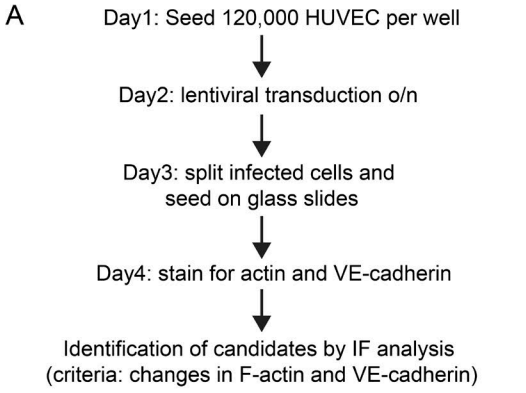


B

Gene name	Clone ID	Stress fibers	Cortical actin	Mixed	Other
XIAP	3785	+	-	-	-
XIAP	231578	-	-	-	-
BIRC2	3780	-	-	+	-
BIRC2	320867	-	-	-	-
CBL	288695	-	-	-	elongated cells
TRIP12	273210	-	-	-	-
TRIP12	273135	-	-	-	-
SMURF1	3471	+	-	-	-
SMURF1	3473	+	-	-	-
SMURF2	3478	-	-	-	-
SMURF2	272880	-	-	-	-
HACE1	3415	-	-	-	-
HACE1	415313	-	-	-	-
HACE1	427070	-	-	-	-
NEDD4	7550	-	-	-	-
NEDD4	7552	-	-	-	-
NEDD4L	904	-	-	-	-
NEDD4L	905	-	-	-	-
KIA0317	280035	-	-	-	-
RABGEF1	47237	+	-	-	-
RABGEF1	419718	-	-	-	-
RABGEF1	422664	-	-	-	-
TNFAIP3	50961	-	-	-	-
BTRC	6543	-	-	-	-
BTRC	6545	-	-	+	-
FBXW7	355641	-	+	-	-
FBXW7	355643	-	+	-	-
FBXW7	355644	-	+/-	-	-
FBXL19	62336	-	-	+	-
FBXL19	359134	-	-	-	-
USP8	284767	-	-	-	-
USP8	284769	-	+/-	-	-
USP17L2	376547	-	+/-	-	-
CYLD	230278	-	+	-	stellar actin
CYLD	230279	-	+	-	stellar actin
CYLD	230280	-	-	-	-
CYLD	230281	-	-	-	-
OTULIN	275410	+	-	-	-
OTULIN	275411	+	-	-	-
CUL1	318414	-	-	-	-
CUL1	318416	-	-	-	-
CUL3	288625	-	-	+	-
CUL3	307983	-	-	+	-
CAV1	8002	-	-	-	-
CAV1	11218	-	-	-	-

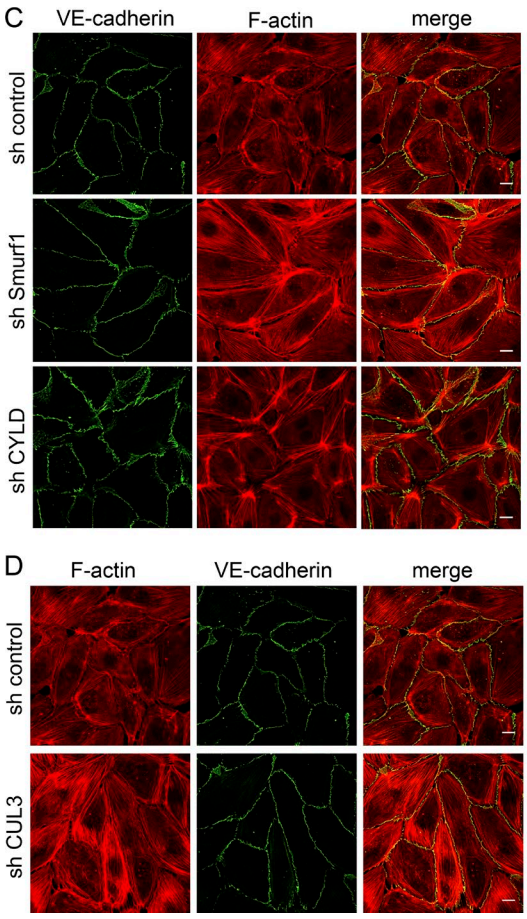


Figure S1. **(A) Workflow of the lentiviral shRNA screen for identification of ubiquitin ligases and associated proteins involved in regulation of the endothelial actin cytoskeleton. (B)** Summary table of the shRNA clones tested in the screen and phenotype analysis. shRNA targeted proteins were grouped into E3 ligases (black), substrate recognition receptors (red), ubiquitin proteases (blue), and adaptor proteins directly (green) or indirectly (orange) involved in ubiquitination. Based on changes in F-actin organization, the different phenotypes were grouped into (1) pronounced actin stress fiber formation, (2) induction of cortical actin bundles, (3) a mixed group in which both changes could be observed, and (4) other phenotypes, which include changes in actin morphology, that do not fit into the previous phenotype groups. **(C)** Confocal immunofluorescence images of HUVECs infected with lentiviruses carrying nontargeting control shRNA, Smurf1-targeting shRNA, or CYLD-targeting shRNA. Cells were fixed 72 h after infection and stained with VE–cadherin antibody (green) and phalloidin (red). Bars, 15 µm. Control panels are identical to control in Fig. 1 A. **(D)** HUVECs were infected with lentiviruses carrying control shRNA or Cullin-3 targeting shRNA (clone 288625). At 72 h after infection, cells were fixed and stained with VE–cadherin antibody and phalloidin. Control panels are identical to control in C. Bars, 15 µm.

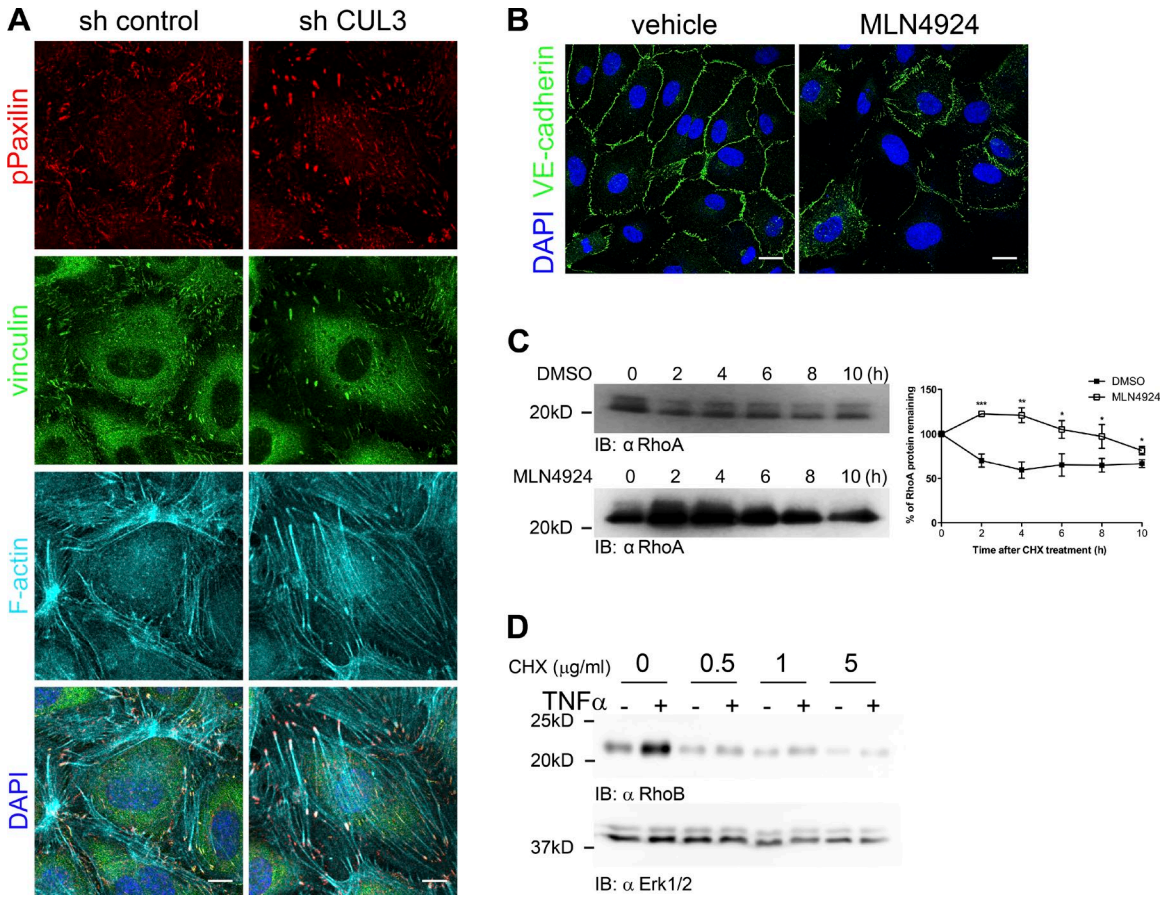


Figure S2. **(A) Confocal immunofluorescence staining of HUVECs transduced with control or CUL3 shRNA and fixed at 72 h after infection.** Cells were stained for pPaxillin and vinculin, F-actin, and nuclei. Bars, 10 µm. **(B)** HUVECs were treated with 500 nM MLN4924 for 20 h. Cells were fixed and stained with VE–cadherin antibody and DAPI. Bars, 20 µm. **(C)** HUVECs were pretreated with 500 nM MLN4924 or DMSO control for 48 h followed by addition of 25 µg/ml cycloheximide for the indicated time points. Cells were lysed, and 20 µg of total proteins was analyzed by immunoblot (IB) with RhoA antibody. Densitometric analysis was performed using ImageJ software (n = 3). **(D)** Immunoblot analysis of RhoB expression in HUVECs treated with 10 ng/ml TNF-α for 4 h in combination with increasing concentrations of cycloheximide. Error bars represent SD. *, P = 0.01–0.05; **, P = 0.01–0.001; ***, P = 0.001–0.0001.

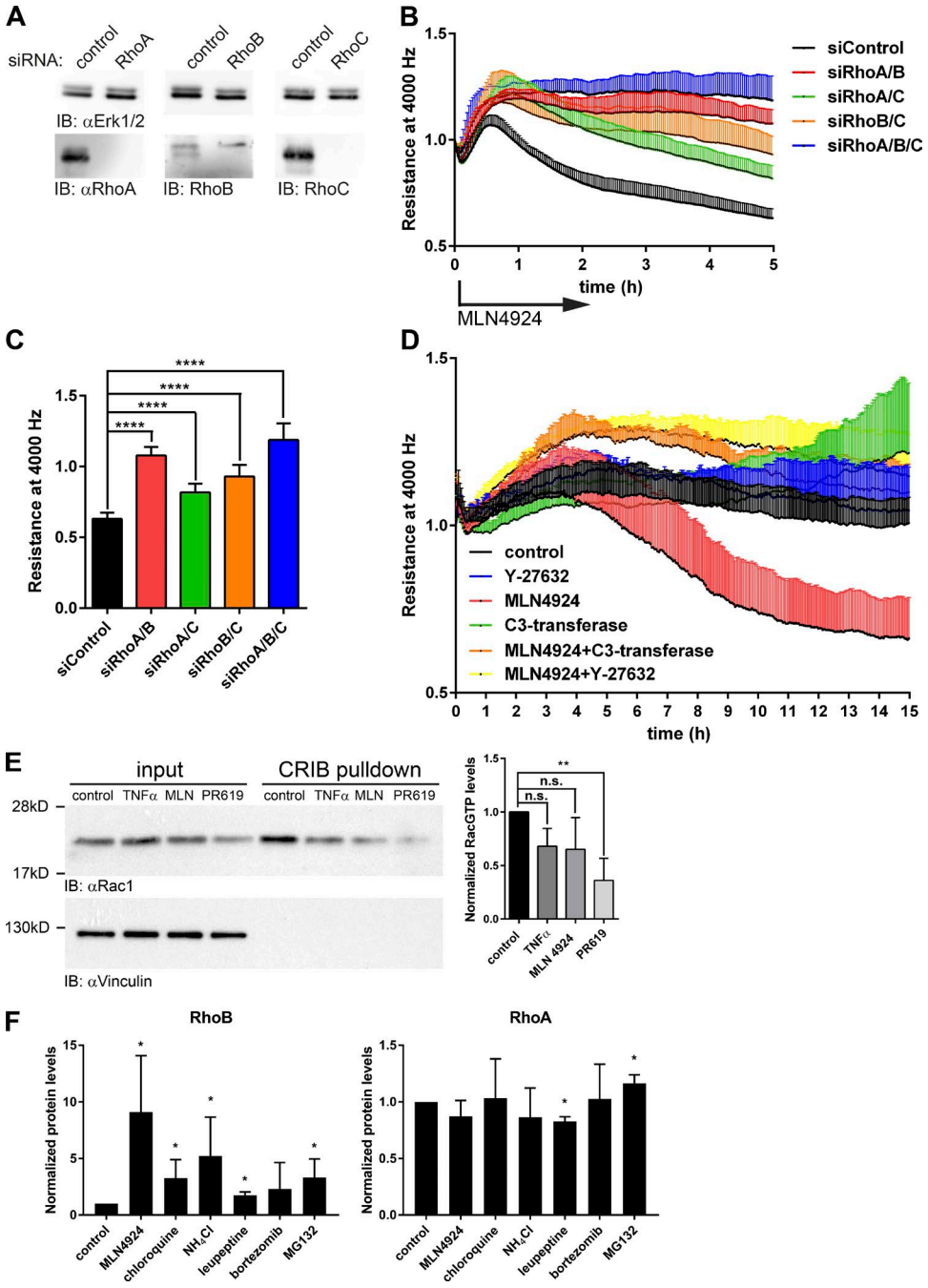


Figure S3. **(A) Immunoblot analysis of RhoA, RhoB, and RhoC expression in HUVECs transfected with siRNAs.** Cells were lysed at 72 h after transfection, and lysates were analyzed by immunoblotting using RhoA, RhoB, or RhoC antibody. Erk1/2 immunoblot was used as loading control. **(B)** HUVECs were transfected with combinations of siRNA targeting RhoA/RhoB, RhoA/RhoC, RhoB/RhoC, RhoA/RhoB/RhoC, or nontargeting control siRNA. 72 h after transfection, cells were starved for 24 h, followed by the addition of 500 nM MLN4924, after which resistance of endothelial monolayers was measured at 4,000 Hz. Resistance was normalized to values measured before the addition of MLN4924. **(C)** Quantification of normalized resistance measured in B at time point 5 h after addition of MLN4924. One-way ANOVA statistical analysis with Tukey's post-tests was performed using GraphPad Prism ($n = 7$). **(D)** MLN4924 effect on the barrier function of HUVECs pretreated with Y-27632 or C3-transferase. Cells were starved for 1.5 h and treated with 10 µM Y-27632 or 1 µg/ml C3-transferase for 30 min, followed by the addition of 500 nM MLN4924 and measurement of electrical resistance at 4,000 Hz using ECIS ($n = 3–8$). **(E)** CRIB pull-down was performed using lysates of HUVECs treated or not with 500 nM MLN4924 or 10 ng/ml human TNF-α for 4 h or with 2.5 µM PR619 for 2 h. Input is equal to 2.5% of the lysate used for the pull-down. Vinculin was used as loading control ($n = 7$). Densitometric analysis of the CRIB pull-down immunoblots is shown on the right. **(F)** Densitometric analysis of RhoB and RhoA blots from Fig. 4 E ($n = 3$). Statistical comparison was calculated using an unpaired Student's *t* test. Error bars represent SD. n.s., $P \geq 0.05$; *, $P = 0.01–0.05$; **, $P = 0.01–0.001$.

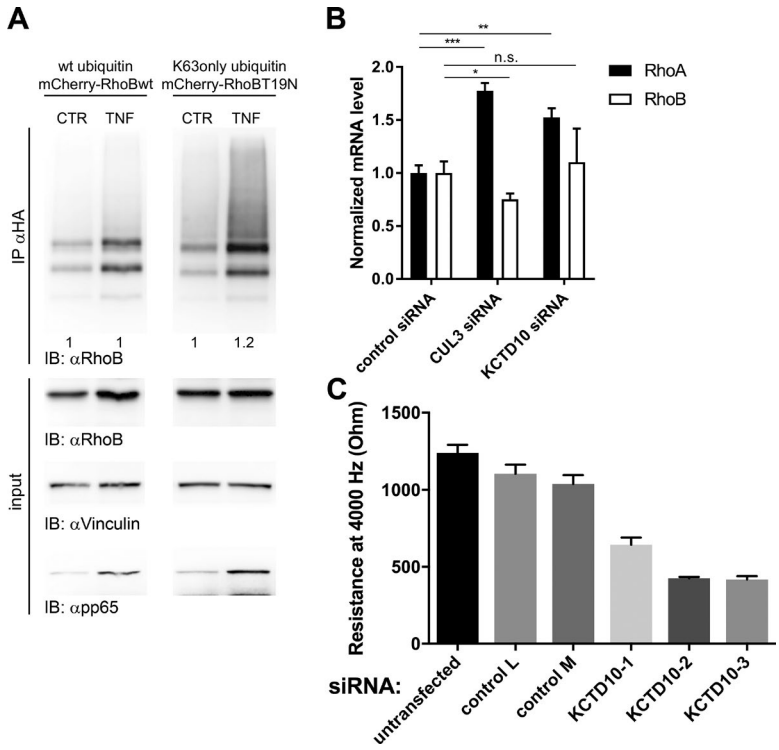


Figure S4. **(A) HEK293T cells were cotransfected with HA-ubiquitin and mCherry-RhoB or HA-ubiquitin-K63only and mCherry-RhoB-T19N.** Cells were treated with 10 ng/ml TNF-α for 6 h and 5 μM MG132 for last 4 h of treatment. Samples were analyzed by immunoblotting (IB) for RhoB. Vinculin was used as a control. Phospho-p65 immunoblot shows activation of the NF-κB pathway. **(B)** RT-PCR analysis of RhoA and RhoB mRNA expression in CUL-3 and KCTD10 knockdown HUVECs. mRNA was isolated 72 h after siRNA transfection and analyzed for RhoA and RhoB transcripts using RT-PCR (n = 3). **(C)** HUVECs were transfected with 25 nM control L or control M siRNA or three different siRNA all targeting KCTD10. Graph represents quantification of endothelial resistance 72 h after transfection measured at 4,000 Hz. Measurement was performed in triplicate. Error bars represent SD. n.s., $P \geq 0.05$; *, P = 0.01–0.05; **, P = 0.01–0.001; ***, P = 0.001–0.0001.

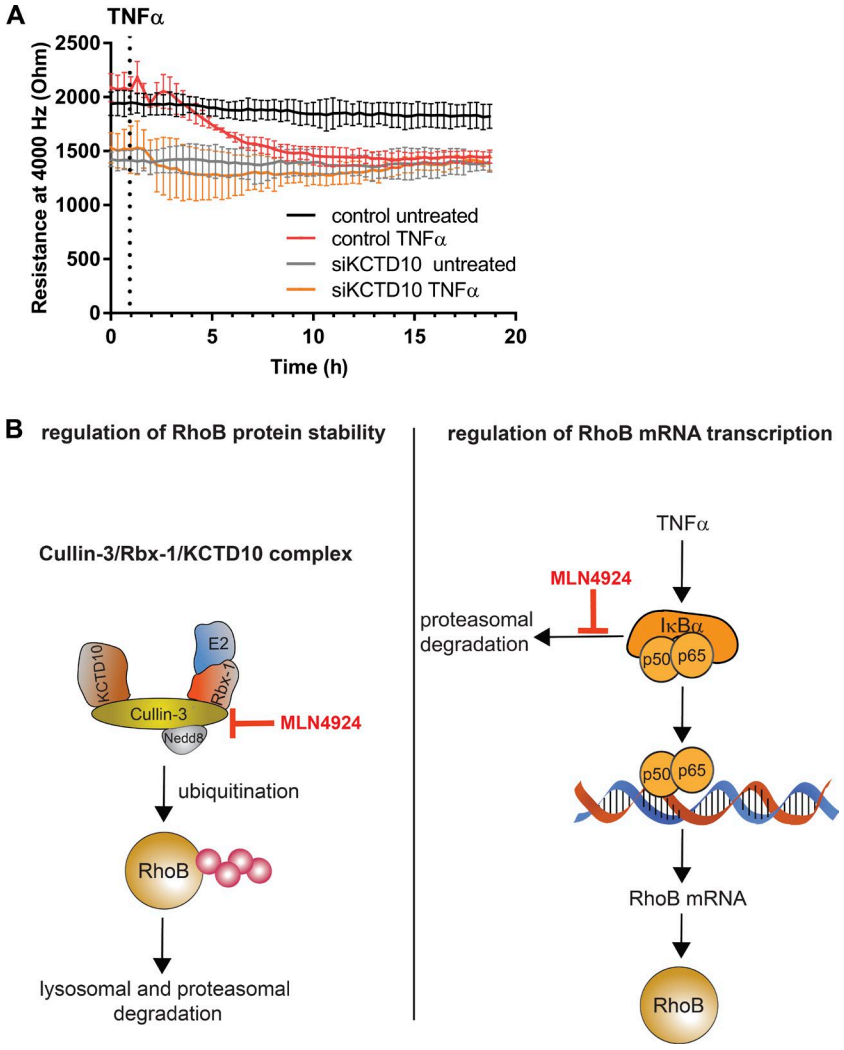
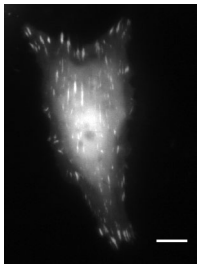
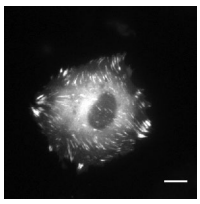


Figure S5. **(A) ECIS measurement (4,000 Hz) of HUVECs transfected with control or KCTD10 siRNA and treated with 10 ng/ml TNF-α at 72 h after transfection.** Error bars represent SD. **(B)** Model of the regulation of RhoB expression in ECs. In unstimulated conditions, Cullin-3–Rbx1–KCTD10 ubiquitinates RhoB, which is subsequently degraded in lysosomes and via the proteasomal pathway. TNF-α induces activation of the proinflammatory NF-κB pathway and increases transcription of RhoB mRNA followed by protein synthesis. A prerequisite for NF-κB activation is degradation of IκBα, which is ubiquitinated by the Cullin-1–β-TRCP complex (Suzuki et al., 1999), which is blocked by MLN4924.



Video 1. **Focal adhesion dynamics in control shRNA-expressing ECs.** Control shRNA-expressing HUVECs were transfected with pEGFP-C3 vinculin and imaged every 20 s for 20 min. Frame rate is five frames per second. Bar, 10 µm.



Video 2. **Focal adhesion dynamics in Cullin-3 knockdown ECs.** Cullin-3 shRNA-expressing HUVECs were transfected with pEGFP-C3 vinculin and imaged every 20 s for 20 min. Frame rate is five frames per second. Bar, 10 µm.

References

Suzuki, H., T. Chiba, M. Kobayashi, M. Takeuchi, T. Suzuki, A. Ichiyama, T. Ikenoue, M. Omata, K. Furuichi, and K. Tanaka. 1999. IkappaBalpha ubiquitination is catalyzed by an SCF-like complex containing Skp1, cullin-1, and two F-box/WD40-repeat proteins, betaTrCP1 and betaTrCP2. *Biochem. Biophys. Res. Commun.* 256:127–132. https://doi.org/10.1006/bbrc.1999.0289