SUPPLEMENTARY MATERIAL

Tacrolimus rescues the signaling and gene expression signature of endothelial ALK1 loss-of-function and improves HHT vascular pathology

Santiago Ruiz, Pallavi Chandakkar, Haitian Zhao, Julien Papoin, Prodyot K. Chatterjee, Erica Christen, Christine N. Metz, Lionel Blanc, Fabien Campagne and Philippe Marambaud

SUPPLEMENTARY METHODS

Materials and antibodies. Recombinant human ALK1-Fc (Cat. #770-MA), BMP9 (#3209-BP), and $VEGF_{165}$ (#293-VE) were obtained R&D Systems. Tacrolimus was from Cayman Chemical (#10007965). For flow cytometry, antibody directed against ALK1 was from R&D Systems (#AF370). For Western blots, antibodies directed against pSmad1/5/8 (#13820), Smad5 (#9517), p-ERK1/2 (#9101), ERK1/2 (#9102), p-p38 (#4511), p38 (#9212), p-T308-Akt (#2965), p-S473-Akt (#4060), Akt (#9272) were from Cell Signaling Technology; anti-ID1 antibody from BioCheck (#BCH-1/195-14); anti-actin antibody from BD Transduction Laboratories (#612656); anti-Dll4 antibody from R&D Systems (#AF1389); anti-HA antibody from Roche (#3F10, used to detect transfected HA-tagged ALK1 in Fig. 6A); anti-ALK1 antibody from Santa Cruz Biotechnology (#sc-101555, used to detect transfected ALK1 in Fig. 6B). For immunohistochemistry (IHC), anti-Dll4 antibody was obtained from R&D Systems (#AF1389) and donkey anti-goat Alexa Fluor 633 secondary antibody from Molecular Probes (#A21082). PCR primers were obtained from IDT and sequencing was performed at Genewiz.

Western blot analyses. Cells were processed as before (1) with the following modifications. Cells were solubilized in RIPA buffer (#20-188, EMD Millipore) supplemented with 1× Complete protease inhibitor mixture (#11697498001, Roche). 5-20 µg of proteins (depending on the primary antibody used) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then probed with primary and secondary antibodies. A standard ECL detection procedure was then used.

Flow cytometry. HUVECs were stained with anti-ALK1 extracellular domain antibody #AF370 (20 µg/mL final concentration) for 15 min at room temperature (RT). After 2 washes in PBS 0.5% (w/v) BSA, cells were incubated with a donkey anti-goat Alexa Fluor 488 (1:2000 dilution) for 15 min at RT. Cells were washed twice in PBS 0.5% BSA and fluorescence was measured using a BD Fortessa cytometer. Subsequent analyzes were performed using FlowJo software v10.0.8.

Mice. To conform to ARRIVE guidelines (2) and control for potential bias, animals were randomized by arbitrary selecting half of each litter to go into the control or tacrolimus treatment group. Researchers who assessed the treatment outcomes (S.R. and P.M.), i.e., by retinal vascularization analyses, were blinded to mouse allocation and treatment, which were performed by H.Z. No allocation concealment, however, was used. Based on sample size calculations, mixed groups of male and female mice (litters of 7-9 pups) were treated on P3 (average weight 2 grams) with anti-BMP9/10 antibodies or isotype controls using the transmammary route and as described in details in Ref. (3). The same mouse groups were also treated daily with tacrolimus (0.5 mg/kg) from P3 to P5 and sacrificed on P6 for analyses. Mice were maintained in regular housing conditions and were allowed free access to water and diet.

Retinal whole-mount histochemistry. After fixation using 4% paraformaldehyde, retinas were dissected, cut four times to flatten them into petal flower shapes, and fixed with methanol for 20

min on ice. After removing methanol, retinas were washed in PBS for 5 min on a shaker at RT, and incubated in blocking solution (0.3% Triton, 0.2% BSA in PBS) for 1h on a shaker at RT. Retinas were then incubated in isolectin GS-IB4 Alexa Fluor 488 (#I21411, Molecular Probes) diluted 1:100 in blocking solution on a shaker overnight at 4°C. Retinas were then washed four times in 0.3% Triton in PBS for 10 min on a shaker, followed by two washes in PBS for 5 min on a shaker before mounting with Vecta Shield (#H-1000, Vector Laboratories). For Dll4 IHC, 5% donkey serum was added to the blocking solution, and an additional incubation step with donkey anti-goat Alexa Fluor 633 secondary antibody (1:2000, Molecular Probes) was performed.

SUPPLEMENTARY REFERENCES

1 Vingtdeux, V., Chandakkar, P., Zhao, H., Blanc, L., Ruiz, S. and Marambaud, P. (2015) CALHM1 ion channel elicits amyloid-β clearance by insulin-degrading enzyme in cell lines and in vivo in the mouse brain. *J Cell Sci*, **128**, 2330-2338.

2 Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M. and Altman, D.G. (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*, **8**, e1000412.

3 Ruiz, S., Zhao, H., Chandakkar, P., Chatterjee, P.K., Papoin, J., Blanc, L., Metz, C.N., Campagne, F. and Marambaud, P. (2016) A mouse model of hereditary hemorrhagic telangiectasia generated by transmammary-delivered immunoblocking of BMP9 and BMP10. *Sci Rep*, **5**, 37366.

SUPPLEMENTARY FIGURES

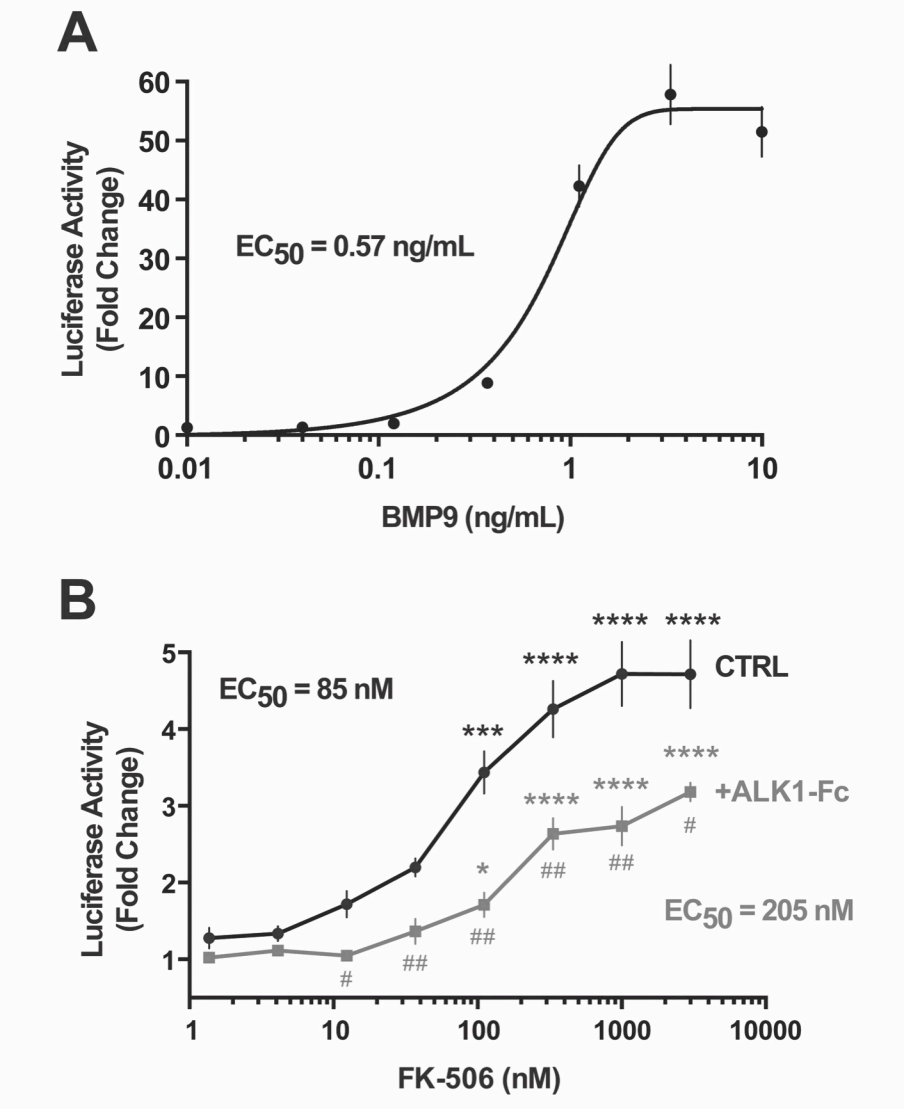

Figure S1. BMP9 and tacrolimus dose-responses in C2C12BRA cells. (**A**) Luciferase activity in C2C12BRA cells treated with different concentrations of BMP9 for 24h in depleted medium (0.1% FBS). Data represent mean ± s.e.m. (n = 5). (**B**) Luciferase activity in C2C12BRA cells treated or not (CTRL) for 24h in complete medium (conditioned for 2 days) with ALK1-Fc (1 µg/mL) and the indicated concentrations of tacrolimus (FK-506). Data in (A) and (B) represent mean ± s.e.m. (n = 4); **P* < 0.05, ****P* < 0.001, *****P* < 0.0001; one-way ANOVA, Dunnett's multiple comparisons test; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 relative to non-ALK1-Fc-treated controls at the corresponding FK-506 concentration, Student's *t*-test.

```
Table MJKWEEE-counts-table.tsv -
File Path
  ${PM_RETINA}/data/MJKWEEE-counts-table.tsv
Columns
  element-id: string [ ID ]
  element-type: [one of: GENE]
  OPRISZN-PM2-15-HUVEC-FK506-Batch2 : numeric [ FK506, Batch=2, counts ]
  CSNTWZP-PM2-11-HUVEC-DMSO-Batch2 : numeric [ DMSO, Batch=2, counts ]
  QHLSNAC-PM2-6-HUVEC-FK506-Batch1 : numeric [ FK506, Batch=1, counts ]
  LPDOCXS-PM2-3-HUVEC-DMSO-Batch1 : numeric [ DMSO, Batch=1, counts ]
  YEHCYXQ-PM2-14-HUVEC-FK506-Batch2 : numeric [ FK506, Batch=2, counts ]
  PUJBRTV-PM2-5-HUVEC-FK506-Batch1 : numeric [ FK506, Batch=1, counts ]
  OQPCQGD-PM2-12-HUVEC-DMSO-Batch2 : numeric [ DMSO, Batch=2, counts ]
  UQBGULA-PM2-10-HUVEC-DMSO-Batch2 : numeric [ DMSO, Batch=2, counts ]
  WFWEZJN-PM2-8-HUVEC-ALK1Fc-Batch1 : numeric [ ALK1Fc, Batch=1, counts ]
  NONOJXZ-PM2-4-HUVEC-FK506-Batch1 : numeric [ FK506, Batch=1, counts ]
  RFLLPJP-PM2-2-HUVEC-DMSO-Batch1 : numeric [ DMSO, Batch=1, counts ]
  LJPAHWR-PM2-9-HUVEC-ALK1Fc-Batch1 : numeric [ ALK1Fc, Batch=1, counts ]
  LAGPRWS-PM2-18-HUVEC-ALK1Fc-Batch2 : numeric [ ALK1Fc, Batch=2, counts ]
  NZGEIUF-PM2-7-HUVEC-ALK1Fc-Batch1 : numeric [ ALK1Fc, Batch=1, counts ]
  PHQEDRW-PM2-16-HUVEC-ALK1Fc-Batch2 : numeric [ ALK1Fc, Batch=2, counts ]
  SWCUEIK-PM2-17-HUVEC-ALK1Fc-Batch2 : numeric [ ALK1Fc, Batch=2, counts ]
  YIGIGJA-PM2-1-HUVEC-DMSO-Batch1 : numeric [ DMSO, Batch=1, counts ]
  DIFDLQR-PM2-13-HUVEC-FK506-Batch2 : numeric [ FK506, Batch=2, counts ]
```

Figure S2. Table annotation for MetaR Analysis.

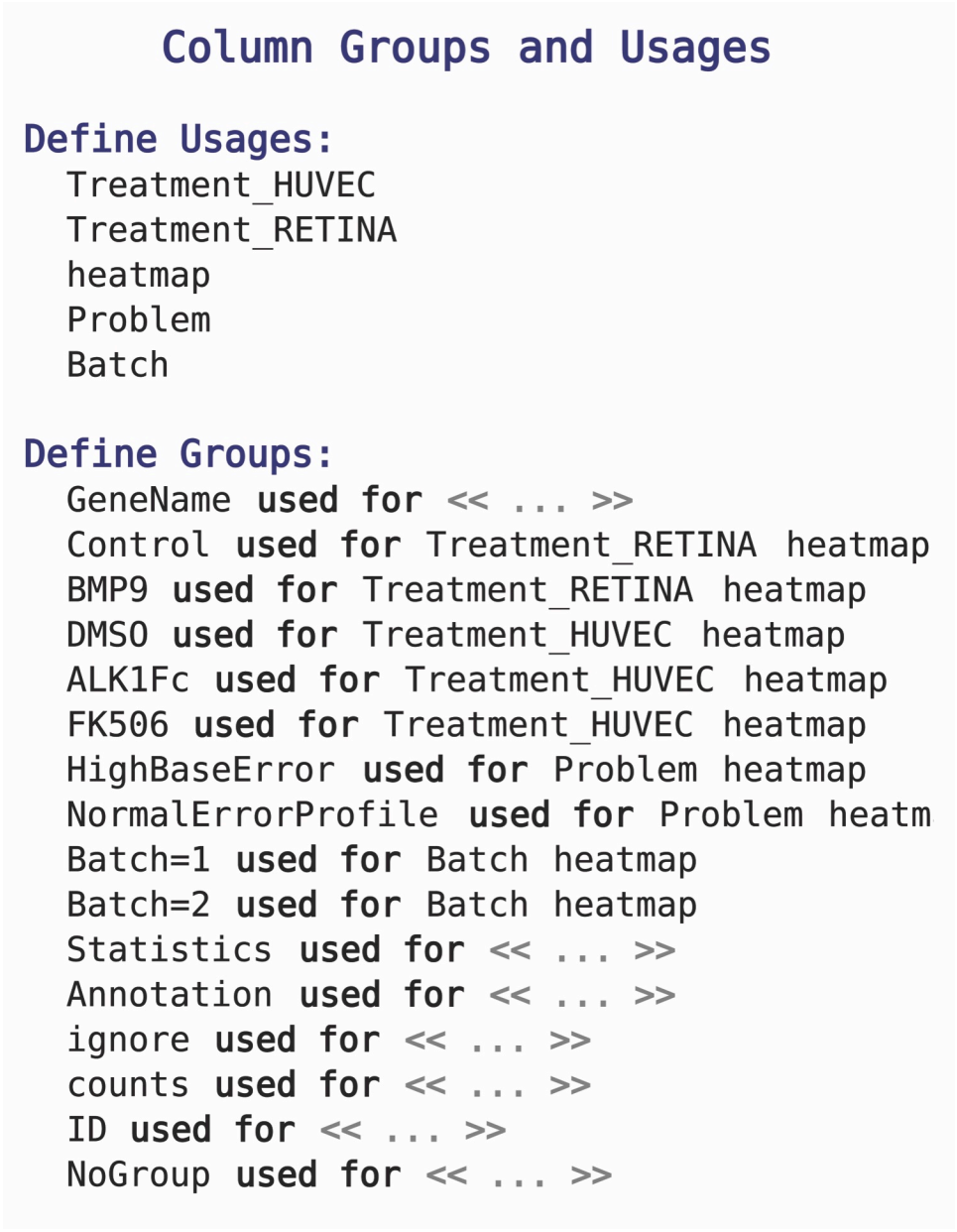

Figure S3. Group definitions for MetaR Analysis.

```
Analysis DiffExp HUVEC2
{
  import table MJKWEEE-counts-table.tsv
  limma voom counts= MJKWEEE-counts-table.tsv  model: ~ 0 + Treatment_HUVEC + Batch
    comparing DMSO - FK506 -> stats: Tacrolimus normalized: nr1 adjusted counts: Adjusted_Tacrolimus
  limma voom counts= MJKWEEE-counts-table.tsv  model: ~ 0 + Treatment_HUVEC + Batch
    comparing ALK1Fc - DMSO -> stats: ALK1Fc normalized: nr2
  join ( Adjusted_Tacrolimus , ALK1Fc, Tacrolimus ) by group ID -> joined
  fit logFC.Tacrolimus  by logFC.ALK1Fc with table joined -> tacrolimus vs. ALK1Fc logFC not filtered  AllGenes
  subset rows joined when true: $(adj.P.Val.Tacrolimus ) <= 0.0001 | $(adj.P.Val.ALK1Fc ) <= 0.0001 ->
      adjusted FDR one or the other

  subset rows joined when true: $(adj.P.Val.Tacrolimus ) <= 0.0001 & $(adj.P.Val.ALK1Fc ) <= 0.0001 ->
      adjusted FDR in both conditions

  fit logFC.Tacrolimus  by logFC.ALK1Fc with table adjusted FDR one or the other  ->
      tacrolimus vs. ALK1FC logFc highly significant  SignificantHits

  multiplot -> comparison [  2 cols x 1 rows ]  Preview
   [ tacrolimus vs. ALK1Fc logFC not filtered  ] [ tacrolimus vs. ALK1FC logFc highly significant  ]

  transform table adjusted FDR in both conditions  -> Keep One GeneId {
    drop column genes.Tacrolimus
    drop column genes.ALK1Fc
  }
  // Retrieve Gene Names:
  // Add Gene names to the table of results:
  import table annotations-all-genes.tsv
  join ( Keep One GeneId , annotations-all-genes.tsv  ) by group ID -> ResultBiomart
  // Plot the heatmap with gene names
  heatmap with ResultBiomart  select data by one or more group DMSO, group FK506, group ALK1Fc -> heatmap no style [
    show names using group  GeneName
    annotate with these groups:  Treatment_HUVEC ,  Batch
    scale values:  scale by row
    cluster columns:  false cluster rows:  true
  ]
  render heatmap as PDF named "${PM_RETINA}/heatmaps/HUVEC/heatmap-HUVEC2-oneless-adjusted.pdf "  -  no style
  render comparison as PDF named "${PM_RETINA}/scatter-plots/HUVEC/comparison-significant-vs-all-genes.pdf  "  -
      no style
  transform table adjusted FDR in both conditions  -> transformedTable {
    rename column:  element-id  -> elementId
  }
  write transformedTable  to "selected-HUVEC2-lessone-normalized-adjusted-counts.tsv "  -
  subset rows adjusted FDR one or the other  when true: $(logFC.ALK1Fc ) > 2 & $(logFC.Tacrolimus ) < 1 ->
      Changed with ALK1Fc but not Tacrolimus
  write Changed with ALK1Fc but not Tacrolimus  to "ChangedWithALK1Fc_notTacrolimus.tsv "  -

  UpSet { ids from table when true: Tacrolimus$adj.P.Val  < 0.0001 name of set:  Tacrolimus } -> upset plot
          ids from table when true: ALK1Fc$adj.P.Val  < 0.0001 name of set:  ALK1Fc
  render upset plot as PDF named "${PM_RETINA}/upset/HUVEC/upset-tacrolimus-ALK1Fc.pdf "  -  no style

  join ( Tacrolimus , ALK1Fc, annotations-all-genes.tsv  ) by group ID -> merged with gene names
  define Set of IDs GenesOfInterest {
    ANGPT2 CXCR4 DLL4 WNT9A SMOC1 KDR PPM1H PGF
  }
  MA plot with stats from  merged with gene names  red when FDR<= 0.0001 label set:  GenesOfInterest  with GeneName  ->
      MA tacrolimus TacroMAPlot
  MA plot with stats from  merged with gene names  red when FDR<= 0.0001 label set:  GenesOfInterest  with GeneName  ->
      MA ALK1Fc ALK1MAPlot

  multiplot -> MA plots [  2 cols x 1 rows ]  Preview
   [ MA tacrolimus ] [ MA ALK1Fc ]
  render MA plots as PDF named "${PM_RETINA}/ma-plots/HUVEC/Tacrolimus_ALK 1Fc_MAPlots.pdf "  -  no style
}
```

Figure S4. MetaR analysis script for RNA-Seq analysis.

Table S1. RNA-Seq analyses in HUVECs. Gene expression changes were analyzed in HUVECs treated or not (DMSO) for 24h in complete medium (conditioned for 2 days) with ALK1-Fc (1 µg/mL) or tacrolimus (FK-506, 0.3 µM). Table presents the annotated results (selected-HUVEC2-lessone-normalized-adjusted-counts.tsv, imported into Excel, produced by the analysis script shown in Fig S4) and describes differentially expressed genes with a FDR less or equal to 0.0001 (n = 5-6).