Mitofusin-2 stabilizes adherens junctions and suppresses endothelial inflammation via modulation of β-catenin signaling

Endothelial barrier integrity is ensured by the stability of the adherens junction (AJ) complexes comprised of vascular endothelial (VE)-cadherin as well as accessory proteins such as β-catenin and p120-catenin. Disruption of the endothelial barrier due to disassembly of AJs results in tissue edema and the influx of inflammatory cells. Using three-dimensional structured illumination microscopy, we observe that the mitochondrial protein Mitofusin-2 (Mfn2) co-localizes at the plasma membrane with VE-cadherin and β-catenin in endothelial cells during homeostasis. Upon inflammatory stimulation, Mfn2 is sulfenylated, the Mfn2/β-catenin complex disassociates from the AJs and Mfn2 accumulates in the nucleus where Mfn2 negatively regulates the transcriptional activity of β-catenin. Endothelial-specific deletion of Mfn2 results in inflammatory activation, indicating an anti-inflammatory role of Mfn2 in vivo. Our results suggest that Mfn2 acts in a non-canonical manner to suppress the inflammatory response by stabilizing cell–cell adherens junctions and by binding to the transcriptional activator β-catenin.
One devastating manifestation of the disassembly of endothelial adherens junctions (AJs) is acute lung injury (ALI), in which an excessive immune response triggers the disruption of the lung endothelial barrier, fluid, and protein leak into the alveolar space resulting in compromised oxygen exchange and an unhinged state of inflammatory activation\textsuperscript{12}. Understanding the mechanisms leading to the breakdown of endothelial barrier function as well as the inflammatory pathways within the endothelium are essential for developing therapeutic strategies to treat ALI patients\textsuperscript{3}. The vascular endothelium maintains an intact barrier to prevent leakage of circulating nutrients, solutes, and fluid into the tissues as well as tightly regulating the influx of immune cells\textsuperscript{4}. However, during severe infections and subsequent inflammatory responses, the endothelial barrier is compromised due to the breakdown of endothelial AJs. The plasma membrane AJ protein complex in endothelial cells consists of VE-cadherin as well as members of the catenin family such as β-catenin and p120-catenin\textsuperscript{5}. The influx of immune cells following AJ disassembly exacerbates the inflammatory process, leading to a feed-forward activation of inflammation and further destruction of the endothelial barrier\textsuperscript{1}.

There are multiple mechanisms underlying the breakdown of AJs such as signaling induced by the pro-inflammatory cytokine TNFs which promotes inflammation via the generation of reactive oxygen species (ROS)\textsuperscript{6,7}. ROS mainly mediate reversible or irreversible oxidative modification at cysteine residues\textsuperscript{8}. Reactive cysteine thiol (SH) converts cysteine sulfenic acid (Cys-SOH), termed as protein sulfenylation, a key initial mediator of redox signaling\textsuperscript{9}. Prior studies of inflammatory redox signaling and barrier function have understandably focused on known junctional proteins at the plasma membrane. However, recent studies on inflammatory signaling implicate the involvement of mitochondria\textsuperscript{10-12}.

The mitochondrial GTPases Mitofusin-1 (Mfn1) and Mitofusin-2 (Mfn2) are key regulators of mitochondrial function by mediating mitochondrial network fusion which allows for the distribution of proteins, mitochondrial DNA, and metabolites to maintain network connectivity\textsuperscript{13,14}. Mitofusin 1 and Mitofusin 2 are located in the outer membrane of mitochondria and thus may facilitate interactions with other organelles\textsuperscript{13}. The function of Mfn1/2 can be regulated by post-translational modifications such as ubiquitination, acetylation, or phosphorylation\textsuperscript{13}. Mfn1/2 also acts as tethers for mitochondria with each other or with other organelles such as ER and as mitochondrial anchoring proteins\textsuperscript{15}. Although Mfn1 and Mfn2 demonstrate approximately 80% sequence homology, double mutant embryos die earlier than either single mutant, and the post-natal Mfn1 knockout (KO) mice do not exhibit significant pathology whereas Mfn2 KO mice show rapid lethality\textsuperscript{16}. It has thus been suggested that non-redundant functions of Mfn1 and Mfn2 that may go beyond the traditionally ascribed mitochondrial fusion roles of these proteins. Non-fusion unique roles of Mfn2 include the mediation of mitophagy and apoptosis\textsuperscript{13}, or regulation of contact sites with ER\textsuperscript{14,16,17}. However, the potential roles of Mfn2 in the formation or stabilization of adherens junctions are not yet known.

In this study, we identified a non-canonical function of Mfn2 as a stabilizer of endothelial adherens junction complexes during homeostasis. Mfn2 is sulfenylated during inflammation and accumulates in the nucleus where it acts as an endogenous suppressor of inflammation by binding the transcriptional regulator β-catenin.

Results

Mfn2 binds to the adherens junction complex during homeostasis. To identify binding partners of Mfn2 in an unbiased manner, we performed a comprehensive proteomic analysis in human lung microvascular endothelial cells (HLMVECs). Mfn2 was overexpressed in HLMVECs using lentiviral GFP-Mfn2 and GFP-Mfn2 was immunoprecipitated with GFP-trap magnetic agarose to reduce non-specific binding that may be present with the use of anti-GFP antibodies. Overexpression of lentiviral GFP in HLMVECs was used as a control to assess protein partners that non-specifically bind to GFP (Supplementary Fig. 1a, b). We focused on protein partners unique to the expression of GFP-Mfn2 versus the GFP-control. The obtained proteins were analyzed using high-affinity liquid chromatography with tandem mass spectrometry (LC-MS/MS). We considered candidates which are identified in at least two samples among three independent experiments with a >1.5-fold increase threshold. We found that 25 proteins specifically interacted with Mfn2 in ECs (see Source data) and all identified protein partners were classified based on their function using gene ontology enrichment analysis (Supplementary Fig. 1c). We found that Mfn2 interacted with the expected mitochondrial protein partners but, we also identified non-mitochondrial protein partners. The non-mitochondrial proteins interacting with Mfn2 in included cadherin binding partners and cell–cell adhesion partners such as KRT18, ENO1, or MACF1 (Supplementary Fig. 1c). In order to understand the functional significance of this intriguing finding, we focused on studying the role of non-mitochondrial Mfn2.

Next, we investigated whether Mfn2 co-localizes with cadherin proteins in ECs using three-dimensional structured illumination microscopy (3D-SIM) which provides high spatial resolution to resolve individual protein complexes\textsuperscript{18,19}. 3D-SIM demonstrated the presence of non-mitochondrial Mfn2 complexes, especially at the plasma membrane where Mfn2 co-localized with the endothelial adherens junction (AJ) protein VE-cadherin (Fig. 1a±c and Supplementary Fig. 1d, e). As expected, the majority of Mfn2 co-localized with the mitochondrial membrane protein Tom20 (R2 in Fig. 1a, b) as previously reported\textsuperscript{20}. However, we found that Mfn2 additionally co-localized with the endothelial AJ protein VE-cadherin but not with Tom20, which indicated that non-mitochondrial Mfn2 was present at the AJs (R1 in Fig. 1a, b). Manders’ overlap coefficient indicates that 54% of Mfn2 (M1 = 0.54 ± 0.045) co-localizes with VE-cadherin and 33% of VE-cadherin (M2 = 0.33 ± 0.055) co-localizes with Mfn2 at AJs area (Fig. 1c). Confocal microscopy experiments involving the expression of GFP-Mfn2 independently confirmed the presence of non-mitochondrial Mfn2 (not co-localizing with Tom20) (R1 in Fig. 1d, e), indicative of an unrecognized non-mitochondrial role consistent with the proteomic analysis (Supplementary Fig. 1c). In contrast, its homolog Mfn1 did not co-localize with VE-cadherin at the plasma membrane (R1 in Fig. 1f, g, R1 in Supplementary Fig. 1f, h, i) and instead always co-localized with the Tom20 (R2 in Fig. 1f, g, R1 in Supplementary Fig. 1h, g) as evidenced by the low Manders’ overlap coefficient (M1 = 0.0019 ± 0.0024 for Mfn1, M2 = 0.0074 ± 0.0031 for VE-cadherin) (Fig. 1h) and low Spearman coefficient (R = 0.195) (Supplementary Fig. 1j) between Mfn1 and VE-cadherin at AJs.

To understand the specific roles of Mfn2 in AJ protein complexes, we next investigated potential binding partners of Mfn2 within AJs in HLMVECs. Total Mfn2 proteins were immunoprecipitated and followed by immunoblotting with antibodies for AJ complex proteins VE-cadherin or β-catenin. Mfn2 significantly interacted with VE-cadherin and β-catenin in resting ECs (Fig. 1i, j). To verify the specificity of the Mfn2 interactions at AJs, Mfn2 was depleted in HLMVECs with a doxycycline inducible lentiviral Mfn2 shRNA. Mfn2 depletion (Mfn2-KD) decreased the interaction of Mfn2 with VE-cadherin and β-catenin (Supplementary Fig. 1k). Importantly, these biochemical approaches independently confirmed that Mfn2 interacts with the endothelial AJ proteins VE-cadherin and
β-catenin at the plasma membrane, consistent with the 3D-SIM findings (Fig. 1a).

Mfn2 localized at the plasma membrane promotes endothelial barrier integrity during endothelial homeostasis. To determine whether the presence of Mfn2 at AJs affects AJ function as manifested by endothelial barrier integrity, we next investigated endothelial barrier function in control and Mfn2-depleted HLMVECs. We confirmed that our Mfn2 depletion strategy was specific to Mfn2 and did not affect Mfn1 levels (Supplementary Fig. 2a, b). Mfn2 depletion disrupted adherens junctions (AJs) as visualized by VE-cadherin and β-catenin immunostaining (Fig. 2a), whereas Mfn1 depletion did not affect the endothelial
barrier (Supplementary Fig. 2c), indicating that the observed barrier stabilizing effect was specific for Mfn2. The area and fluorescence intensity of VE-cadherin or β-catenin at AJs were quantified with ImageJ (Fig. 2b and Supplementary Fig. 2d). Next, we used a transendothelial resistance (TER) assay and a FITC-conjugated albumin permeability assay to assess endothelial barrier integrity. Mfn2 depletion significantly decreased transendothelial resistance (TER) (Fig. 2c and Supplementary Fig. 2e). Similarly, the transendothelial permeability for the FITC-conjugated albumin tracer was increased in Mfn2-depleted HLMVECs (Fig. 2d). Moreover, we examined whether the loss of barrier integrity could be rescued by GFP-Mfn2 overexpression. We designed an Mfn2 shRNA to target 3′-UTR regions of Mfn2 gene instead of the coding sequences (CDS), thus making GFP-Mfn2 impervious to Mfn2 shRNA. We observed that the increased permeability in Mfn2-KD cells was significantly rescued by overexpressing GFP-Mfn2 as assessed by FITC-conjugated albumin permeability (Fig. 2d). We also assessed the barrier by confocal microscopy and found that the disrupted barrier in Mfn2-depleted ECs was restored after GFP-Mfn2 overexpression (Fig. 2e, f).

It is known that filamentous actin (F-actin) plays a critical role in stabilizing cell–cell contacts at AJs21. Our proteomic analysis had shown that Mfn2 interacts with an actin binding protein, MACF1 (Microtubule Actin Crosslinking Factor 1) (Supplementary Fig. 1c). Thus, we examined whether the effects of Mfn2 on the endothelial AJ stability may in part relate to its interaction with F-actin. Control endothelial cells showed a regular F-actin structure, whereas Mfn2-KD ECs clearly demonstrated decreased sites of cell–cell contact and a disassembly of F-actin contact points using LifeAct-GFP live cell imaging of actin filaments (Supplementary Fig. 2f). Furthermore, we found that F-actin co-localization with VE-cadherin was dependent on Mfn2 (Supplementary Fig. 2g, h). These results suggest that Mfn2 may help anchor F-actin structures at AJs and thus promote endothelial barrier integrity.

In addition, we investigated the possibilities that our observed effects of Mfn2 depletion on the loss of endothelial barrier integrity may reflect a form of generalized cellular stress that would increase cell death, oxidative stress, or reduce cell proliferation. We found that Mfn2 depletion in homeostatic ECs did not decrease cell proliferation or induce apoptosis (Supplementary Fig. 3a–i). Furthermore, Mfn2 depletion did not increase mitochondrial ROS production (Supplementary Fig. 3g, h) and also had no significant effect on mitophagy (Supplementary Fig. 3i, j), ER stress (Supplementary Fig. 3k), and total AJs protein levels in homeostatic ECs (Supplementary Fig. 2l). Next, we examined whether changes in the mitochondrial morphology could affect EC barrier integrity, as Mfn2 is a key regulator of mitochondrial fusion and therefore any effects on EC barrier integrity may be mediated by changing mitochondrial dynamics. We used siRNA to specifically deplete the mitochondrial fission mediator Drp1 or the mitochondrial fusion mediators Opal1, Mfn1, and Mfn2 (Supplementary Fig. 3m, n). Even though the siRNA depletions were sufficient to modify mitochondrial network structure, as seen in the increase of mitochondrial fragmentation following Opal1 depletion or the increase in mitochondrial elongation with Drp1 depletion, EC barrier integrity was only significantly decreased with Mfn2 depletion suggesting that the observed Mfn2 effects on EC barrier integrity were not primarily related to the Mfn2 role in mitochondrial fusion. Taken together, these data suggest that Mfn2 binds to AJ complexes at the plasma membrane where it specifically stabilizes the endothelial barrier and regulates F-actin filament structures.

Endothelial-specific deletion of Mfn2 increases inflammatory injury in vivo. We next studied whether Mfn2 regulates the endothelial barrier integrity in vivo and used a tamoxifen-inducible EC-specific conditional Mfn2 knockout (Mfn2EC−/−) mice (Supplementary Fig. 4a, b). First, we investigated mRNA or protein expression levels of Mfn2 in whole lungs from Mfn2fl/fl (littermate controls) and Mfn2EC−/− mice. The mRNA levels of Mfn2 showed about 50% knockdown efficacy in whole lungs from Mfn2EC−/− mice (Supplementary Fig. 4b). To verify specific deletion of Mfn2 in ECs, we examined protein levels of Mfn2 in ECs isolated from the lungs of Mfn2fl/fl and Mfn2EC−/− mice. ECs from Mfn2EC−/− mice demonstrated greater than 80% reduction of Mfn2 levels compared to ECs from Mfn2fl/fl (Fig. 3a, b). Next, we examined the functional role of endothelial Mfn2 in the regulation of in vivo vascular permeability. Mfn2EC−/− and
Mfn2^{fl/fl} mice were injected intravenously with the Evans blue-albumin dye to assess lung vascular permeability\(^{22}\). The lungs from Mfn2^{EC−/−} mice demonstrated significantly higher leakiness than lungs from Mfn2^{fl/fl} control mice (Fig. 3c, d). We also examined the expression levels of pro-inflammatory genes in the lungs of Mfn2^{fl/fl} or Mfn2^{EC−/−} mice. As shown in Fig. 3e, the expression of IL-β, IL-6, TNFα, or IFNγ genes was significantly increased in lungs of Mfn2^{EC−/−} mice than in those of Mfn2^{fl/fl} control mice (Fig. 3e).

Furthermore, the lungs from Mfn2^{EC−/−} mice demonstrated significant accumulation of immune cells in the proximity of venous blood vessels but not arterial blood vessels when compared to the lungs of control Mfn2^{fl/fl} mice (Fig. 3f). Finally, to determine the phenotype of infiltrating immune cells in the...
lungs of Mfn2EC/−/− mice, we generated a single cell suspension of the whole lung tissues obtained from Mfn2fl/fl and Mfn2EC/−/− mice, and performed flow cytometry after staining for cell type-specific markers. We found that the lungs of Mfn2EC/−/− mice were significantly enriched for myeloid cells such as interstitial macrophages (IMs) and neutrophils (Neu), as well as lymphoid B + T cells and natural killer T (NKT) cells when compared to the lungs of Mfn2fl/fl mice (Fig. 3g, h). However, B cells and T cells were decreased in the lungs from Mfn2EC/−/− mice, suggesting an activation of the innate immune response in resting lung endothelial cells upon endothelial Mfn2 deletion (Supplementary Fig. 4c, d). These data indicate that endothelial Mfn2 is a critical regulator of the endothelial barrier integrity during homeostasis and that its absence induces spontaneous vascular leakiness as well as inflammation.

Mfn2 interaction with β-catenin is enhanced following their disassociation from adherens junctions. After establishing a key role of Mfn2 in stabilizing the endothelial barrier during homeostatic conditions, we next examined the role of Mfn2 during inflammation because inflammatory stimulation promotes AJ disassembly. HLMVECs were stimulated with TNFα and the AJ complex proteins VE-cadherin or β-catenin were immunoprecipitated, followed by immunoblotting. We found that inflammatory stimulation increased the interaction between Mfn2 and β-catenin, but not between Mfn2 and VE-cadherin (Fig. 4a, b and Supplementary Fig. 4e, f). We confirmed these results by using a GFP-tagged Mfn2 construct that was expressed in ECs and found a six-fold increase in the interaction between Mfn2 and β-catenin following inflammatory activation with TNFα (Fig. 4c, d). We also used an in situ proximity ligation assay (PLA), which creates a spatial fluorescent signal within a 30–40 nm maximum distance and can thus establish close proximity interactions between protein partners. The known interaction between VE-cadherin and β-catenin was used as a positive control. The interaction between VE-cadherin and β-catenin (red dots) was present under homeostatic conditions but rapidly decreased following TNFα stimulation (Fig. 4e, f). However, the binding of Mfn2 and β-catenin (green dots) was markedly increased in the cytosol (Fig. 4g, h). Moreover, EC barrier impairment in Mfn2-KD ECs was further increased by TNFα stimulation, and rescued by overexpressing GFP Mfn2 (Supplementary Fig. 4g). Based on multiple lines of inquiry, we concluded that Mfn2 disassociates from VE-cadherin and there is a concomitant increase of Mfn2 interaction with β-catenin in the cytosol following inflammatory stimulation.

TNFα-induced ROS increase the binding of Mfn2 to β-catenin. We next investigated the mechanism by which inflammatory activation with TNFα could affect the interaction between Mfn2 and β-catenin. ROS are key mediators of inflammatory signaling by increasing reversible oxidative modifications. To investigate whether TNFα-induced cysteine modifications may post-translationally regulate interactions of Mfn2 with partner proteins, ROS production increased within 15 min of inflammatory stimulation with TNFα (Fig. 5a). Exogenous ROS (H2O2) increased the interaction of Mfn2 and β-catenin (Fig. 5b), consistent with the notion that Mfn2 modified by inflammation-induced ROS promotes the interaction of Mfn2 with cytosolic β-catenin when both are disassociated from AJ complexes, but that Mfn2 no longer interacts with VE-cadherin. Moreover, the complex of Mfn2 and β-catenin exhibited band shifts that were dependent on Mfn2 in a non-reducing SDS-PAGE gel (Supplementary Fig. 5a) and decreased in Mfn2-KD ECs in reducing SDS-PAGE gels (Supplementary Fig. 5b). These data suggest that their interaction may be mediated via disulfide bond formation that was dependent on the presence of ROS. Then, we performed a rescue experiment using the ROS scavenger N-Acetyl-L-cysteine (NAC) which reduces disulfide bond formation. As shown in Fig. 5c, d, the increased interaction of Mfn2 and β-catenin by H2O2 was reversed by NAC treatment. These data suggest that during inflammation, ROS mediate the interaction of Mfn2 and β-catenin. Moreover, we investigated whether the complex formation of Mfn2 and β-catenin was associated with cysteine sulfenylation, a key initial step for cysteine oxidation. To examine whether TNFα-induced ROS modulate cysteine sulfenylation of Mfn2 and β-catenin, TNFα stimulated-ECs were lysed with lysis buffer containing DCP-Bio1, a cell permeable biotin-labeled Cys-OH trapping probe. The captured proteins were used to determine sulfenylation of Mfn2 or β-catenin by Western blotting with their specific antibodies. Importantly, sulfenylation of both Mfn2 and β-catenin was significantly increased 1 h after TNFα stimulation and subsequently decreased in a time-dependent manner (Fig. 5e, f). We then addressed the role of sulfenylation in the binding of Mfn2 and β-catenin. Control and Mfn2-depleted ECs were stimulated with 500 µM H2O2 for 30 min and subjected to a DCP-Bio1 assay. H2O2 treatment of control ECs induced cysteine sulfenylation of...
Fig. 3 Increase in lung vascular permeability following endothelial-specific genetic deletion of Mfn2. a, b ECs were isolated from the lungs of Mfn2/fl or Mfn2EC/− mice using CD31 antibody. Mfn2 knockdown efficiency was determined by Western blotting (a) and protein levels for Mfn2 were quantified using ImageJ (b). Uncropped blots can be found in the Source Data file. Data are mean values ± SEM for n = 3 mice. **p = 0.0017 by unpaired, two-tailed t-test. c Mfn2/fl and Mfn2EC/− mice in resting condition were i.v. injected with Evans blue albumin (40 mg/mL). After 45 min, the mice were perfused with PBS and lung permeability was evaluated by measuring Evans blue albumin contents in lung. d Evans blue albumin contents in lungs was measured at OD620 and the transvascular flux of albumin in filtration is presented (in µg) after normalizing by the wet lung weight (in g). Data are mean values ± SEM for at least n = 3–4 mice. **p = 0.0056 by unpaired, two-tailed t-test. e Lungs from Mfn2/fl and Mfn2EC/− mice in homeostatic conditions were used to evaluate the mRNA levels of pro-inflammatory genes such as IL-1β (n = 4–6), IL-6 (n = 4), TNFα (n = 4), and IFNγ (n = 6) by qRT-PCR. Data are mean values ± SEM for at least n = 4 mice. *p = 0.0208 for Mfn2, **p = 0.035 for IL-1β, *p = 0.0314 for IL-6, **p = 0.0127 for TNFα, **p = 0.0075 for IFNγ by paired, two-tailed t-test. f Lungs from Mfn2/fl (n = 3) and Mfn2EC/− (n = 3) mice in homeostatic conditions were used to evaluate infiltration of inflammatory cells. The sections from paraffin fixed lungs were represented by H&E staining. Biological replicates (n = 3 mice) from each group showed similar results. A: artery, V: vein. Red arrow heads represent infiltrated inflammatory cells. g–h Lungs from Mfn2/fl and Mfn2EC/− mice in homeostatic conditions were homogenized and inflammatory cells were stained with indicated specific antibodies by following FACS analysis (g). h The inflammatory cells were represented by percent (%) of whole lung cells in (g). Data are mean values ± SEM for n = 3 mice. IM2: Interstitial macrophages 2, Neu: neutrophil, B: B cells, T: T cells, NKT: Natural killer T cell. **p = 0.0011 for IM2, **p = 0.0018 for Neu, **p = 0.0073 for B + T, **p = 0.0014 for NKT by unpaired, two-tailed t-test. The sequential gating strategies were presented at Supplementary Fig. 4d. All Mfn2/fl and Mfn2EC/− mice received tamoxifen and were rested for one month before experiments.
Fig. 4 Mfn2 binding to the AJ complex protein β-catenin is increased following inflammatory stimulation. Confluent HLMVECs were treated with TNFα (10 ng/mL) for 6 h. a Mfn2 was immunoprecipitated with Mfn2 specific antibody followed by Western blotting for β-catenin or Mfn2 antibodies. Uncropped blots can be found in the Source Data file. b The band intensities for Mfn2 and β-catenin interactions in (a) were quantified with ImageJ. Data are mean values ± SEM for three independent experiments. *p = 0.0403 by unpaired, one-tailed t-test. c HLMVECs expressing GFP or GFP-Mfn2 were stimulated with TNFα (10 ng/mL) for 6 h. The GFP-Mfn2 was immunoprecipitated with GFP-trap magnetic agarose followed by Western blotting for β-catenin and Mfn2 antibodies. Uncropped blots can be found in the Source Data file. d The band intensities of the Western blot were quantified with ImageJ. Data are mean values ± SEM for three independent experiments. *p = 0.0097 by unpaired, one-tailed t-test. e–h Proximity ligation assay (PLA). e The interaction of endogenous VE-cadherin and β-catenin in HLMVECs was examined using mouse VE-cadherin antibody and rabbit β-catenin antibody for positive control. The red dots show an interaction between VE-cadherin and β-catenin. f Quantification of the number of interactions (red dots) per field in (e). Data are mean values ± SEM from n = 6 for basal and n = 7 for TNFα treatment in independent biological replicates samples. **p = 0.0018 by paired, two-tailed t-test. g The interaction of endogenous Mfn2 and β-catenin in HLMVECs was examined with mouse Mfn2 antibody and rabbit β-catenin antibody. The green dots show interaction between Mfn2 and β-catenin, and DAPI (blue) indicates nuclei. h Quantification of the number of interactions (green dots) per cell in (g). Data are presented with box and whiskers plot and whiskers are Min to Max. n = 8 for basal and n = 14 for TNFα. ****p < 0.0001 by paired, two-tailed t-test. The images in (e) and (g) are representative from at least six independent biological replicates.
Mfn2 and β-catenin, whereas Mfn2 depletion of ECs resulted in the inhibition of β-catenin sulfenylation (Supplementary Fig. 5c).

To investigate whether sulfenylation of Mfn2 also occurs during inflammation in vivo, we used the experimental model of endotoxemia which induces profound inflammatory injury. C57/BL6 mice were intraperitoneally injected with the bacterial endotoxin lipopolysaccharide (8 mg/kg sublethal LPS) or PBS (control) and we monitored Mfn2 sulfenylation at 6 h and 24 h in whole lungs. LPS induced inflammation significantly increased Mfn2 sulfenylation at 6 h (Fig. 5g, h), indicating that Mfn2 sulfenylation is a post-translational modification that also occurs in vivo. However, we found that Mfn2 depletion or TNFα stimulation did not induce phosphorylation at serine 33 and 37 residues of β-catenin which are known to promote β-catenin...
Fig. 5 TNFα induced ROS production mediates the interaction of Mfn2 and β-catenin. a HLMVECs were stimulated with TNFα (10 ng/mL) for indicated durations (0, 15 min, 3 h, and 6 h). TNFα-induced total ROS production was measured using a DCFH-DA probe which detects intracellular H2O2. The ROS images in upper panel were taken with confocal microscope (Zeiss LSM880, Plan 1.45NA, ×63 magnification). The relative fluorescence of DCF was quantified with ImageJ (lower panel) and presented fold change to basal. Data are mean values ± SD from n = 4. b Left panel, HLMVECs were stimulated with H2O2 (500 μM) for 30 min and immunoprecipitated with Mfn2 antibody followed by Western blotting with β-catenin antibody under reducing conditions (with β-mercaptoethanol, ME). Mouse normal IgG was used as a negative control for immunoprecipitation. Uncropped blots are provided in the Source Data file. Right panel, the quantification of band intensities for Mfn2 and β-catenin interactions with ImageJ. Data are mean values ± SEM for three independent experiments. *p = 0.050 by unpaired, one-tailed t-test. c HLMVECs were pretreated with 20 mM NAC for 30 min. The cells were stimulated with H2O2 (500 μM) for 30 min and immunoprecipitated with Mfn2 antibody followed by Western blotting with β-catenin antibody. Uncropped blots are in the Source Data file. d Quantification of band intensity for Mfn2 and β-catenin interactions in (c) with ImageJ. Data are mean values ± SEM for three independent experiments. *p = 0.0287 for Basal vs H2O2, **p = 0.0281 for H2O2 vs H2O2 + NAC by unpaired, two-tailed t-test. e Confluent HLMVECs were stimulated with TNFα (10 ng/mL) for the indicated time course (0, 1, 3, or 6 h) and lysed with lysis buffer containing DCP-Bio1. The sulfenylated (DCP-Bio1 conjugated) proteins were pull down with streptavidin and sulfenylated of Mfn2 or β-catenin was determined by Western blotting with their specific antibodies. Actin was used as a negative control for TNFα induced sulfenylation. Uncropped blots are provided in the Source Data file. f Quantification of sulfenylation levels of Mfn2, β-catenin, and actin in (e) with ImageJ. Data are mean values ± SEM for 3–4 independent experiments. *p = 0.0068 for Mfn2-SOH in TNFα 0 h vs 1 h, ****p < 0.0001 for β-catenin-SOH in TNFα 0 h vs 1 h, ns: not significant (p = 0.5061) for Actin-SOH in TNFα 0 h vs 1 h by unpaired, two-tailed t-test. g, h C57BL/6L control mice were administrated with PBS or sub lethal LPS (8 mg/kg i.p.) and sacrificed after 6 h or 24 h. The whole lungs after PBS perfusion were used for DCP-Bio1 assay and followed by streptavidin pulldown and Western blotting (g). Uncropped blots are provided in the Source Data file. h Quantification of sulfenylated Mfn2 in the lungs shown in (g) with ImageJ. The levels of Mfn2-SOH were normalized by the total Mfn2 loading amount and further normalized with controls (PBS), and are presented as fold change. Data are mean values ± SEM for n = 6 mice for each condition. *p = 0.0126 for PBS vs LPS 6 h, p = 0.1142 for PBS vs LPS 24 h by unpaired, two-tailed t-test.

degradation (Supplementary Fig. 5d, e). TNFα (10 ng/mL) stimulation had no effect on total protein levels of AJs complex until 24 h (Supplementary Fig. 5f). Taken together, these data suggest that inflammation-induced Mfn2 sulfenylation increases its interaction with sulfenylated-β-catenin.

Transcriptional activity of β-catenin is negatively regulated by Mfn2 during inflammation. To address the potential functional roles of the Mfn2-β-catenin interaction during inflammation, we investigated whether the presence of Mfn2 affects the expression of key pro-inflammatory genes such as Intercellular cell adhesion molecule-1 (ICAM-1), Interleukin-6 (IL-6), and –18 (IL-18). Control and Mfn2-KD ECs were stimulated with TNFα and mRNA expression levels of pro-inflammatory genes were evaluated by quantitative real-time PCR (qRT-PCR). Mfn2 depletion in ECs significantly increased TNFα-induced mRNA levels of ICAM-1, IL-6, and IL-18, indicating that Mfn2 suppresses the TNFα induced pro-inflammatory response in ECs (Fig. 6a, b).

Upon disassociating from AJs complexes at plasma membrane, β-catenin translocates to the nucleus where it acts as a key co-factor for the transcription factor, T-cell factor (TCF) and thereby mediates Wnt signaling. Since we had found that Mfn2 binds β-catenin after inflammation-induced disassociation from AJs, we next investigated whether inflammation affects β-catenin transcriptional activity using a β-catenin luciferase assay. TNFα stimulation significantly increased β-catenin transcriptional activity in a time-dependent manner (Supplementary Fig. 6a). Interestingly, β-catenin transcriptional activity was significantly increased by Mfn2 depletion and further enhanced by TNFα stimulation in ECs (Fig. 6c), thus suggesting that Mfn2 acted as a suppressor of β-catenin-mediated transcriptional activation during inflammation. The exaggerated β-catenin transcriptional activity was reset by restoring Mfn2 expression (Fig. 6d).

We then examined whether β-catenin directly regulates the expression of pro-inflammatory genes. HLMVECs were transfected with siRNA for β-catenin (Supplementary Fig. 6b) and mRNA levels of pro-inflammatory genes were examined with or without TNFα stimulation. The TNFα-induced expression of ICAM-1, IL-β, or IL-18 genes was significantly inhibited in β-catenin knockdown ECs (Fig. 6e). Taken together, these data indicate that Mfn2 functions as a suppressor for β-catenin-mediated transcriptional activation during inflammation.

TNFα triggers nuclear accumulation of Mfn2. Next, we investigated whether Mfn2 accumulates in the nucleus during inflammation. The precise subcellular localization of Mfn2 was determined by a subcellular fractionation assay as well as by confocal microscopy at baseline and following TNFα stimulation. We first confirmed nuclear translocation of the pro-inflammatory transcription factor NF-kB after TNFα stimulation to validate the degree of inflammatory activation induced by TNFα in ECs (Supplementary Fig. 6d). Mfn2 was mainly localized in the cytosolic/mitochondrial fraction but a portion of Mfn2 clearly accumulated in the nuclei at 6 h after TNFα stimulation (Fig. 7a, b), whereas Mfn1 showed no such accumulation (Supplementary Fig. 6d). It came as a surprise because Mfn2 does not have a nuclear localization sequence (NLS) even though the presence of mitochondrial proteins in the nucleus has been recently described. To verify nuclear accumulation of Mfn2 during inflammation, we investigated whether exogenously over-expressed GFP-Mfn2 is also found in the nucleus along with endogenous Mfn2 during inflammation using confocal microscopy with three-dimensional Z-stacking. GFP-Mfn2 accumulated in the nucleus along with endogenous Mfn2 after 6 h of TNFα stimulation (Fig. 7c, d). Moreover, it was confirmed by 3-dimensional image analysis using all sections (average 91 sections) and ortho analysis using one section to show co-localization of GFP-Mfn2, Mfn2 (red color), and DAPI (Fig. 7e). We found that β-catenin translocated into the nucleus at 6 h after TNFα stimulation, mirroring the nuclear accumulation pattern we had observed for Mfn2 (Fig. 7f, g and Supplementary Fig. 6d, e). The persistence of the Mfn2/β-catenin interaction in the nuclei by TNFα stimulation was further demonstrated by a proximity ligation assay (PLA) (Fig. 7h). We next examined whether Mfn2 affected nuclear accumulation of β-catenin using confocal microscopy. The TNFα-induced nuclear accumulation of β-catenin was not affected by Mfn2 depletion (Supplementary Fig. 6f, g). Taken together, these results indicate that Mfn2 and β-catenin both accumulate in the nucleus and that nuclear Mfn2 may suppress the transcriptional activity of β-catenin.

Discussion
Our goal was to identify binding partners of Mfn2 and potential non-mitochondrial roles of Mfn2 in the endothelium. The non-mitochondrial roles for Mfn2 we identified in the
endothelium include: (i) stabilization of endothelial AJ junctions by binding to the AJ proteins complex VE-cadherin and β-catenin under homeostatic conditions, (ii) Sulfenylation of Mfn2 disassociated from the AJ complex during inflammation and accumulation of Mfn2 in the nucleus, and (iii) the binding of Mfn2 to the transcriptional regulator β-catenin and inhibition of β-catenin transcriptional activity during inflammatory activation.

Mfn2 is typically found in the outer mitochondrial membrane and its structural motifs contain a cytosolic N-terminal GTPase domain, a proline-rich region (PR), two coiled-coil heptad-repeat domains (HR1 and HR2), and a transmembrane domain (TM) which allows Mfn2 anchorage in the outer mitochondrial membrane. Mfn1 and Mfn2 are key mediators of mitochondrial fusion but only Mfn2 has a PR domain, thought to be responsible for specific protein–protein interactions suggesting the
Fig. 6 Mfn2 suppresses β-catenin transcriptional activity which requires TNFα induced pro-inflammatory gene expression. a, b Control and Mfn2-KD ECs were stimulated with or without TNFα (10 ng/mL) for 6 h and total RNA was extracted. a The knockdown efficiency of Mfn2 mRNA was determined under basal and TNFα stimulation by qRT-PCR with its specific primers. Data are mean values ± SEM for n = 6 independent biological replicates. **p < 0.0001 for Mfn2 in basal control vs Mfn2-KD. b The mRNA levels of pro-inflammatory genes, ICAM-1, IL-6, and IL-18 were determined by qRT-PCR with their specific primers. **p = 0.0001 for ICAM-1 of TNFα 6 h in control vs Mfn2-KD; p = 0.016 for IL-6 of TNFα 6 h in control vs Mfn2-KD; **p < 0.0001 for IL-18 in basal control vs Mfn2-KD by unpaired, two-tailed t-test. Data are mean values ± SEM for n = 4–6 independent biological replicates. c Control and Mfn2-KD ECs were transfected with 1 μg Topflash (β-catenin reporter containing the TCF promoter) and 35 ng of PRL/TK for 48 h. The cells were stimulated with or without TNFα (10 ng/mL) for 6 h. Firefly and renilla-luciferase activity were determined by the dual luciferase reagent assay system and the firefly luciferase activity was normalized by renilla-luciferase activity. Transcriptional activity of β-catenin was presented for fold change by further normalizing with value of control basal (TNFα 0 h). Data are mean values ± SEM for n = 3 independent experiments. ***p = 0.0008 for control in TNFα 0 h vs 6 h, p = 0.025 for basal in control vs Mfn2-KD, p = 0.0394 for TNFα 6 h in control vs Mfn2-KD by paired, two-tailed t-test. d HLMVECs expressing doxycycline inducible lentiviral Mfn2 shRNA were transfected Topflash and PRL/TK with the same method in (c) for 24 h, and treated with or without doxycycline for 72 h. The rescue experimental group (Mfn2-KD → control) was treated with doxycycline for 48 h and further incubated in media without doxycycline for 24 h. The cells were stimulated with or without TNFα (10 ng/mL) for 6 h. Data are mean values ± SEM for n = 6–7 biological independent samples. **p = 0.0447 for TNFα 6 h in control vs Mfn2-KD, p = 0.0356 for TNFα 6 h in Mfn2-KD vs Mfn2-KD → control by unpaired, one-tailed t-test. e HLMVECs were transfected with siRNA of control or β-catenin for 48 h and stimulated with TNFα (10 ng/mL) for 6 h. The mRNA levels of β-catenin and pro-inflammatory genes such as ICAM-1, IL-1β, and IL-18 were evaluated by qRT-PCR. Data are mean values ± SEM for n = 6–7 independent biological replicates. ***p < 0.0001 for β-catenin by one-way ANOVA, p = 0.0116 for ICAM-1 of TNFα 3 h in siCont vs si-β-cat, ****p < 0.0001 for ICAM-1 of TNFα 6 h in siCont vs si-β-cat, **p = 0.004 for IL-1β of TNFα 3 h in siCont vs si-β-cat, **p = 0.0025 for IL-1β of TNFα 3 h in siCont vs si-jun, p = 0.0419 for IL-18 of TNFα 6 h in siCont vs si-jun by unpaired, two-tailed t-test.

We next investigated the mechanism by which Mfn2 disassociated from the adherens junctions during inflammation. Post-translational modifications are regulatory switches which modify the activity of proteins, and oxidation is one of the most frequently occurring post-translational modification29. Especially during inflammation, ROS levels can acutely increase and result in oxidative modification of proteins. Cysteine (Cys) is an amino acid that is susceptible to several types of oxidative post-translational modification including sulfenylation, disulfide formation, S-glutathionylation, and S-nitrosylation36,40. Oxidative modifications of proteins are critical mediators of compartmentalized ROS signaling41. Recently, it has been shown that increasing concentrations of xanthine oxidase, a cytosolic source of ROS inhibits Mfn2 activity by inducing disulfide linked oligomerization via oxidation of C-terminal Cys 684, 700 residues in vitro22,43. However, it is unknown whether redox-dependent modifications of Mfn2 could affect its non-mitochondrial roles as a stabilizer of AJ protein complexes at the plasma membrane in ECs. We found that TNFα-induced ROS increased Mfn2 sulfenylation in ECs. Our results suggest that the sulfenylation step might be required for the disassociation of Mfn2 from adherens junctions and that Mfn2 sulfenylation may constitute a form of post-translational regulation of Mfn2 activity during inflammation. Future studies could identify specific cysteine residues that serve as sulfenylation targets and whether such sulfenylation would also impact other aspects of Mfn2 function such as its GTPase activity and mitochondrial fusion.

Interestingly, we also found Mfn2 presence in the nucleus during inflammatory activation. This surprising nuclear localization of Mfn2 was independently confirmed by several different approaches including biochemical subcellular fractionation, immunofluorescence with 3D analysis, and proximity ligation assays. We also confirmed TNFα-induced accumulation of Mfn2 using exogenously overexpressed GFP-Mfn2. However, there are important questions regarding the mechanisms of Mfn2 nuclear accumulation during inflammation that still need to be addressed in future studies. Recent work suggests that several mitochondrial enzymes can translocate into the nucleus and constitute a form of mitochondria-to-nucleus communication29 and that mitochondria-derived vesicles or chaperone proteins and nuclear transcription factors may promote the entry of selected mitochondrial proteins into the nucleus via the nuclear pores35,44. It is possible that the Mfn2 interaction with β-catenin or other proteins may facilitate the entry via nuclear pores during inflammation but this will need to be addressed in future studies targeting nuclear transport mechanisms. Although Mfn2 lacks a nuclear localization sequence (NLS), we found that its binding to the adherens junction protein β-catenin, which is known to translocate to the nucleus where it acts as a transcriptional corepressor36, was increased following inflammatory activation. Sequence alignment analysis indicated that Mfn2 has a putative β-catenin binding motif such as “SxxSSSxSxS” or “Dxθθθθθθθθx2,7E” possibilities for additional roles beyond mitochondrial fusion for Mfn2. In line with this, it has been recently reported that the mitochondria–ER–cortex anchor (MECA) interacts directly with mitochondria and the plasma membrane via core protein component, Num1 in budding yeast35. Moreover, it has been appreciated that many organelles communicate by using molecular tethers36 and the function of the mitochondria–plasma membrane contact extends beyond the mitochondrion itself35,37.

Our proteomic analysis revealed unexpected Mfn2 binding partners located outside of the mitochondria such as cell junction proteins. We took advantage of super-resolution microscopy which resolves individual protein complexes18,19 to validate the extra-mitochondrial localization of Mfn2. Super-resolution imaging identified the expected mitochondrial localization of the bulk of Mfn2 but also clearly visualized Mfn2 localization at the plasma membrane. Biochemical immunoprecipitation assays identified the plasma membrane binding partners of Mfn2 which included the adherens junction proteins VE-cadherin and β-catenin in homeostatic ECs. Loss of Mfn2 resulted in the disruption of the endothelial barrier, thus indicating that Mfn2 is not only localized at the junctions but also plays a functional role by stabilizing barrier integrity. We also found that Mfn2-depleted ECs showed an impaired F-actin structure which is important for cell–cell interaction. Importantly, during inflammatory stimulation, Mfn2 was disassociated from the junctions and this likely contributes to the disruption of barrier integrity that is typically observed during inflammation3,38. It is plausible that Mfn2 may play a role as an anchor between AJs proteins at plasma membrane and F-actin at cytosol to maintain EC barrier integrity in homeostatic ECs. We also did not find any broad effects on cell stress, cell death or cell proliferation following Mfn2 deletion, which suggested that our observations were most likely due to a specific role for Mfn2 in AJs and endothelial barrier integrity that is independent of generalized cellular health functions of Mfn2.

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motifs (θ and Φ are hydrophobic and aromatic residues, respectively) between its heptad repeat (HR) domains. The nuclear translocation of β-catenin is essential for canonical Wnt signaling pathway because β-catenin binds DNA and acts as a transcriptional regulator during embryonic development and adult tissue homeostasis. It has been shown that β-catenin translocates into the nucleus despite the lack of an NLS although the underlying mechanisms are not well understood. It is possible that the nuclear accumulation of Mfn2 utilizes a similar mechanism because we found that Mfn2 remains bound to β-catenin when they both disassociate from adherens junctions. This raised the intriguing question whether Mfn2 could ...
functionally regulate gene expression, possibly by affecting the transcriptional activity of β-catenin. We found that nuclear Mfn2 indeed inhibits β-catenin transcriptional activity for key pro-inflammatory cytokines such as IL-6 or IL-18 as well as an adhesion molecule ICAM-1 during inflammation. Interestingly, β-catenin depleted ECs demonstrated significant decreases in the gene expression of selected pro-inflammatory genes such as ICAM-1, IL-1β, or IL-18, but β-catenin depletion did not impact other pro-inflammatory genes such as IL-6, caspase1, or VCAM-1, indicating a specificity of the inflammatory genes regulated by β-catenin. A non-canonical role for transcriptional regulators in endothelial barrier regulation has also recently been reported for Notch signaling. Possible future studies could address whether Mfn2 also modulates the role of Notch in regulating the endothelial barrier.

Although mitochondria contain an independent genome, the vast majority of mitochondrial proteins are encoded in the nuclear genome. However, less is known about how mitochondria engage in retrograde communication with the nucleus and could potentially modulate nuclear gene expression. Even though it came as a surprise that Mfn2 accumulated in the nucleus, recent studies are increasingly finding mitochondrial proteins in nuclei. Multiple proteins that were previously thought to be exclusively mitochondrial have recently been shown to also localize within nuclei, yet their nuclear function is not fully understood.

Importantly, we demonstrate here that Mfn2 and β-catenin are both necessary for EC barrier integrity in vitro and in vivo. Genetic in vivo deletion of Mfn2 specifically in the endothelium resulted in a disruption of vascular homeostasis, as evidenced by increased lung vascular permeability and an increase in inflammatory gene expression along with increasing inflammatory immune cells even in the absence of any additional pathogenic stimuli. The lung endothelium may be especially vulnerable to inflammatory activation as a recent comparative analysis of vascular endothelial cells in distinct organs found that the lung endothelium had the highest expression of inflammatory and immune response genes even during homeostasis when compared to the endothelium of other organs such as the brain or the heart. The association between Mfn2 and vascular permeability may thus represent promising therapeutic targets, to reduce the influx of inflammatory cells, as well as to reduce tumor metastasis or tumor growth. A recent study also identified an important link between angiogenesis and endothelial barrier maturation by demonstrating that the transcriptional regulator YAP/TAZ was involved in both processes. Our study established the barrier stabilization role for Mfn2 in the endothelium, but it is possible that Mfn2 also may regulate angiogenesis.

In summary, our findings reveal intriguing non-mitochondrial roles for Mfn2 as a stabilizer of the vascular barrier and an endogenous suppressor of inflammatory gene expression. These insights could help us better understand the role of “mitochondrial” proteins in the nucleus and pave the way for future anti-inflammatory therapies.

**Methods**

Reagents. CM-H2DCFDA [5-(and-6)-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate, acetyl ester, Invitrogen C6827] was obtained from Molecular Probes. DCP-Bio1 was obtained from EMD Millipore (Keraxact, NS12653MG). Catalase (#219008) was obtained from Calbiochem (USA). Other reagents were obtained from Sigma.

si/shRNA or plasmid construct. Lenti-GFP-Mfn2 or lenti-shMfn2 RNA constructs were generated in our laboratory. shMfn2 RNA oligos were inserted into a (sense 5′-CCG GGC TCA GTC CCT CAT CCC ATT TCT CGA GAA ATG GGA TGA AGC ACT GAG CTT TTT -3′, anti-sense 3′- AATATAA AAA CCT CAG TGC TCC TTC ACC CTA GGA TGG GAT GAA CGA GCT AGC AGC -5′, target of 3′UTR from 3237-3257) lentiviral Tet-pLKO-puro backbone vector (Addgene, #21915). GFP-Mfn2 was subcloned into a mammalian lentiviral pWPXl expression vector (Addgene, #12357). A β-catenin reporter (M50 super 8x TOP-flash, Addgene, #12456) was provided by Dr. Kishore K Wary at University of Illinois at Chicago. pRL/TK (renilla-luciferase) was provided by Dr. Chinnaswamy Tiruppathi at University of Illinois at Chicago. pRL-LifeAct-GFP was provided by Dr. Peter Carmeliet at the Center for Cancer Biology, VIB, Leuven, Belgium. We obtained siRNA for beta-catenin (sc-29209), Mfn2 (sc-43928), Mfn1 (sc-43927), Drp1 (sc-43732), Opa1 (sc-106808) or control (sc-57007) from Santa Cruz Biotechnologies.

Lentivirus production and purification. HEK293T (CRL-11268, ATCC) cells were used to produce lentivirus and the cells were transfected with DNAs (2.5 µg pMD2.G, 5 µg of psPAX2, and 7.5 µg of DNA expression vector) with 30 µg polyethyleneimine (PEI, Polysciences, 23966, USA) in DMEM media containing 10% FBS without antibiotics overnight and changed with fresh DMEM media supplemented with 10% FBS and 1% Pen/Strep and then incubated for 48 h at 37 °C. The media containing secreted lentivirus was collected and the virus was purified using Lenti-X concentrator (Clonetech, 631252).

Cell culture. Human lung microvascular endothelial cells (HLMVECs, CC-2527, Lonza) were obtained from Lonza and cultured with EGM2 (Lonza) including all supplements and 10% FBS (HyClone) until passage 8. The cells were transfected with doxycycline inducible lentiviral shMfn2 RNA with 1:2000 dilution polybrene (Millipore, TR-1003-G) for 24 h after which the media was changed. After 48 h, the cells were treated with doxycycline (200 ng/mL) or DMSO (control) for 72 h to
knockdown endogenous Mfn2. As a control, we used lentivirus-Mfn2 shRNA expressing cells which were treated with DMSO.

Three-dimensional structured illumination microscopy (3D-SIM). To visualize Mfn2 in those proteins complex, we used the recently developed technology of 3D-SIM which provides high spatial resolution.13,14 HLMVECs were cultured on #1.5 coverslips (~170 µm thickness) with restricted thickness-related tolerance (+5 µm) (MatTek: high tolerance coverslips, pcs-170-1818). Confluent ECs were fixed with 100% cold (~20 °C) methanol for 5 min on ice or with 4% paraformaldehyde (PFA) for 10 min at room temperature. The fixed cells were permeabilized with 0.25% Triton X-100 at room temperature for 10 min and then blocked with blocking buffer (1X PBS, 2% BSA, and 0.05% Tween 20) for 1 h followed by immunostaining with 1:250 dilution of anti-Mfn2 (ab56889, mouse; 12186-1-AP, rabbit), anti-Tom20 (sc-14415, rabbit; sc-17784, mouse), or anti-Mfn1 (CST-14739, rabbit) overnight at 4 °C and then stained with 1:500 of secondary antibodies for Alexa488 (mouse, Alexa647, Alexa488, or rabbit Alexa488) for 2 h at room temperature (RT). After secondary antibodies for Mfn2 or Tom 20, the VE-cadherin was immunostained with 1.25 dilution of Alexa Fluor647 mouse anti-human CD144 (DSB61567) for 1 h at room temperature. The nuclei were visualized by separately staining with DAPI. After mounting with Prolong gold antifade reagent without DAPI, the images were taken using confocal fluorescence imaging using confocal microscopy

Immunofluorescence imaging using confocal microscopy. To evaluate EC barrier integrity, confluent HLMVECs in 6 well plates containing coverslips were treated with or without TNFs (10 ng/ml) for 6 h and fixed with 4% PFA for 10 min at RT and then blocked with blocking buffer (1X PBS, 2% BSA, and 0.05% Tween 20) without permeabilization for 1 h. To evaluate co-localization of Mfn2, Tom20 and AJs proteins, the cells were treated with or without TNFs (10 ng/ml) for 6 h and fixed with 4% PFA for 10 min at RT. The fixed cells were permeibilized with 0.25% Triton X-100 at room temperature for 10 min and then blocked with blocking buffer (1X PBS, 2% BSA, and 0.05% Tween 20) for 1 h. The primary antibodies (VE-cadherin; Cayman #160840, β-catenin; ab32572, SC-7963, or Mfn2; 12186-1-AP, Mfn1; CST-14739 or Tom20; SC-17764) were diluted 1:250 with antibody dilution solution (1X PBS, 1% BSA, and 0.05% Tween 20) overnight at 4 °C and then incubated with 1:500 dilution of secondary antibody ( Goat anti-Rabbit or anti-mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, 1:250 dilution) and Alexa488 or Alexa546 for 1 h at room temperature. To evaluate co-localization of exogenous GFP-Mfn2 and endogenous Tom20, HLMVECs expressing GFP-Mfn2 were immunostained with Tom20 specific antibody (sc-14415, rabbit, 1:250 dilution) and anti-rabbit secondary Alexa647 (1:500 dilution). The nuclei were visualized by separately staining with DAPI. After mounting with Prolong gold antifade reagent without DAPI, the images were taken using confocal microscopy (Zeiss LSM880, Plan Apo 1.46NA, 63x objective). To confirm Mfn2 nuclear localization, the samples were Z sectioned (average 91 slices) with equal Z-axis sections (125 nm step size) were taken at full-frame structured illumination mode (1024 x 1024 pixel, sequential acquisition). Softworx (Applied Precision) was used to reconstruct 3D-SIM images. The projection images are presented and the protein co-localization was analyzed with plot profiles and Manders’ overlap coefficient using ImageJ (NIH, USA).

Immunoprecipitation assay and western blotting. To evaluate the interaction between VE-cadherin (SC-9989, mouse) and β-catenin (ab32572, rabbit), or between Mfn2 (ab68898, mouse) and β-catenin (ab32572, rabbit) was determined with1:200 dilution by PLA (the Duolink In situ Red Starter Kit, U092101, Sigma), following manufacturer’s instructions with minor modifications.

Evaluation for total ROS. HLMVECs were stimulated with TNFs (10 ng/ml) for the indicated time (0, 15 min, 30 min, 3 h or 6 h) and then incubated with 20 µM CM-H2DCFDA (Invitrogen, C6827) for 6 min at 37 °C. DCF fluorescence was measured by confocal microscopy (Zeiss LSM880) with x63 magnification using the same exposure conditions in each experiment. Relative DCF fluorescence was measured by ImageJ and presented as fold change.

DCP-Bio1 assay. To determine sulfenic acid (Cys-OSH) of target protein, we used an innovative cell permeable biotin-labeled Cys-OSH trapping probe, (DCP-Bio1). Briefly, TNFα stimulated-control or Mfn2-KD ECs were lysed with degassed-specific isox buffer (50 mM HEPES, pH 7.0, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 10 mM sodium pyrophosphate, 5 mM IAA, 100 µM DTA, 1% Triton-X-100, protease inhibitor, 50 unit catalase, 200 µM DCP-Bio1), and then pull-downed with streptavidin beads overnight. All steps were performed in the dark. DCP-Bio1 conjugated sulfenylated-proteins were measured with specific antibodies (1:1000 dilution) for Mfn2, β-catenin, or acting as Western blotting.

Subcellular fractionation. Confluent HLMVECs in 100 mm dishes were stimulated with TNFs for the indicated times (0, 1, 3, 6 h) by subcellular fractionation. Briefly, the cells were lysed with 500 µl lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and EDTA free protease inhibitor cocktail), collected in Eppendorf tubes, and incubated in ice for 10 min. The lysate was freshly added to 60 µl 10% NP-40 and then vortexed for 10 s followed by centrifugation at 16,000g for 1 min at 4 °C. The supernatant (cytoplasmic fraction) was carefully transferred to a second tube. The pellet was washed once with lysis buffer and 200 µl nuclear extract buffer (50 mM HEPES, 0.4 M NaCl, 0.5 mM EDTA, 0.5 mM EGTA, and EDTA free protease inhibitor cocktail) was added by tapping gently and incubated in ice for 30 min with vortexing every 5 min. The nuclear fraction was collected by centrifugation at 16,000g for 5 min at 4 °C. The cytosolic and nuclear fraction was loaded in a SDS-PAGE gel followed by Western blotting. GAPDH (1:1000 dil, 10494-1-AP, rabbit) and p44 (nuclear matrix protein, 1:1000 dil, sc-514212, mouse) were used as controls for the cytosolic or nuclear fraction, respectively.

β-catenin reporter luciferase assay. Control and Mfn2-KD ECs were transacted with 1 µg of a β-catenin reporter (MSO super 8x TOP flash containing TCF/LEF sites upstream of a luciferase reporter)15 and 35 ng of pKLTK using PEI tranfection reagent (Polyethylenimine). At 48 h after transfection, the cells were stimulated with TNFα (10 ng/ml) for 6 h and then 100 µL of cell lysate from each sample was used to measure reporter gene expression. Firefly and Renilla luciferase activity were determined by the dual luciferase reagent assay system (Promega). The relative luciferase activity represents the mean value of the firefly/Renilla luciferase.

Quantitative real-time PCR. Total RNA was isolated by using phenol/chloroform and Trizol Reagent (Invitrogen, 15596026) as described. Reverse transcription was carried out using high capacity cDNA reverse transcription kit (Applied Biosystems, 4368814) using 2 µg of total RNA. Quantitative PCR was performed with fast start universal SYBR Green master mix (ROX) PCR kit (Roche, 0491391401) using the reaction mix was all run in triplicate to reduce variability. Expression of human genes of Albumin, Mfn1, Mfn2, β-catenin, ICAM-1, IL-6, IL-18, or IL-1β was determined using the following primers: Mfn2, sense 5′-CATCCCTAGGTTGCTTCCACTCATGACCCCA-3′ and antisense 5′-GATGACGTTGGGCGTTGGGG-3′.
CAAG -3’, anti-sense 5’-CAAGCCGCTTATCATGTGCTG-3’, ICAM-1, sense 5’-CCTGCGACAGTGCTGTCG-3’, CT-3’, sense 5’-CACCCTTCAGACGTCTC-3’, anti-sense 5’-GGGCACTTCTTACGAGAAG-3’, IL-1β, sense 5’-CAAGCCGCTTATCATGTGCTG-3’, anti-sense 5’-CAACGGGACAGATATGAGCA-3’, GAGCA-3’, anti-sense 5’-TTTATT-3’, anti-sense 5’-CAAGCCGCTTATCATGTGCTG-3’, IL-6, sense 5’-TACCCGCGCGCAGACAGACAG-3’, anti-sense 5’-GGGCACTTCTTACGAGAAG-3’, TGFβ-3’, sense 5’-CAAGCCGCTTATCATGTGCTG-3’, anti-sense 5’-CAACGGGACAGATATGAGCA-3’, GM-CSF, sense 5’-CTTCCATTCACAGCTTACGT-3’, anti-sense 5’-TGGCAGTAAACAGCCAGAACAA-3’. Human primers for β-catenin (QT00778822) or mouse primers for HPRT (QT00166768) were purchased from Qiagen. Expression of genes was normalized and expressed as fold-changes relative to HPRT

**Data availability**

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files. Source data files provided with this paper include proteomic datasets and microscopy images. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024620 and 10.6019/PXD024620. Source data are provided with this paper.

**Code availability**

No new code was generated for this work.

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Author contributions

Y.M.K. and J.R. developed the overall study design, Y.M.K., S.K. and H.G.M. performed the experiments, Y.M.K., S.K., H.G.M., AJ and IS performed the overall data analysis, P.T.T. performed the image analysis, Y.M.K. and J.R. wrote the initial manuscript draft. G.Y.P. designed the experiments to study immune cell phenotypes. All authors provided critical feedback and revisions for the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Conflict of interest

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