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PECAM-1 supports leukocyte diapedesis by tension-dependent dephosphorylation of VE-cadherin

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1st Editorial Decision 4th Aug 2020

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you will see from the comments, the reviewers appreciate the study and the presented model of PECAM1-dependent regulation of leukocyte extravasation via tension-sensitive VE-Cad dephosphorylation. However, they also indicate a number of issues that would have to be addressed and clarified before they can support publication of the manuscript, in particular regarding the used FRET sensor (reviewers #1 and #3), the role of PECAM1 in regulation of SHP2-mediated VE-Cad dephosphorylation (reviewers #2 and #3) and data quantification and analysis (reviewers #1 and #3). From my side, I find the reviewer comments generally reasonable. Therefore, I would invite you to address the concerns raised by the reviewers in a revised manuscript.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Referee #1:

The study "PECAM-1 supports leukocyte diapedesis by tension-dependent de-phosphorylation of VE-cadherin" by Arif et. al., investigates potential molecular mechanisms involved in immune cell migration across the endothelial barrier. These authors have previously published extensively on the phosphorylation of VEC- tyrosine 731, which they showed was dephosphorylated in concert with leukocyte diapedesis. The current study attempts to uncover the mechanism regulating such dephosphorylation. Here a series of biochemical studies together with mutagenesis, siRNA and pharmacologic interventions were applied during in vitro modeling of diapedesis. These seem to establish new linkage between PECAM-1 and VEC at the level of regulating SHP2 and Y321 dephosphorylation. The authors then perform 2 experiments (Fig.7) to establish relationship of the above to cellular mechanics. From these the authors ultimately conclude that 'leukocytes destabilize endothelial junctions by interacting with PECAM-1, which provides SHP2 for targeting VE-cadherin in a force-dependent manner.' Overall, this work provides some advancement, but does not seem to adequately support the overall conclusions (detailed below)

My main concerns revolve around the tension experiments in Figure 7. These seem to be the critical

data necessary to support the over conclusions, model and novelty as indicated in the title. Unfortunately, these are not found to be particularly extensive or convincing, and in fact are problematic in some respects.

Fig.7a - Substrate stiffness experiments. Substrate stiffness is known to directly promote cellular stiffness of the adherent cells by upregulating the contractile apparatus (e.g., Rho, myosin, stress fibers). This also tends to 'prime' or 'potentiate' contractile responses to other stimuli (e.g., leukocyte binding). Here, ECs were grown on relatively soft (0.2 kPa) and stiff (20 kPa) substrates, then preconditioned for 17 hr with TNFa, followed by addition of HL60 cells for 20 min and blotting for VEC phospho-Y731. The presented data show that upon addition of HL60, Y731 phosphorylation exhibits a trend of ~25% increased phosphorylation on the 0.2 kPa substrate, sharply contrasted by ~50% decrease of phosphorylation on the 20 kPa surface. However, only the latter was statistically significant ($p<0.01$) after the three replicates performed. Thus, the authors focus on the latter. Yet, the sizeable magnitude and opposite sign of the signal on 0.2 kPa is hard to ignore and one is left to wonder what the statistical significance of this trend might be after a few more replicates. It should be noted that both of these stiffnesses are within physiologic ranges, though the majority of health tissues exhibit stiffness less than 20kPa [e.g., brain, bone marrow, fat (~0.2-0.5 kPa); lung (~0.5-2 kPa); liver, skin (2-6 kPa); muscle, kidney, heart (~8-16 kPa); cornea, tendon, bone (>20 kPa)]. These experimental also miss opportunities to further probe mechanistic relationships (e.g., with tension modulating pharmacologic agents).

Fig.7b-e - FRET measurement studies. As researcher with extensive expertise and knowledge with FRET, including the biosensor used herein, I do not find the demonstrated differences in FRET efficiency of ~31% before PMN vs ~30% after PMN, to be particularly compelling. While this seems to have reached statistical significance (despite replicate variations ~10-fold greater than the actual difference (~1%); see analysis/statistical concerns below), it is not convincing as being biologically meaningful, particular since this biosensor is shown previously to exhibit ~2-fold dynamic range in FRET signal in similar endothelial cells. In relation to above paragraph, it should be noted also that these FRET experiments were done glass or plastic substrates that have supra-physiologic Giga-Pascal stiffness, which if anything should strongly potentiate expected tension responses to PNM.
Furthermore, even if one conceded that the minute FRET signal differences were biologically meaningful, the experimental design suffers from confounding variables. The 'before' condition was under static conditions in the absence of IL-8, while the 'after' condition was after addition of IL-8 and induction of shear, as well as addition of PMN. Thus, it cannot be determined if the reported 'change' in tension is PMN-related or a direct effect of IL-8 or shear forces. Indeed, both acute shear onset and IL-8 exposure are well established to each independently drive Rho signaling, stress fibers and contractility. (e.g., Schraufstatter, 'IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways' Am J Physiol Lung Cell Mol Physiol. 2001 Jun;280(6):L1094-103). Moreover, studies with precisely this biosensor were shown to exhibit diminished FRET in response to shear flow, though the range of shears used in the current study were not evaluated.

Image Analysis and Statistic Considerations: As described by the authors (see below) for each experiment and endothelial junctional segment the authors generated a 'baseline' or 'before PMN' value and a later 'after PMN'. But in reality each 'before' and 'after' value is derived from a sequential series of imaging frames (a window of ~7 frames for 'before' and an extended window of 44 frames for 'after'). First, since the authors are not reporting data as continuous time course, it is not clear how the multiple before and after frames were combined. In principle the approach seems analogous to rapidly making triplicate baseline and endpoint reads, for example on a plate reader for

a chromogenic assay, followed by averaging reads in corresponding wells to reduce influence of instrument noise. This is acceptable when the time between reads is small compared to the experiment and biologic response. However, assuming use of sequential imaging frames for was meant to be used analogously here, the authors have not described how this was done. For example, were frames separately analyzed for FRET signal and then averaged or was pixel-by-pixel image averaging performed first followed by a single FRET measurement, another approach? This in nontrivial. Furthermore, and more problematic the 'after' measurement was made over an extended period, which as I interpret included the last 8 min of the total 12 minutes of PMN exposure over a total of 44 sequential frames (6 times longer than the 'before' sampling duration). Even more unusual, the authors (seemingly arbitrarily) chose to split these frames into 4 groups (11 frames each) that were each handled separate 'datasets'(see below). If I interpret this at face value this would have effectively created an artificial 4-fold expansion of the apparent number of measurements made, which would ultimately overestimate the statistical significance.

The authors state: In methods: "Measurements were started 5-11 frames prior to IL-8 addition to assess the baseline FRET efficiency."... "The same junctions were analyzed before and after application of PMNs under flow. To exclude altered fitting results upon differences in the number of photons included in the analysis before and after PMN addition, the 44 frames after PMN addition were splitted into 4 times 11 frames with FRET efficiency calculation for each of the four splitted datasets." In legend: "Graphs represent data obtained from ten (d, e) independent experiments with 50 (d) and 51 (e) measurements."

Referee #2:

The authors report that VE-cadherin Y731 is dephosphorylated by SHP2, which is provided by PECAM-1 when leukocytes bind ECs. Interestingly, the Y731 phosphorylation happens in the VE-cadherin proform, before it reaches the surface. In mature VE-cadherin, the Y731 site is inaccessible, presumably because catenin masks it. This explains why overexpressing SHP2 in ECs does not dephosphorylate VE-cadherin Y731. The site becomes accessible when ECs contract in response to calcium flux. MAPTAM shows that the calcium flux is required for myosin-II mediated tension. The authors also constructed a VE-cadherin FRET-based force sensor with a threshold of 4 pN. This reagent will be very useful for the endothelial cell field.

The study is novel, well-controlled, precise, conclusive and elegant (substrate trapping, new FRET probe). It is also mostly well-written, with the exception of some typos and incorrect commas.

1. An alternative hypothesis could be that the SHP2 coming from PECAM-1 is in a special compartment, maybe right next to VE-cadherin, and therefore works. This can be tested by triggering myosin-II mediated contraction in ECs and testing whether this makes overexpressed SHP2 effective, which would support the authors' original hypothesis.
2. Many experimental systems are used: primary ECs and endothelioma and HUVEC, primary lymphocytes and HL60 cells. The authors should provide a rationale for their choices in each experiment.
3. It is very interesting that VE-cadherin Y731 dephosphorylation does not work on soft substrates (0.2 kPa). The endothelial basement membrane stiffness in a venule can be above 20 kPa, which should be discussed.
4. The first 3 paragraphs of the discussion recount the results without putting them in perspective. This is excessive. Instead, the discussion of how this new mechanism relates to Muller's LBRC would seem more interesting.

5. The authors write about VE-cadherin "uptake". Do they mean "internalization"?
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Referee #3:

General summary and opinion about the the study:
The manuscript by Arif, Zinnhardt and Vestweber et al. describes a role for PECAM-1 in the regulation of VE-cadherin during the diapedesis of leukocytes. The manuscript continues on a groundbreaking previous study from this group which revealed that specific dephosphorylation of VE-cadherin at Tyr 731, in a SHP2 dependent manner, is needed for leukocyte migration (Wessel et al, Nature Immunology, 2014). To date, the underlying endothelial molecular mechanism for this event still remained unknown. In the current manuscript the authors conclude that PECAM-1 delivers the phosphatase SHP2 to VE-cadherin to mediate direct Y731 dephosphorylation and promote subsequent VE-cadherin endocytosis for leukocyte transmigration. In addition, evidence is provided that leukocytes activate the actomyosin cytoskeleton to control Y731 dephosphorylation and confer tension on VE-cadherin and change the accesibility of the Y731 residue upon the interaction of leukocytes.

Overall the manuscript is clearly written and the presented data are of high quality and well controlled. The manuscript contains some significant new findings, although it remains quite difficult to comprehend whether we are looking at a coherent mechanism or parallel pathways that are involved in junction breakdown during transmigration. In particular the claim that dephosphorylation of Y731 by SHP2 depends on tension on VE-cadherin remains to be shown. Although the authors elegantly demonstrate that there is a correlation between the Y731 phosphorylation status and tension on VEC during transmigration, they could also be the result of separate cellular processes. The manuscript contains several key new findings (1. VE-cadherin is direct target of the phosphatase SHP2; 2. Phosphorylation of Y731 is modulated by substrate stiffness; and 3. Leukocytes enhance tension on VE-cadherin) that are of significant importance to the field and would warrant publication in EMBO Journal. There are a few remaining concerns for the other claims in the manuscript.

Major concerns:
- The authors conclude that PECAM-1 delivers SHP2 to VE-cadherin. The experiments in Figure 2 clearly show that during T-cell transmigration the interaction between SHP2 and PECAM-1 is reduced and more VE-cadherin and SHP2 interaction is found. The other experiments in this figure demonstrate the importance of PECAM-1 during the interaction of endothelial cells with T-cells/neutrophils. However, direct evidence that PECAM-1 brings SHP2 proteins (thus the same pool of proteins!) to VE-cadherin is lacking. The Y663, 686F mutations in PECAM-1 do support a role for these SHP2 binding residues in the process, but the mutations may affect the PECAM-1-mediated interactions between the leukocytes with endothelial cells and thereby prevent the initial trigger for Y731 dephosphorylation already. Therefore the conclusion that PECAM-1 directly provides SHP2 proteins to VE-cadherin remains uncertain.
- If the hypothesis is true that PECAM-1 provides SHP2 to VE-cadherin and dephosphorylate Y731, then how come basal Y731 phosphorylation of VE-cadherin is lower in siPECAM-1 cells compared to control? (Figure 2C). Please explain.
- In Figure 1a the authors show that SHP2 siRNAs inhibit transmigration in WT cells, but not in Y731F expressing endothelial cells. Based on this result the authors conclude that the contribution

of SHP2 to leukocyte transmigration depends on the Y731 residue in VE-cadherin. Since transmigration across Ctrl siRNA treated Y731F endothelial cells is already lower compared to Ctrl siRNA treated WT cells, an alternative explanation might be that most of the paracellular transmigration is already inhibited by the Y731F mutation itself and that SHP2 can not inhibit any further VE-cadherin-associated transmigration. Similarly, MAPTAM and blebbistatin treatments may not be able to reduce transmigration as transmigration is already much lower across Y731F cells compared to controls. Other, VE-cadherin independent mechanisms, may be at play during transmigration over Y731F cells.

Minor concerns:
-Is the observed increase in tension on VE-cadherin dependent on SHP2? Also, it would be extremely interesting to known how tension sensing occurs in the VE-cadherin Y731F mutant.
- Different FRET probes for measuring VE-cadherin tension are used compared to previous studies. For instance in the Conway et al. paper (Current Biology 2017) a different version of the tension sensor was used. Can the authors elaborate on why the new tension sensors were preferred? Are the current probes better/improved? This may be very relevant information for the field. Can the authors provide further characterization for the proper functioning of the new FRET probes? Please also provide corresponding example fluorescence images. Does FRET efficiency change upon with blebbistatin/thrombin treatments? And what is the dynamic range during these experiments?
- Figure panels in 1d, 2a and 2b are representative blots of multiple experiments. The fold change in co-IPs needs quantification. In addition could the authors confirm that the co-immunoprecipitation of SHP2 with VE-cadherin IPs is reduced upon the incubation with T-cells?
- Fig 7a shows that endothelial cells on top of soft substrates prevents HL60 induced dephosphorylation of pY731. The interaction between, and transmigration of, leukocytes and the endothelium is notoriously different on softer substrates (Stroka et al, Blood 2011). As a consequence it remains unclear whether the primary PECAM-1 derived signal to SHP2 is not initiated or whether tension across endothelial cells is needed to stimulate Y731 dephosphorylation.

Additional non-essential suggestions for improving the study:
- The data point towards a model in which conformational changes within VE-cadherin or the catenins are responsible for the ability of SHP2 to dephosphorylate Y731. How does this relate to other interactions such as with adaptins or known tension imposed changes at endothelial junctions?
-Subcellular information regarding the protein interactions between SHP2, PECAM-1 and VE-cadherin would be helpful to tease out some of the remaining questions.

Detailed reply to reviewers:

We thank the reviewers for their competent, fair and very constructive comments that we have addressed below as follows:

Referee #1:

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We agree that the slight increase of the VEC phospho-Y731 signal upon addition of HL60 cells on low stiffness substrates in our original results was puzzling. Whereas there was no such increase in the blot shown in the original figure 7a (if standardized to the total amount of immunoprecipitated VE-cadherin), a ~25% increase (although not significant) was seen for the quantification of three independent experiments. This was mainly due to one experiment,

where the pY731 signal was untypically increased. We have now performed four more experiments. Quantification over all seven experiments (shown in new figure 8F) shows that there is no significant change of the pY731 signal upon adding HL60-derived neutrophils to endothelial cells grown on low stiffness substrates, whereas the leukocytes induced a highly significant reduction of the pY731 signal for endothelial cells grown on high stiffness substrates. We have newly scanned the X-ray film of the immunoblot we showed before, this time with higher resolution, and show this in the new figure 8F.

Fig.7b-e - FRET measurement studies. As researcher with extensive expertise and knowledge with FRET, including the biosensor used herein, I do not find the demonstrated differences in FRET efficiency of ~31% before PMN vs ~30% after PMN, to be particularly compelling. While this seems to have reached statistical significance (despite replicate variations ~10-fold greater than the actual difference (~1%); see analysis/statistical concerns below), it is not convincing as being biologically meaningful, particular since this biosensor is shown previously to exhibit ~2-fold dynamic range in FRET signal in similar endothelial cells. In relation to above paragraph, it should be noted also that these FRET experiments were done glass or plastic substrates that have supra-physiologic Giga-Pascal stiffness, which if anything should strongly potentiate expected tension responses to PNM.

We agree with the reviewer that the reduction in FRET efficiency caused by PMNs, which we documented in the old Fig. 7d (now new Fig. 8B), was not very strong. [Nevertheless, it is important to note that we saw no such effect for the tailless sensor (old Fig. 7e, now new figure 8C)]. The reason for the weak effect is that we did not just analyze the direct vicinity of single transmigrating leukocytes, but recorded signals from the complete cell junctions surrounding several endothelial cells in a region of interest. Since we imaged live cells, the analysis of the vicinity of single, rapidly transmigrating leukocytes did not yield sufficient photon counts for reliable measurements. Therefore, areas of endothelial cells with multiple PMNs were analyzed, which had the disadvantage that signals of large junctional areas were included, which were devoid of transmigrating neutrophils. This “diluted” our measured effects of PMNs on FRET efficiency.
To circumvent this problem, we have now performed additional experiments, where we fixed the endothelial monolayer with transmigrating neutrophils. This allowed us to analyze endothelial junctions within the direct vicinity of single neutrophils for longer time and collect sufficient photon quantities. The change in FRET efficiency increased considerably when we compared FLIM measurements at sites of PMN transmigration with signals at endothelial junctions of control endothelial cells which were also exposed to flow but in the absence of PMNs. FRET efficiency was reduced from 25.9 % (without PMNs) to 21.9 % (at sites of transmigrating PMNs), which is a relative reduction of 15.4 %. These results are now shown in the new figure 8D. We are now also showing representative fluorescence images of the change in FRET efficiency in the junctional vicinity of a transmigrating neutrophil in the new figure 8E.

As response to referee 3, we also demonstrate now that thrombin reduces FRET efficiency of our VE-cadherin tension sensor by about 20% (new Fig. 7E) and we show that the construct is properly expressed at cell junctions and associates with the catenins (new figure 7C and D).

Furthermore, even if one conceded that the minute FRET signal differences were biologically meaningful, the experimental design suffers from confounding variables. The 'before' condition was under static conditions in the absence of IL-8, while the 'after' condition was after addition of IL-8 and induction of shear, as well as addition of PMN. Thus, it cannot be

determined if the reported 'change' in tension is PMN-related or a direct effect of IL-8 or shear forces. Indeed, both acute shear onset and IL-8 exposure are well established to each independently drive Rho signaling, stress fibers and contractility. (e.g., Schraufstatter, 'IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways' Am J Physiol Lung Cell Mol Physiol. 2001 Jun;280(6):L1094-103). Moreover, studies with precisely this biosensor were shown to exhibit diminished FRET in response to shear flow, though the range of shears used in the current study were not evaluated.

We agree with the reviewer, that we should have addressed in the paper whether IL-8 or flow would affect FRET efficiency of our VE-cadherin tension sensor. In fact, we had actually tested this at the beginning of our studies, but since we found no effects, we had not paid much attention to this issue anymore and missed to include it in the manuscript. We apologize for this oversight. We are now showing these data in the new figure 8A. We found that FRET efficiency determined for endothelial cells under static conditions, in the absence of IL-8 was unchanged when the very same cells were analyzed under flow conditions (1 dyn/cm^2) in the presence of IL-8 (5ng/ml).

We are aware of the fact that Conway et al. (Curr Biol. 2013, 23:1024-1030) have found that flow induces an increase of FRET efficiency (less tension across VE-cadherin) for a VE-cadherin tension sensor. This was the first such sensor generated for VE-cadherin. We made a similar construct, inserting our force sensing module at the same site into VE-cadherin. However, the force sensing peptide linking the two chromophores was a different one, with a different force sensitivity. Whereas the construct used by Conway et al., reacts gradually to forces from below 1pN to 6 pN, the sensor we inserted into VE-cadherin responds to forces above 4 pN (Ringer et al., Nat Methods 14: 1090-1096, 2017). In addition, our flow conditions (1 dyn/cm^2) differed from the conditions in the Conway et al. paper (15 dyn/cm^2). These are two reasons, which may explain why we did not detect an effect of flow on tension release across VE-cadherin. We mentioned this now in the discussion on page 17.

We would like to add, that the effect of flow on tension across VE-cadherin, which was detected by Conway et al., caused a release of tension, whereas the leukocyte-induced effect we describe here causes enhanced tension across VE-cadherin. Thus, both effects are opposite to each other. Therefore, effects by flow would rather lead to an underestimation of the leukocyte-induced effects we report here.

With respect to the findings about IL-8 inducing stress fibers (Schraufstätter et al., Am J Physiol Lung Cell Mol Physiol. 2001 Jun;280(6):L1094-103), this study reported effects on filamentous actin formation (based on staining experiments) at a range of 0.3 to 100 nM IL-8. However, direct functional responses like cell retraction were only observed at 100 nM. We assume that the concentration of 5ng/ml IL-8 we used (0.6 nM) may have been too weak, to see effects on VE-cadherin.

Image Analysis and Statistic Considerations: As described by the authors (see below) for each experiment and endothelial junctional segment the authors generated a 'baseline' or 'before PMN' value and a later 'after PMN'. But in reality each 'before' and 'after' value is derived from a sequential series of imaging frames (a window of ~7 frames for 'before' and an extended window of 44 frames for 'after'). First, since the authors are not reporting data as continuous time course, it is not clear how the multiple before and after frames were combined. In principle the approach seems analogous to rapidly making triplicate baseline and endpoint reads, for example on a plate reader for a chromogenic assay, followed by averaging reads in corresponding wells to reduce influence of instrument noise. This is

acceptable when the time between reads is small compared to the experiment and biologic response. However, assuming use of sequential imaging frames for was meant to be used analogously here, the authors have not described how this was done. For example, were frames separately analyzed for FRET signal and then averaged or was pixel-by-pixel image averaging performed first followed by a single FRET measurement, another approach? This in nontrivial. Furthermore, and more problematic the 'after' measurement was made over an extended period, which as I interpret included the last 8 min of the total 12 minutes of PMN exposure over a total of 44 sequential frames (6 times longer than the 'before' sampling duration). Even more unusual, the authors (seemingly arbitrarily) chose to split these frames into 4 groups (11 frames each) that were each handled separate 'datasets'(see below). If I interpret this at face value this would have effectively created an artificial 4-fold expansion of the apparent number of measurements made, which would ultimately overestimate the statistical significance.

The authors state: In methods: "Measurements were started 5-11 frames prior to IL-8 addition to assess the baseline FRET efficiency."... "The same junctions were analyzed before and after application of PMNs under flow. To exclude altered fitting results upon differences in the number of photons included in the analysis before and after PMN addition, the 44 frames after PMN addition were splitted into 4 times 11 frames with FRET efficiency calculation for each of the four splitted datasets." In legend: "Graphs represent data obtained from ten (d, e) independent experiments with 50 (d) and 51 (e) measurements."

A major aim of our life FLIM measurements was to analyze the same endothelial cells and their junctions in the absence of neutrophils (‘before adding neutrophils’) and during neutrophil transmigration. Therefore, we recorded the same cells before adding neutrophils and then again after adding neutrophils. For each measurement, a small group of 2-3 endothelial cells (within a monolayer of EC) was recorded before adding neutrophils and the identical 2-3 EC were recorded for 44 video frames in the presence of PMNs (flowing over the monolayer with some of them transmigrating). Such a measurement of a specific small group of endothelial cells was defined as one measurement. 50 of such measurements (with 50 different small groups of endothelial cells in 50 different chamber slide lanes) was performed for the data in old Fig. 7d (now new Fig. 8B) and 51 of such experiments were performed for the data in old Fig. 7e (now new Fig. 8C). These 50 and 51 measurements were obtained in 10 different experiments with EC transduced with the VE-cad-FL and 10 different experiments with EC transduced with the VE-cad FL-TS control construct, respectively. Therefore, the number n which was used for statistical analysis and for determining statistical significance was 50 and 51, respectively.

Thus, the multiple ‘before PMN’ and ‘after PMN’ video frames were obtained with the identical small group of EC. The larger number of 44 video frames with PMNs was chosen in order to increase the chances of observing leukocyte transmigration events. The data (decay curve) was obtained from these 2-3 cells from 11 combined frames and then fitted to determine fluorescence lifetime, to make sure that approximately similar numbers of photons were used for data fitting to determine lifetime values. Thus, four comparable values for τ_{DA} were determined ‘after PMN addition’ and combined to obtain an average value. This value was compared to the ‘before PMN addition’ value. Importantly, this whole procedure was done in the same way for the ‘no-tension’ negative control construct (old Fig. 7e, now new Fig. 8C). Again, such an analysis was done 50 times for the tension sensing construct and 51 times for the ‘no-tension’ negative control construct.

Referee #2:

The authors report that VE-cadherin Y731 is dephosphorylated by SHP2, which is provided by PECAM-1 when leukocytes bind ECs. Interestingly, the Y731 phosphorylation happens in the VE-cadherin proform, before it reaches the surface. In mature VE-cadherin, the Y731 site is inaccessible, presumably because catenin masks it. This explains why overexpressing SHP2 in ECs does not dephosphorylate VE-cadherin Y731. The site becomes accessible when ECs contract in response to calcium flux. MAPTAM shows that the calcium flux is required for myosin-II mediated tension. The authors also constructed a VE-cadherin FRET-based force sensor with a threshold of 4 pN. This reagent will be very useful for the endothelial cell field.

The study is novel, well-controlled, precise, conclusive and elegant (substrate trapping, new FRET probe). It is also mostly well-written, with the exception of some typos and incorrect commas.

1. An alternative hypothesis could be that the SHP2 coming from PECAM-1 is in a special compartment, maybe right next to VE-cadherin, and therefore works. This can be tested by triggering myosin-II mediated contraction in ECs and testing whether this makes overexpressed SHP2 effective, which would support the authors' original hypothesis.

Following this excellent suggestion of the reviewer, we have overexpressed SHP2 (or as neg. control lacZ) by adenoviral transduction of HUVEC and exposed the cells to thrombin. Whereas simply overexpressing SHP2 did not affect VE-cadherin-Y731 phosphorylation (old Fig. 5A), we now found that thrombin indeed enabled overexpressed SHP2 to dephosphorylate VE-cadherin-Y731 very efficiently (new Fig. 6E). In control cells overexpressing lacZ, thrombin had a weak but not significant effect (Fig. 6E). Since we also show, that thrombin treatment of EC increases tension across VE-cadherin (see new results in Fig. 7E) these additional results strongly support our hypothesis that tension across the VE-cadherin-catenin complex induce accessibility of pY731 of VE-cadherin.

2. Many experimental systems are used: primary ECs and endothelioma and HUVEC, primary lymphocytes and HL60 cells. The authors should provide a rationale for their choices in each experiment.

We have used HUVEC for all experiments where we transduced cDNAs with the help of adenovirus vectors, since in our hands these vectors did not work well for mouse primary endothelial cells or endothelioma cells. This was valid for experiments in Fig. 1B and 5A (SHP2 expression), Fig. 2E and Fig. 3 (PECAM1-Y/F mutant expression), Fig. 7 and 8 (expression of VE-cadherin FRET constructs). Testing the effects of substrate stiffness on leukocyte-induced VE-cadherin Y731F dephosphorylation (new Fig. 8 F) was done in HUVEC since mouse endothelial cells had difficulties to grow on polyacrylamide hydrogels.

In all experiments with mouse endothelial cells expressing VE-cadherin Y731F instead of VE-cadherin, we used primary isolated cells from our VE-cadherin Y731F knock in mice. This was the case for experiments shown in Fig. 1A, 1C, 4B, 6A, and 6C.

In all other experiments we used mouse endothelioma cells: Fig. 1D and 2A: SHP2 association with VE-cadherin and dissociation from PECAM-1; Fig.2B and 2D: the need of PECAM-1 for leukocyte-induced VE-cad-Y731 dephosphorylation (which was also shown for HUVEC, Fig. 2C); Fig. 6: MAPTAM and blebbistatin block leukocyte-induced VE-cad-Y731 dephosphorylation.

Since VE-cadherin is very sensitive to artificial degradation by PMN proteases (Moll, Dejana, Vestweber et al., JCB 1998, 140: 403-407), human primary PMNs were replaced by HL60-derived neutrophils and mouse primary PMNs were replaced by T cells in experiments where VE-cadherin was analyzed by immunoprecipitation or by antibody staining.

3. It is very interesting that VE-cadherin Y731 dephosphorylation does not work on soft substrates (0.2 kPa). The endothelial basement membrane stiffness in a venule can be above 20 kPa, which should be discussed.

We have chosen a stiffness of 0.2kPa for our experiments, since at this stiffness we observed changes in cell morphology resulting in a less extended shape which was a clear indication for lower tension levels. This way, we could create conditions which reduced tension inside the cells and allowed us to test whether tension is a requirement for leukocyte induced dephosphorylation of VE-cadherin-Y731. Below stiffness of 3kPa, polyacrylamide gels lack the mechanical support needed to stabilize endothelial cell layers, in turn impairing the efficiency of leukocyte diapedesis (Stroka et al., Blood 118:1632-1640, 2011).

The high stiffness level of 20 kPa guaranteed a higher tension level which resembled conditions found in biological material. The stiffness of human basement membranes can range from 2 - 80 kPa (Last JA, Liliensiek SJ, et al., J. Struct. Biol. 21:736-722, 2011). It is difficult to know, how far into the supporting tissue (beyond the basement membrane) endothelial cells can sense stiffness. Yet, it is well possible that endothelial cells experience stiffnesses of variable magnitude in tissues and organs of variable stiffness. As outlined by reviewer 1, different organs vary in stiffness: e.g., brain, bone marrow, fat (~0.2-0.5 kPa); lung (~0.5-2 kPa); liver, skin (2-6 kPa); muscle, kidney, heart (~8-16 kPa); cornea, tendon, bone (>20 kPa). These are average values for these organs and it is likely that stiffness varies within each organ dependent on the location within the organ and its microarchitecture. In general, a stiffness between 3 and 20 kPa and in some cases even more is not unlikely to be experienced by endothelial cells of venules in physiological settings.

4. The first 3 paragraphs of the discussion recount the results without putting them in perspective. This is excessive. Instead, the discussion of how this new mechanism relates to Muller's LBRC would seem more interesting.

We have now shortened the first two paragraphs of the discussion and elaborate in the third paragraph on some thoughts about how our new mechanism may relate to the potential role of the LBRC.

5. The authors write about VE-cadherin "uptake". Do they mean "internalization"?

We have now replaced the term “uptake” by “internalization”

Referee #3:

General summary and opinion about the the study:
The manuscript by Arif, Zinnhardt and Vestweber et al. describes a role for PECAM-1 in the regulation of VE-cadherin during the diapedesis of leukocytes. The manuscript continues on a groundbreaking previous study from this group which revealed that specific dephosphorylation of VE-cadherin at Tyr 731, in a SHP2 dependent manner, is needed for leukocyte migration (Wessel et al, Nature Immunology, 2014). To date, the underlying endothelial molecular mechanism for this event still remained unknown. In the current manuscript the authors conclude that PECAM-1 delivers the phosphatase SHP2 to VE-cadherin to mediate direct Y731 dephosphorylation and promote subsequent VE-cadherin endocytosis for leukocyte transmigration. In addition, evidence is provided that leukocytes activate the actomyosin cytoskeleton to control Y731 dephosphorylation and confer tension on VE-cadherin and change the accessibility of the Y731 residue upon the interaction of leukocytes.

Overall the manuscript is clearly written and the presented data are of high quality and well controlled. The manuscript contains some significant new findings, although it remains quite difficult to comprehend whether we are looking at a coherent mechanism or parallel pathways that are involved in junction breakdown during transmigration. In particular the claim that dephosphorylation of Y731 by SHP2 depends on tension on VE-cadherin remains to be shown. Although the authors elegantly demonstrate that there is a correlation between the Y731 phosphorylation status and tension on VEC during transmigration, they could also be the result of separate cellular processes. The manuscript contains several key new findings (1. VE-cadherin is direct target of the phosphatase SHP2; 2. Phosphorylation of Y731 is modulated by substrate stiffness; and 3. Leukocytes enhance tension on VE-cadherin) that are of significant importance to the field and would warrant publication in EMBO Journal. There are a few remaining concerns for the other claims in the manuscript.

Major concerns:
- The authors conclude that PECAM-1 delivers SHP2 to VE-cadherin. The experiments in Figure 2 clearly show that during T-cell transmigration the interaction between SHP2 and PECAM-1 is reduced and more VE-cadherin and SHP2 interaction is found. The other experiments in this figure demonstrate the importance of PECAM-1 during the interaction of endothelial cells with T-cells/neutrophils. However, direct evidence that PECAM-1 brings SHP2 proteins (thus the same pool of proteins!) to VE-cadherin is lacking. The Y663, 686F mutations in PECAM-1 do support a role for these SHP2 binding residues in the process, but the mutations may affect the PECAM-1-mediated interactions between the leukocytes with endothelial cells and thereby prevent the initial trigger for Y731 dephosphorylation already. Therefore, the conclusion that PECAM-1 directly provides SHP2 proteins to VE-cadherin remains uncertain.

We feel that it is highly likely that the dissociation of SHP2 from PECAM-1 provides VE-cadherin with SHP2, which directly mediates dephosphorylation of VE-cadherin Y731. Yet, we do agree with the reviewer that we have not directly demonstrated that the SHP2 molecules, which were dissociated from PECAM-1 are identical with those that associate with VE-cadherin. Therefore, we have toned down our statements in this respect, accordingly

(end of introduction, subtitle on page 6, concluding sentence of the respective chapter (page 7), first paragraph of discussion).

- If the hypothesis is true that PECAM-1 provides SHP2 to VE-cadherin and dephosphorylate Y731, then how come basal Y731 phosphorylation of VE-cadherin is lower in siPECAM-1 cells compared to control? (Figure 2C). Please explain.

We agree that in the absence of PECAM-1 the level of pY731 is lower than in EC with normal PECAM-1 levels. This is also seen, when we analyze EC which express the tyrosine mutant of PECAM-1 instead of wt PECAM-1. This observation was reproducible and not only made occasionally. We assume that PECAM-1 not only provides SHP2 to VE-cadherin, upon stimulation by leukocytes. PECAM-1 may also sequester SHP2 thereby preventing its access to VE-cadherin. From our experience, we can co-immunoprecipitate SHP2 with PECAM-1 much better than with VE-cadherin. Therefore, it is possible that PECAM-1 binds with higher affinity to SHP2. This would mean that cytosolic SHP2 binds preferentially to PECAM-1. It would follow, that in the vicinity of PECAM-1 SHP2 rather binds to PECAM-1 and not to VE-cadherin. If we assume that not all PECAM-1 molecules are associated with SHP2, (i.e. if we have no saturation), PECAM-1 could act as a "protector" for VE-cadherin and capture the majority of SHP2 molecules, sequestering them from VE-cadherin. If this were the case, the absence of PECAM-1 would cause a lower level of Y731 phosphorylation, provided the steady state tension across actin-anchored VE-cadherin molecules allows some low-level access of Y731. In summary, according to this model, low tension and the presence of PECAM-1 in the vicinity of VE-cadherin would cooperate to prevent access of SHP2 to VE-cadherin-Y731.

- In Figure 1a the authors show that SHP2 siRNAs inhibit transmigration in WT cells, but not in Y731F expressing endothelial cells. Based on this result the authors conclude that the contribution of SHP2 to leukocyte transmigration depends on the Y731 residue in VE-cadherin. Since transmigration across Ctrl siRNA treated Y731F endothelial cells is already lower compared to Ctrl siRNA treated WT cells, an alternative explanation might be that most of the paracellular transmigration is already inhibited by the Y731F mutation itself and that SHP2 cannot inhibit any further VE-cadherin-associated transmigration. Similarly, MAPTAM and blebbistatin treatments may not be able to reduce transmigration as transmigration is already much lower across Y731F cells compared to controls. Other, VE-cadherin independent mechanisms, may be at play during transmigration over Y731F cells.

We argue in our study that the inhibitory effects of SHP2 siRNA, blebbistatin, MAPTAM and of anti PECAM-1 antibodies are each dependent on the presence of Y731 of VE-cadherin. In other words, we do not see additive inhibitory effects of any of these reagents with the inhibitory effects caused by the Y731F mutation. Of note, each of these inhibitory effects, including the mutation of Y731, inhibit transmigration partially and to about similar extent. We agree with the reviewer that if the Y731F mutation would completely block the extravasation process, a lack of additivity of other inhibitory effects would not mean much, since there would not be much diapedesis activity left in VE-cadherin Y731F endothelial cells anyway. However, this is not the case. The Y731F mutation of VE-cadherin inhibits leukocyte extravasation partially, *in vitro* as well as *in vivo*. Importantly, we and others have shown before that only 10% of transmigrating neutrophils use the transcellular pathway, whereas 90% of the neutrophils use the paracellular pathway, at least in experimental systems used here. Thus, the Y731F mutation of VE-cadherin and all other inhibitory reagents used here inhibit **paracellular** diapedesis and they do this only partially. Collectively, this suggests that if SHP2 siRNA, blebbistatin, MAPTAM, or anti PECAM-1 would affect the paracellular

transmigration process in a way that is independent of VE-cadherin Y731, they should further reduce residual diapedesis and show an additive effect. This is not the case, instead we see no additivity. Therefore, we believe, that the absence of additive inhibitory effects argues for a dependence of each of the inhibitory effects on the presence of Y731 in VE-cadherin. Or to put it in other words, all the inhibitory effects tested here (anti-PECAM-1, SHP2 siRNA, MAPTAM, blebbistatin) interfere with signaling steps that act in the same signaling cascade which enables the diapedesis process.

Minor concerns:
-Is the observed increase in tension on VE-cadherin dependent on SHP2? Also, it would be extremely interesting to known how tension sensing occurs in the VE-cadherin Y731F mutant.

We have now silenced SHP2 in endothelial cells by siRNA and performed new FLIM-FRET measurements. We found that leukocyte-induced tension across VE-cadherin was not abolished. Thus, the effect of SHP2 on VE-cadherin is downstream of the induction of force on the VE-cadherin catenin complex. These results are now shown in the extended version of figure 8 (Fig. EV8). We agree that testing effects of the Y731F mutation on tension induction would be interesting. However, performing these experiments would have required to construct new Adenovirus packaged FRET constructs. Since we had technical problems with the organization of our S2 facility in recent months, we could unfortunately not do these experiments.

- Different FRET probes for measuring VE-cadherin tension are used compared to previous studies. For instance in the Conway et al. paper (Current Biology 2017) a different version of the tension sensor was used. Can the authors elaborate on why the new tension sensors were preferred? Are the current probes better/improved? This may be very relevant information for the field.

Indeed, our FRET construct is based on a different force sensing module with different force sensitivity. We inserted it at the same site into VE-cadherin as Conway et al (Current Biology 2013 and 2017). Whereas the previously reported F40 tension sensor used by Conway et al. reacts gradually to forces from below 1pN to 6 pN, the sensor we inserted into VE-cadherin responds to forces above 4 pN (Ringer et al., Nat Methods 14: 1090-1096, 2017). The sensor used here allows for higher FRET efficiency at forces below 4 pN, since almost none of the tension sensor molecules would be in the open state. This creates a more binary on/off situation. This was now mentioned in the discussion (page 17).

Can the authors provide further characterization for the proper functioning of the new FRET probes? Please also provide corresponding example fluorescence images. Does FRET efficiency change upon with blebbistatin/thrombin treatments? And what is the dynamic range during these experiments?

We now show novel results that characterize proper functioning of our FRET probes. We show in the new figure 7 that the VE-cadherin tension sensor is normally localized at endothelial cell contacts (Fig. 7C), and associates properly with the catenins, as shown by co-immunoprecipitations of α-catenin (Fig. 7D). In addition, we show that thrombin treatment of endothelial cells expressing the tension sensor triggers a loss in FRET efficiency, as was measured by live analysis (Fig. 7E). FRET efficiency was reduced by 20%.

These thrombin-induced changes in FRET efficiency were stronger than what we had found in our live analysis of endothelial cells in transmigration assays. As outlined in our comments for reviewer 1, the reason for this is that we did not just analyze the direct vicinity of single transmigrating leukocytes, but recorded signals from the complete cell junctions surrounding several endothelial cells in a region of interest. Since we performed live analysis, and each PMN slipped rapidly through the endothelial monolayer, the analysis of the vicinity of single leukocytes would not have yielded sufficient photon counts for reliable measurements. Therefore, we analyzed an area of endothelial cells with several PMNs. This, however, had the disadvantage that we analyzed also the signals of large junctional areas which were not in contact with neutrophils, which "diluted" our measured effect of neutrophil-induced decrease of FRET efficiency.

To circumvent this problem, we have now performed new experiments, where we fixed the endothelial monolayer with transmigrating neutrophils. This allowed us to analyze endothelial junctions within the direct vicinity of single neutrophils for longer time and collect sufficient photon quantities. The change in FRET efficiency increased considerably when we compared FLIM measurements at sites of PMN transmigration with signals at endothelial junctions of control endothelial cells which were also exposed to flow, but in the absence of PMNs. FRET efficiency was reduced from 25.9 % (without PMNs) to 21.9 % (at sites of transmigrating PMNs), which is a relative reduction of slightly more than 15.4 %. These results are now shown in the new figure 8D. As requested, we are now also showing representative fluorescence images of the change in FRET efficiency in the junctional vicinity of a transmigrating neutrophil in the new figure 8E.

- Figure panels in 1d, 2a and 2b are representative blots of multiple experiments. The fold change in co-IPs needs quantification. In addition, could the authors confirm that the co-immunoprecipitation of SHP2 with VE-cadherin IPs is reduced upon the incubation with T-cells?

We have now quantified the multiple immunoblots for each of the mentioned experiments and show the corresponding quantifications for figures 1D, 2A and 2B.

As requested, we have attempted to perform SHP-2/VE-cadherin co-IPs (figure 1D) in a reverse manner, by blotting for SHP2 after VE-cadherin IP instead of blotting for VE-cadherin after SHP2 IP. However, this way the co-immunoprecipitation did not work. Since the anti-VE-cadherin antibodies which worked best in our hands for immunoprecipitations, were directed against the cytoplasmic part of VE-cadherin, it may be possible that they interfered with the co-IP of SHP2.

- Fig 7a shows that endothelial cells on top of soft substrates prevents HL60 induced dephosphorylation of pY731. The interaction between, and transmigration of, leukocytes and the endothelium is notoriously different on softer substrates (Stroka et al, Blood 2011). As a consequence it remains unclear whether the primary PECAM-1 derived signal to SHP2 is not initiated or whether tension across endothelial cells is needed to stimulate Y731 dephosphorylation.

We have now tested, whether SHP2 dissociation from PECAM-1 can also be seen with EC grown on soft substrate. Using HUVEC, we found that HL60-derived neutrophils could trigger the dissociation of SHP2 from PECAM-1 on stiff substrate (20 kPa) as well as when

cells were grown on a substrate with 0.2 kPa. These results are now shown in the extended figure 8 (Fig. EV 8).

Additional non-essential suggestions for improving the study:
- The data point towards a model in which conformational changes within VE-cadherin or the catenins are responsible for the ability of SHP2 to dephosphorylate Y731. How does this relate to other interactions such as with adaptins or known tension imposed changes at endothelial junctions?

It is presently not yet known how force assisted SHP2 mediated Y731 dephosphorylation of VE-cadherin affects interactions with other factors and how this relates to other tension induced effects on junctions. It will certainly be interesting and important to investigate such questions in future studies. Besides the effects on Y731 dephosphorylation and subsequent internalization of VE-cadherin, which we report here, leukocyte induced pulling forces on the VE-cadherin-catenin complex may also have other consequences. Not the least of them might be an active support of junction de-stabilization by possibly weakening trans-interactions between VE-cadherin.

-Subcellular information regarding the protein interactions between SHP2, PECAM-1 and VE-cadherin would be helpful to tease out some of the remaining questions.

This is indeed a very good suggestion for further studies of the process whereby leukocytes modulate the function of VE-cadherin. Intermolecular FRET approaches to show local dissociation of SHP2 from PECAM-1 and association of the phosphatase with VE-cadherin might be a possible avenue for future studies. Likewise, proximity proteomics might be useful to label/tag SHP2 within the molecular vicinity of PECAM-1 and test, whether these tagged SHP2 molecules can be found associated with VE-cadherin.

Thank you for submitting a revised version of your manuscript. I apologise for the delay in the processing of your manuscript due to the holiday period. Your revised study has now been seen by all of the original referees, who find that their main concerns have been addressed and recommend publication of the manuscript. Therefore, I would like to invite you to address the remaining editorial issues before I can extend the official acceptance of the manuscript.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Referee #1:

The authors have addressed my queries.

Referee #2:

adequately revised

Referee #3:

The authors have in improved their manuscript significantly. In particular the connection between tension-induced changes across VE-cadherin in relation to local transmigration of neutrophils have been strenghtened. (Fig 6E and following Fig 7). As I mentioned earlier, it is expected that this research will have a large impact on the current view of the transmigration process and how the endothelial junctions are remodeled.

All my questions were addressed in a satisfactory manner, and I want to thank the authors for their efforts put in this work.

2nd Authors' Response to Reviewers 15th Jan 2021

Authors performed the requested changes.

2nd Revision - Editorial Decision 27th Jan 2021

Editor accepted the manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dietmar Vestweber
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-106113

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was not predetermined by statistical methods, instead it was determined based on our previous experiences with similar methodologies. The sample size was assumed to be sufficient to identify differences between groups.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	An empirical estimate of at least three mice per group were analyzed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude any animals from the experiments. For in vitro transwell transmigration assays, samples were excluded when subsequent filter stainings revealed that the endothelial monolayer was not intact.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For animal experiments, grouping was based on genotypes and different treatments. For in vitro studies, where the test subjects (cells or protein-complex) received different treatments, the test subjects were allocated into different experimental groups based on treatments.
For animal studies, include a statement about randomization even if no randomization was used.	Animals of same gender, age, genetic background and treatment were randomly allocated to groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Animals were randomly allocated to groups by a person different from the investigator to minimize the effects of subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For analysis of intra vital microscopy experiments, the investigators were blinded to the identities of the samples during data analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normalized distribution was visually assessed based on distribution of single data points in a boxplot + mean.
Is there an estimate of variation within each group of data?	Yes. Data are represented as mean ± SEM
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All commercial antibodies are described in Supplementary Table 1. α-catenin (610194, BD Biosciences), α-tubulin (T6199, Sigma-Aldrich), human β-catenin (H-102, Santa Cruz), mouse β-catenin (610154, BD), GFP fusion proteins (ab6556, Abcam), GST fusion proteins (Z-5, sc-459, Santa Cruz), mouse ICAM-1 (M-19, sc-1511, Santa Cruz), mouse p120 (15D2, sc-23872, Santa Cruz), human PECAM-1 (H-3, sc-376764, Santa Cruz), mouse PECAM-1 (M-20, sc-1506, Santa Cruz; MEC13.3, 550274, BD Biosciences), plakoglobin (610254, BD Biosciences), pY731 of VE-cadherin (our laboratory, Wessel et al., 2014), SH-PTP2 (B1, sc-7384, Santa Cruz), pTyr (4G10, 05-321, Merck Millipore), human VE-cadherin (C-19, sc-6458, Santa Cruz; D87F2, 2500, Cell Signalling; F-8, sc-9989, Santa Cruz; BV6, MABT134, Sigma-Aldrich), mouse proVE-cadherin (VD47, our laboratory), mouse VE-cadherin (C-5, Gotsch et al., 1997; VE-42, Broermann et al., 2011), mouse endomucin (7C7.1, Pharmingen), rabbit IgG (our laboratory), Armenian hamster IgG1 (553968, BD Biosciences), anti-mouse Alexa Fluor 488 (Invitrogen), anti-mouse HRP (Jackson Laboratories), anti-rabbit Alexa Fluor 568 (Invitrogen), anti-rabbit HRP (Jackson Laboratories), anti-rat HRP (Jackson Laboratories) and anti-goat HRP (Jackson Laboratories).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HL60 (originally from ATCC), bEND.3 (ECACC 96091929), bEnd.5 (ECACC 96091930) and LuEnd cells (Graesser. et al., the Journal of Clinical Investigation, 2002) were maintained in our laboratory. Cell lines were routinely tested for mycoplasma contamination in our laboratory.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male mice of C57BL/6 background at an age of 14 - 16 weeks were used for the experiments. Mice were raised in a barrier facility under special-pathogen free conditions with food and water ad libitum and a 12 h light/ dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were carried out under the German legislation for the protection of animals and were approved by the Landesamt fuer Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance is confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA