Springer showed impressively that monocytes, neutrophils and also lymphocytes were partially surrounded by ICAM-1-containing docking structures while a part of the same leukocyte had already protruded through the endothelial layer and was spreading at the basal side of the endothelial cells.[21] Furthermore, quantifying whether ICAM-1-containing projections were present with adhering and transmigrating leukocytes clearly showed that they were more frequently associated

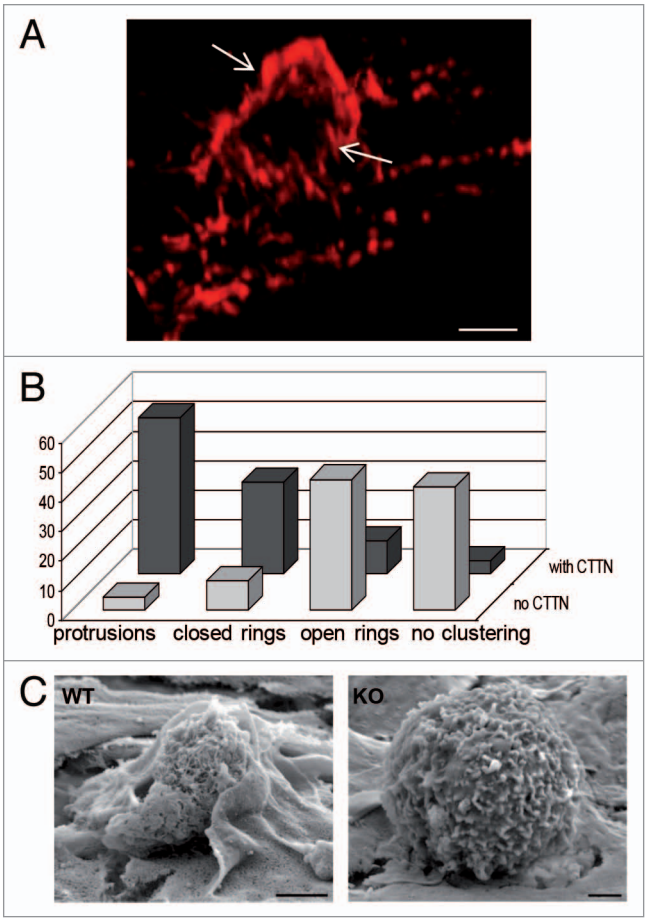


**Figure 2.** Cortactin-deficiency leads to impaired formation of ICAM-1-dependent docking structures. (**A**) HUVEC transfected with control siRNA show formation of ICAM-1-enriched protrusions (arrows) surrounding an adhering neutrophil (reflected light channel omitted for clarity). Adhesion of primary human neutrophils to TNF-α-activated HUVEC was analyzed as described with some modifications.[19] Briefly, further activation of endothelial cells by platelet-activating factor and neutrophils by manganese chloride was omitted. Neutrophils were added to the inflamed endothelial cell layer in the presence of 10 ng/ml IL8 in the lower chamber as chemokine gradient. After 20 min, cells were fixed in 4% paraformaldehyde and stained for ICAM-1 (clone R6–5-D6, BioXCell) using standard protocols. Three independent preparations were examined using an Axiovert 200 M LSM510 confocal laser scanning microscope (Zeiss) and docking structures were quantified as described.[19] Bar = 5 μm. The depicted 3D-image is a representative still image derived from **Video S1** and was generated using Imaris software (Bitplane). (**B**) ICAM-1-enriched endothelial structures surrounding adhering neutrophils were classified into the four indicated groups and counted from three independent preparations. For details see text. (**C**) Docking structures can also be observed in scanning electron microscopy. Briefly, TNF-inflamed primary MLEC isolated from cortactin-deficient and littermate wild-type mice and grown on 3 μm transwell filters were incubated for 20 min with murine primary bone-marrow neutrophils in the presence of 40 ng/ml keratinocyte-derived chemokine (KC) in the bottom chamber as gradient. Subsequently, cells were fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4 and post-fixed with 1% osmium tetroxide for one hour. After dehydration, samples were critical point dried (CPD300, Leica, Austria), sputtered with 2 nm platin and stabilized by carbon before analysis on a Leo scanning electron microscope (Zeiss). SEM micropraphs revealed endothelial membrane structures engulfing adhering neutrophils only in WT MLEC (three independent preparations were analyzed). Bar = 1 μm. For more experimental detail please refer to our original paper.[1]

with transmigrating than with adhering cells.[21] This was the reason why this study suggested the name "transmigratory cups." In other studies, it was reported that initially formed docking structures would rapidly vanish as lymphoblasts or lymphocytes began to migrate through the endothelial monolayer.[18] Others could confirm that ICAM-1-enriched ring-like structures formed around transmigrating leukocytes, but the formation of actin-rich projections by endothelial cells was not observed.[25,26]

Collectively, we conclude from published results and from our own experience that the docking structures do form with different types of endothelial cells and leukocytes. Their formation can be enhanced, but does not require additional activation of leukocyte integrins beyond what is achieved by chemokines added to the bottom chamber of transwell filters. The central question of course is whether docking structures do indeed form in vivo and whether they are relevant to the transmigration process. There are studies based on electron microscopy of serial sections of the fMLP inflamed skin, which do not report on these structures.[27] However, very recently it was shown that docking structure-like protrusions can be formed by endothelial cells in isolated blood vessels, induced by beads loaded with anti ICAM-1 antibodies.[28] This does of course not provide proof that transmigrating leukocytes induce the formation of docking structures, but it does at least show that endothelial cells within the environment of a blood vessel and embedded on a physiological basement membrane are able to extend ICAM-1-enriched protrusions toward ICAM-1 binding partners. Finally, it has clearly been shown in studies based on electron microscopy of tissues that endothelial cells do form microfilament-rich endothelial protrusions on their apical surface with which they engulf extravasating leukocytes in vivo.[29] Whether these protrusions are necessary for the extravasation process or in which way they support this process is presently unknown.

In conclusion, despite the continuous debate on the in vivo relevance of docking structures for leukocyte extravasation, there is clear agreement that there are at least ICAM-1 enriched zones around transmigrating leukocytes. The formation of these structures requires ICAM-1-triggered stimulation of RhoG activation.[23] The Luscinskas group was the first to show that cortactin is necessary for the formation of the ICAM-1 ring-like structures.[16,17] We confirmed this and extended the finding by demonstrating that cortactin acts in this process by supporting the activation of RhoG. Furthermore, we could show that cortactin is in vivo required for leukocyte extravasation by acting in two ways: First, by supporting the function of ICAM-1 as endothelial counterpart during integrin-mediated slowing down of leukocyte rolling; and second, by supporting leukocyte adhesion and thus subsequent diapedesis. Since blocking of ICAM-1-integrin interactions wiped out the contribution of cortactin to slowing down rolling or to supporting adhesion and diapedesis,[1] we conclude that cortactin is required in vivo to allow proper functioning of ICAM-1 in each of these steps. For slowing down leukocyte rolling, it might be that ICAM-1 microclusters are necessary for optimal function as integrin ligands. During diapedesis, it is likely that the formation of ICAM-1-enriched zones around leukocytes, which requires cortactin, is necessary to support the actual transmigration step.

### Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.


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### Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/tissuebarriers/articles/23862

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