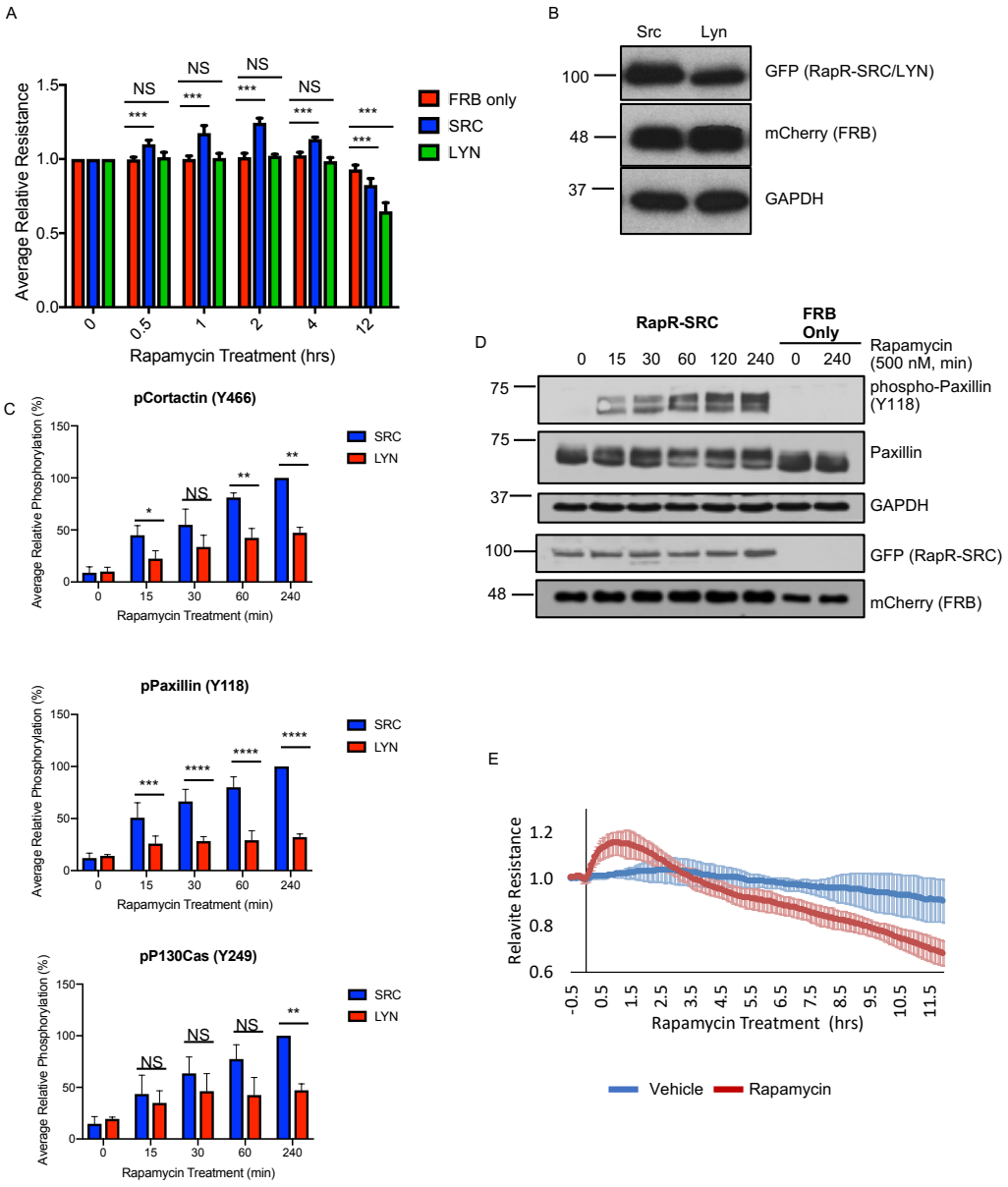
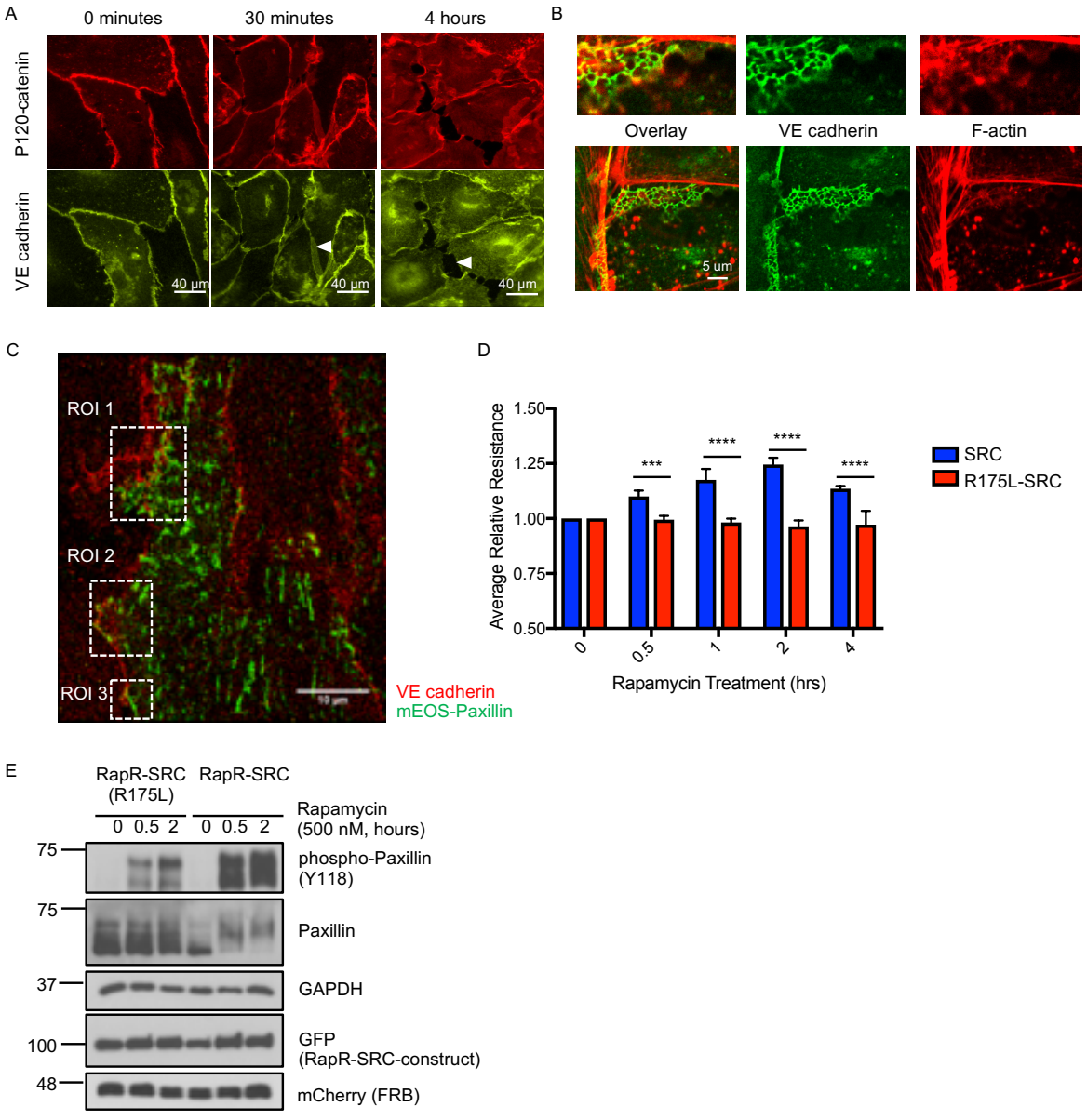
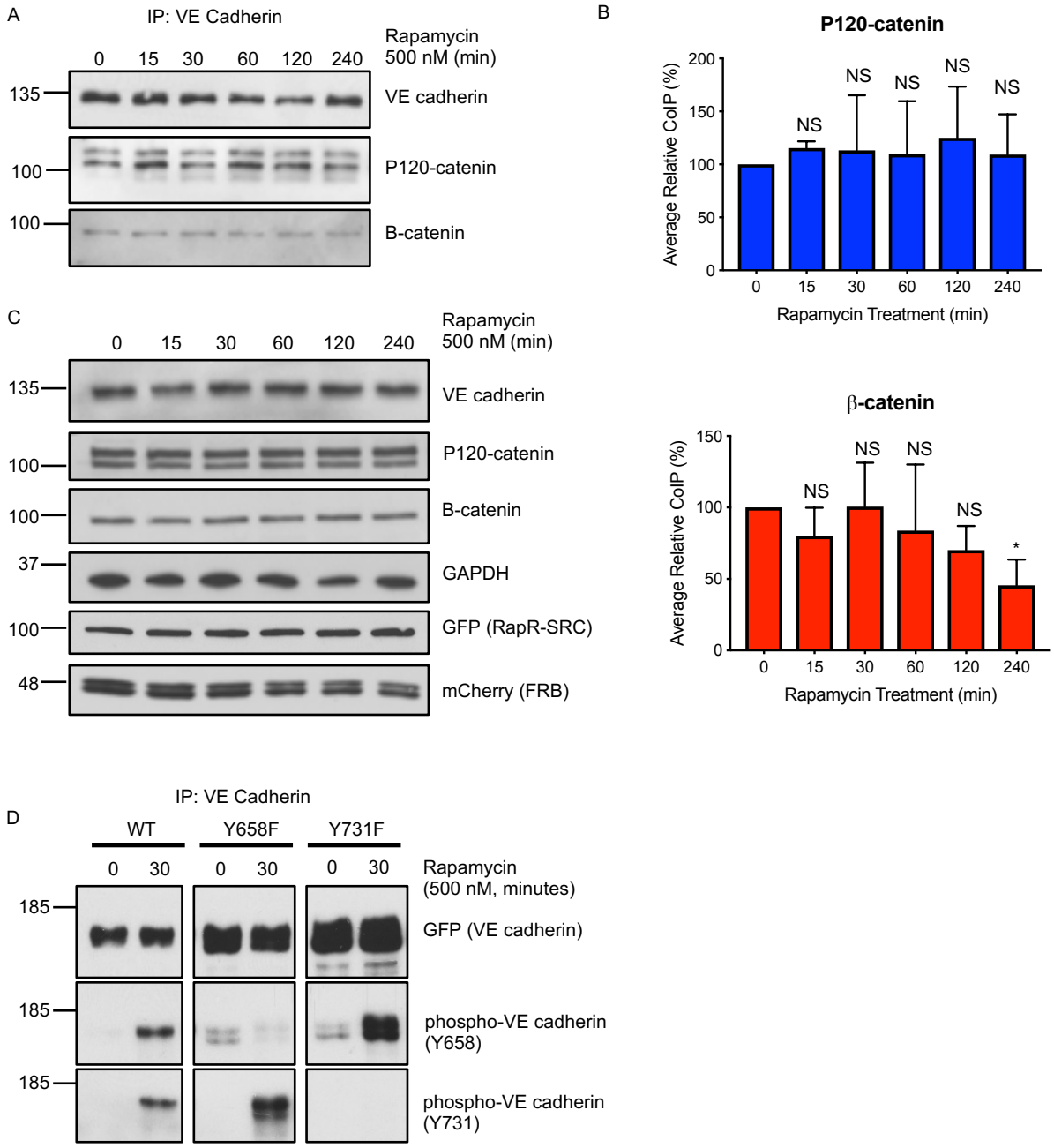


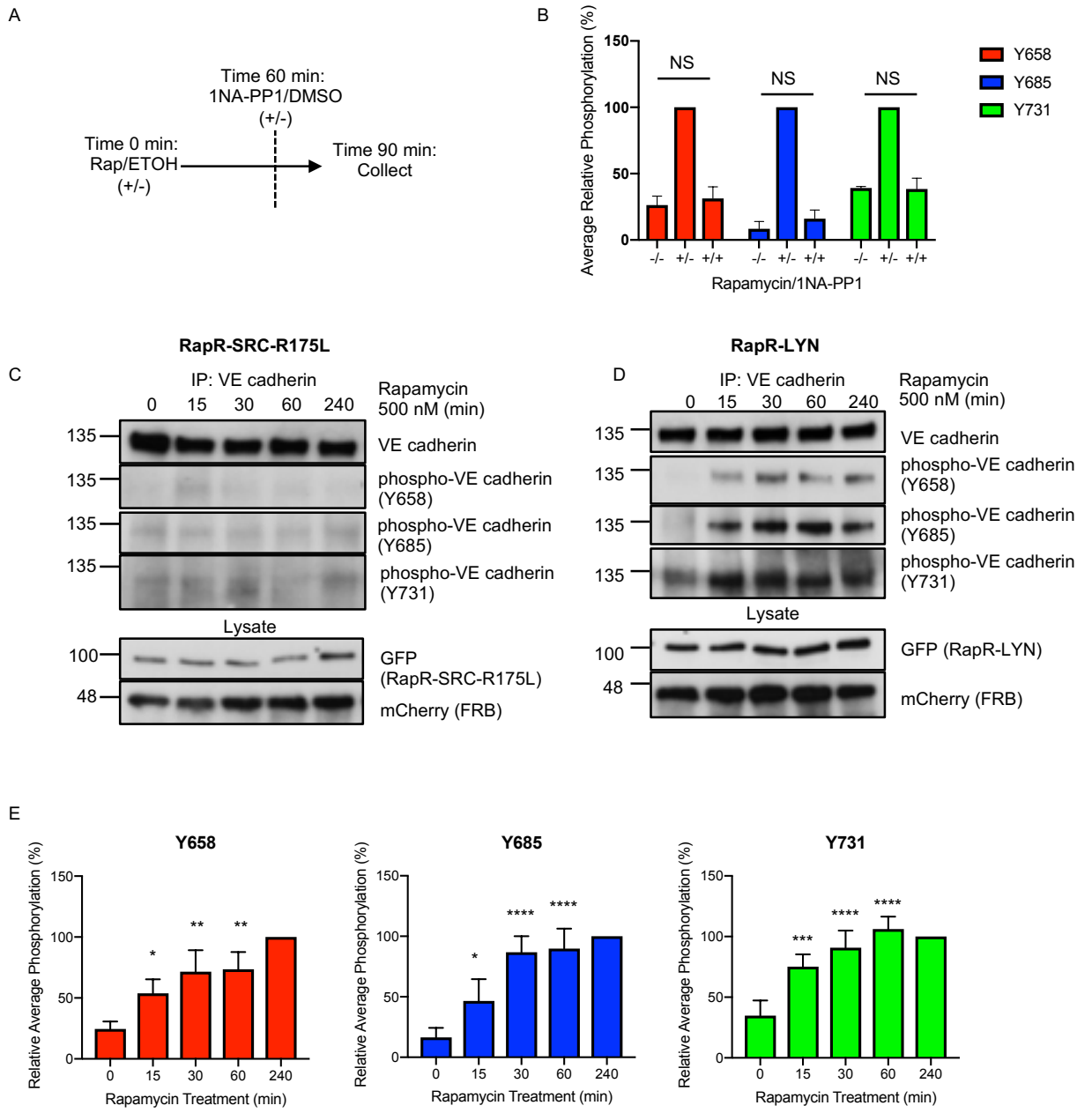
Supplementary Figure 1: Effect of SRC activation on endothelial permeability. (Relates to Figure 1). (A-B) HPAE cells co-expressing RapR-SRC-cerulean (SRC) and mCherry-FRB, RapR-LYN-cerulean (LYN) and mCherry-FRB, or expressing mCherry-FRB alone (FRB) were analyzed using TER at a constant voltage. Rapamycin (500 nM) was added at time point 0. The average relative resistance at the indicated time points was determined from 3 independent experiments and compared to FRB only samples using a two-way ANOVA with repeated measures and a post-hoc test with a Bonferroni's multiple comparisons adjustment; error bars show standard deviation. (B) Representative blot comparing expression levels of RapR-SRC and RapR-LYN collected in parallel to the running of TER. (C) Comparison of SRC and LYN induced phosphorylation of endogenous HPAE substrates. The graphs depict the average relative change (N = 3) from biological replicates. A two-way ANOVA with repeated measures and a post-hoc test with a Bonferroni's multiple comparisons adjustment was used to compare SRC and LYN induced phosphorylation changes for the 0, 15, 30, and 60 minute time points. A Wilcoxon-Mann-Whitney test was used to compare the 4 hour time point between SRC and LYN. The error bars represent standard deviation. (A and C) Statistical significance is indicated using the following notations: *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. (D) Activation of RapR-SRC in HPAE cells. Cell lysates were collected at the designated time points and immunoblotted for the indicated proteins. (E) HUVE cells co-expressing RapR-SRC-cerulean and mCherry-FRB were analyzed using TER at a constant voltage. Rapamycin (500 nM) or vehicle (ethanol) was added at time point 0. Values were normalized to the average prior to treatment and error bars represent the 90% confidence interval, n=2 biological replicates. (A-E) All exogenous proteins were expressed using adenoviral transduction.



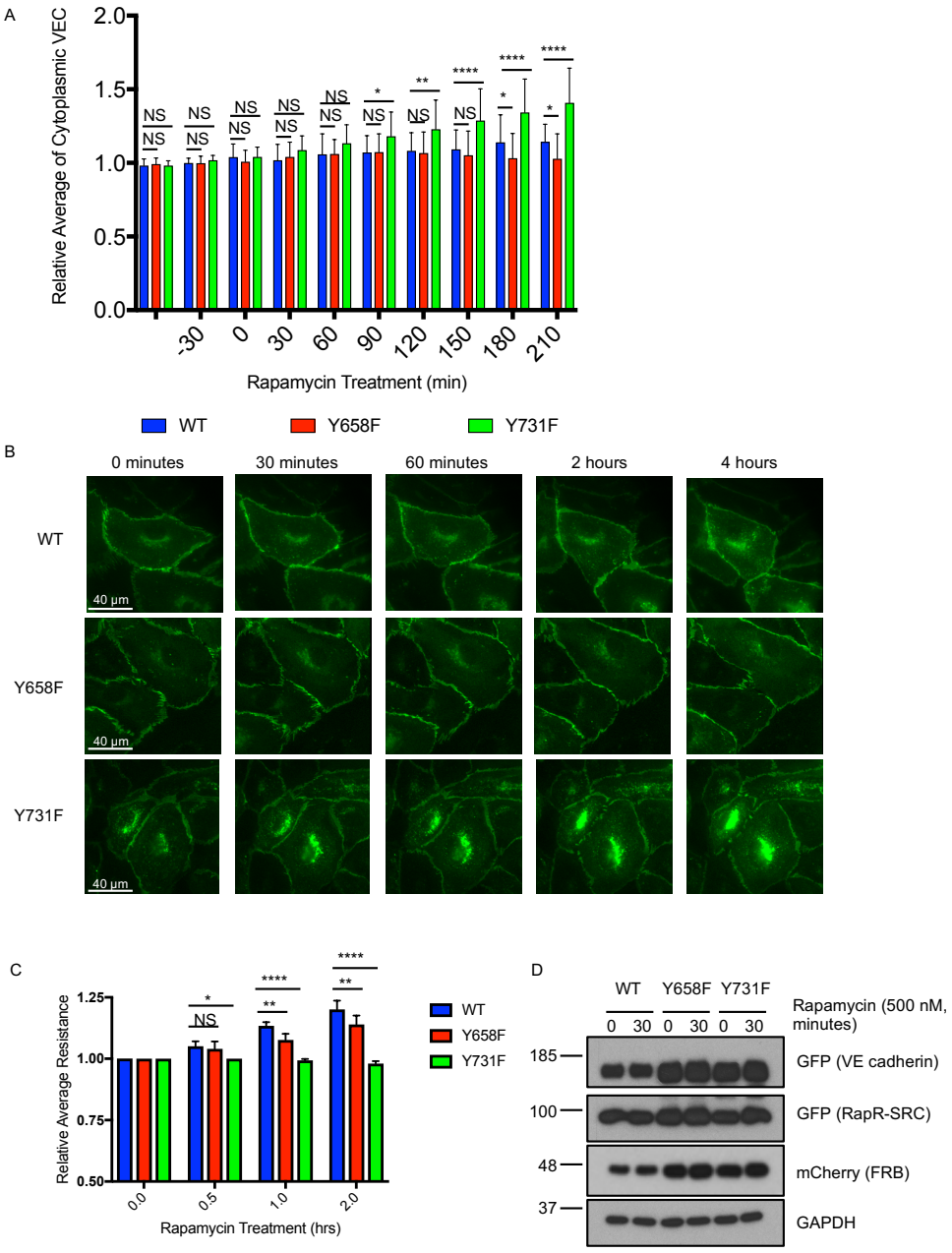
Supplementary Figure 2: SRC activation induced VE cadherin changes. (Relates to Figures 2-4). (A) Broadening of adherens junctions following SRC activation. HPAE cells co-expressing RapR-SRC-cerulean and mCherry-FRB were treated with rapamycin (500 nM) for the designated amount of time, fixed, stained for endogenous P120-catenin (Alexa647) and VE cadherin (Alexa488), and imaged using wide-field fluorescence microscopy. The white triangle indicates an example of broadening of the adherens junctions at 30 min and an example of gap formation at 4 hours following SRC activation. (B) Images of reticular adherens junctions obtained using confocal microscopy. HPAE cells co-expressing RapR-SRC-cerulean and mCherry-FRB were treated with rapamycin (500 nM) for 30 minutes, fixed, and stained for endogenous VE cadherin (Alexa488) and F-actin (Phalloidin-Alexa647). Images were collected using a Zeiss LSM 880 confocal microscope. Upper images show magnified area outlined by the white rectangle in the lower images. (C) Analysis of paxillin and VE cadherin localization in reticular adherens junctions. HPAE cells co-expressing RapR-SRC-cerulean, FRB, and mEOS-paxillin were treated with rapamycin (500 nM) for 30 minutes, fixed, and stained for endogenous VE cadherin (Alexa-647). Samples were imaged using an iPALM microscope. Quantification of the indicated regions is depicted in Figure 5C. (D and E) The role of SRC SH2 domain in regulation of endothelial permeability. (D) HPAE cells co-expressing RapR-SRC-cerulean (SRC) and mCherry-FRB or RapR-SRC-R175L-cerulean (R175L) and mCherry-FRB were analyzed using TER at a constant voltage. Rapamycin (500 nM) was added at time point 0. The average relative resistance at the indicated time points for cells expressing RapR-SRC and R175L mutant was determined from 3 independent experiments and compared using a two-way ANOVA with repeated measures and a post-hoc test with a Bonferroni's multiple comparisons adjustment. Error bars show standard deviation. (E) RapR-SRC expression and activation in HPAE cells. Cell lysates were collected at the indicated time points following rapamycin treatment and analyzed using the designated antibodies. Statistical significance is indicated using the following notations: *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. All exogenous proteins were expressed using adenoviral transduction except for mEOS-Paxillin which was tranfected as indicated in methods.



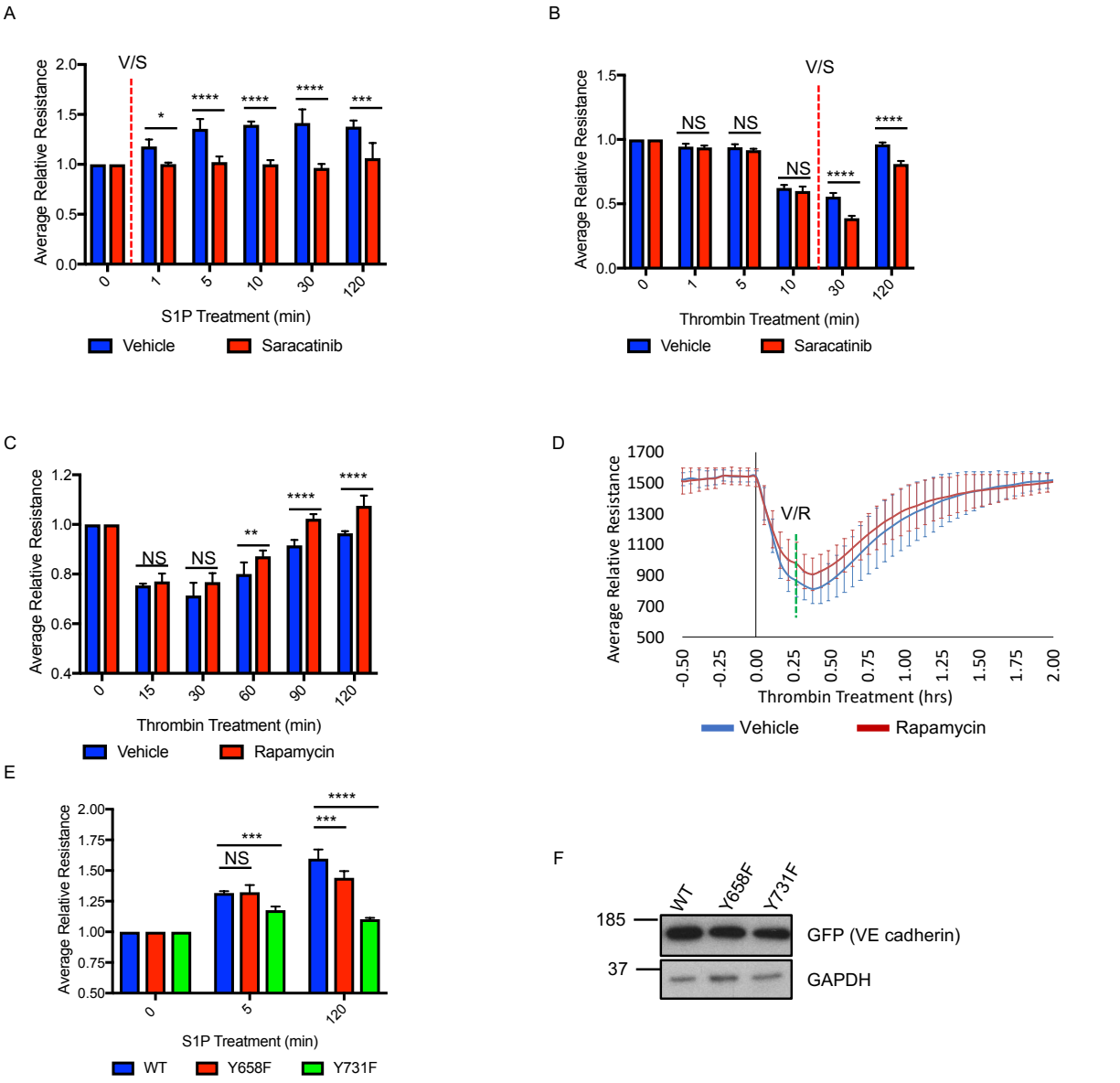
Supplementary Figure 3: SRC regulation of cadherin complexes. (Relates to Figure 5). HPAE cells co-expressing RapR-SRC-cerulean-myc and mCherry-FRB were treated with rapamycin (500 nM) for the specified duration. (A and B) Cell lysates were collected and endogenous VE cadherin was immunoprecipitated. VE cadherin immunoprecipitation samples were immunoblotted for total VE cadherin as well as P120- and β-catenin. The relative amount of P120- and β-catenin for each time point was normalized to time 0. The average of 4 experiments was graphed with error bars showing standard deviations and a one-way ANOVA with a Dunnett's multiple comparisons test was used to compare each time point to time 0. (C) Cell lysates were collected and immunoblotted for the designated proteins. (D) Characterization of antibodies recognizing phospho-Y658 and phospho-Y731 VE cadherin. HPAE cells co-expressing RapR-SRC-mCherry, mCherry-FRB, and GFP tagged VE cadherin (WT, Y658F, or Y731F mutant) were treated with rapamycin (500 nM) for 0 or 30 minutes. GFP tagged VE cadherin constructs were immunoprecipitated via GFP. Immunoprecipitated samples were immunoblotted for GFP, Phospho-Y658, and Phospho-Y731. Statistical significance is indicated using the following notations: p* <0.05, p** <0.01, p*** < 0.001, p**** < 0.0001. All exogenous proteins were expressed using adenoviral transduction.



Supplementary Figure 4: Comparison of SRC and LYN phosphorylation of VE cadherin. (Relates to Figure 5). (A) Schematic of SRC activation and inactivation using rapamycin and 1NA-PP1. (B) Quantification of phosphorylation changes on VE cadherin following inactivation of SRC via 1NA-PP1 from 3 independent biological replicates. Representative blots are shown in Figure 5D. (C-D) Representative blots, from 3 independent biological replicates, evaluating phosphorylation of VE cadherin following activation of the SH2 mutant of RapR-SRC (R175L) (C) and RapR-LYN (D). HPAE cells expressing RapR-SRC-R175L-cerulean (SH2 mutant) and mCherry-FRB or RapR-LYN-cerulean and mCherry-FRB were activated with rapamycin for the indicated amount of time. Cell lysates were collected and endogenous VE cadherin was immunoprecipitated and analyzed via Western blot with the indicated antibodies. (E) Quantification of RapR-LYN phosphorylation of VE cadherin (N=3). Each experiment was standardized to the 4 hour time point. A one-way ANOVA with a Dunnett's multiple comparisons test was used to compare the 15, 30, and 60 minute measurements to time 0, error bars represent standard deviations. Statistical significance is indicated using the following notations: *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. All exogenous proteins were expressed using adenoviral transduction.



S Supplementary Figure 5: VE cadherin localization is regulated by phosphorylation. (Relates to Figure 5). (A-D) The role of VE cadherin phosphorylation on Tyr658 and Tyr731 in regulation of its localization and endothelial permeability following activation of SRC. (A and B) HPAE cells co-expressing RapR-SRC-cerulean, mCherry-FRB, and the designated VE cadherin-GFP construct were imaged live every 2 minutes. Rapamycin (500 nM) was added at time point 0. (A) The amount of cytoplasmic VE cadherin was determined and all data were normalized to the average cytoplasmic VE cadherin prior to rapamycin treatment. Data was collected from 3 independent experiments. Error bars represent 90% confidence intervals. A two-way ANOVA with repeated measures and a post-hoc test with a Bonferroni's multiple comparisons adjustment was used to compare results at the designated time point. (B) Images of the indicated VE cadherin constructs taken at the designated times before and after activation of RapR-SRC. (C) HPAE cells co-expressing RapR-SRC-cerulean, mCherry-FRB, and the designated VE cadherin-GFP construct were analyzed by TER and were treated with rapamycin (500 nM) at time 0. Graph represents the average relative resistance of 3 independent experiments. The average relative resistance was compared using a two-way ANOVA with repeated measures and a post-hoc test with a Bonferroni's multiple comparisons adjustment was used to compare results at the designated time point. Error bars show standard deviation. (D) Western blots of samples prepared for TER assay in D. Statistical significance is indicated using the following notations: *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. All exogenous proteins were expressed using adenoviral transduction.



Supplementary Figure 6: The role of SFK activity via physiological stimuli. (Relates to Figures 6 and 7). (A) The role of SFK activity in S1P regulation of endothelial permeability. HPAE cells were serum starved for 1 hour and then analyzed via TER at a constant voltage. S1P (1 μM) and Saracatinib (100 nM) or vehicle (DMSO) were added at time point 0. (B) The role of SFK activity in endothelial barrier recovery following thrombin treatment. HPAE cells were serum starved for 1 hour and then analyzed using TER at a constant voltage. Thrombin (50 nM) was added at time point 0 and Saracatinib (100 nM) (S) or vehicle (ethanol, V) was added 15 minutes later. (C) Effect of SRC activation on endothelial barrier recovery following thrombin treatment. HPAE cells co-expressing RapR-SRC-cerulean and mCherry FRB were analyzed using TER at a constant voltage. Thrombin (50 nM) was added at time point 0, and rapamycin (500 nM) or vehicle (ethanol) was added 15 minutes later (dashed line). (D) HPAE cells expressing mCherry-FRB were analyzed using TER at a constant voltage. Thrombin (50 nM) was added at time point 0, and rapamycin (500 nM) or vehicle (ethanol) was added 15 minutes later (dashed green line). The graph shows an average of 3 experiments and the error bars depict the 90% confidence intervals. (E-F) The role of VE cadherin phosphorylation in S1P-mediated regulation of endothelial permeability. HPAE cells were infected with the indicated VE cadherin-GFP adenoviral constructs (wild-type (WT), Y658F, or Y731F) and analyzed by TER. Samples were serum starved for 1 hour and then treated with S1P (1μM) (time 0). (F) Western blot comparing expression of the different VE cadherin constructs analyzed via TER in E and F. (A-C, E) Graphs show the average relative resistance from 3 independent experiments each, taken at the designated time points, error bars represent the standard deviation between the 3 experiments. Significance was evaluated using a two-way ANOVA with repeated measures and a post-hoc test with a Bonferroni's multiple comparisons adjustment. Statistical significance is indicated using the following notations: *p <0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. All exogenous proteins were expressed using adenoviral transduction.