Factor VIII promotes angiogenesis and vessel stability regulating extracellular matrix proteins

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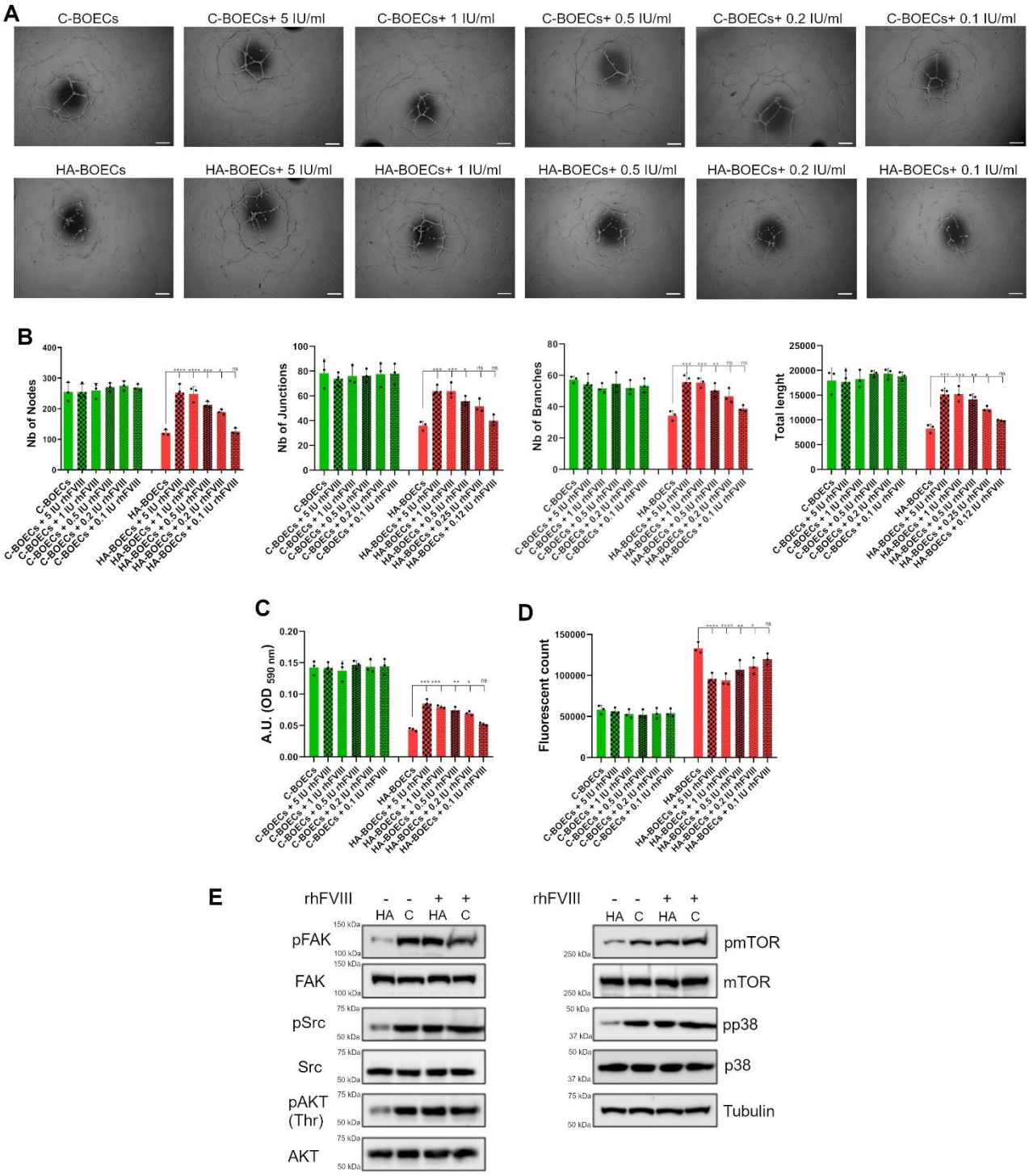
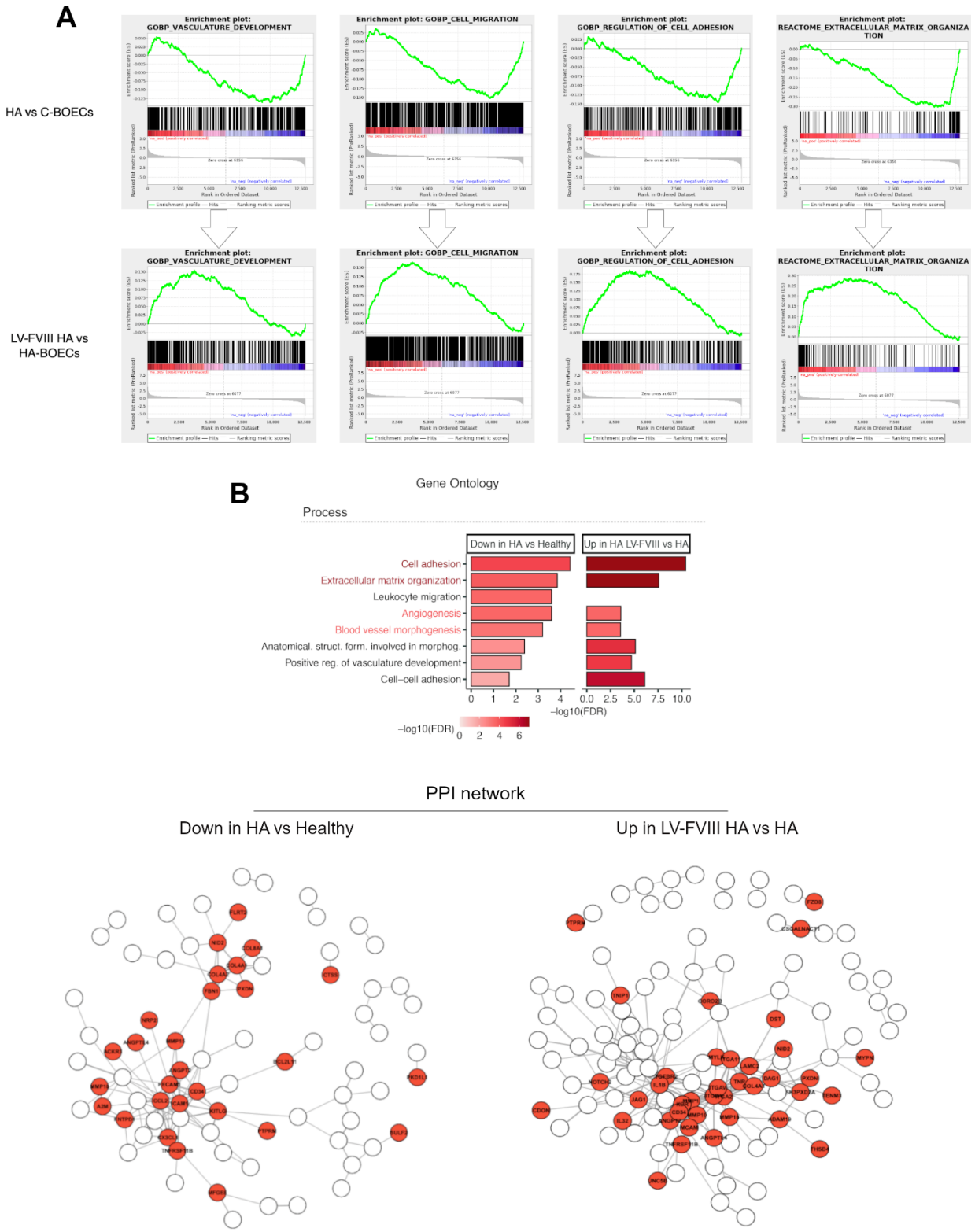
Supplementary figures



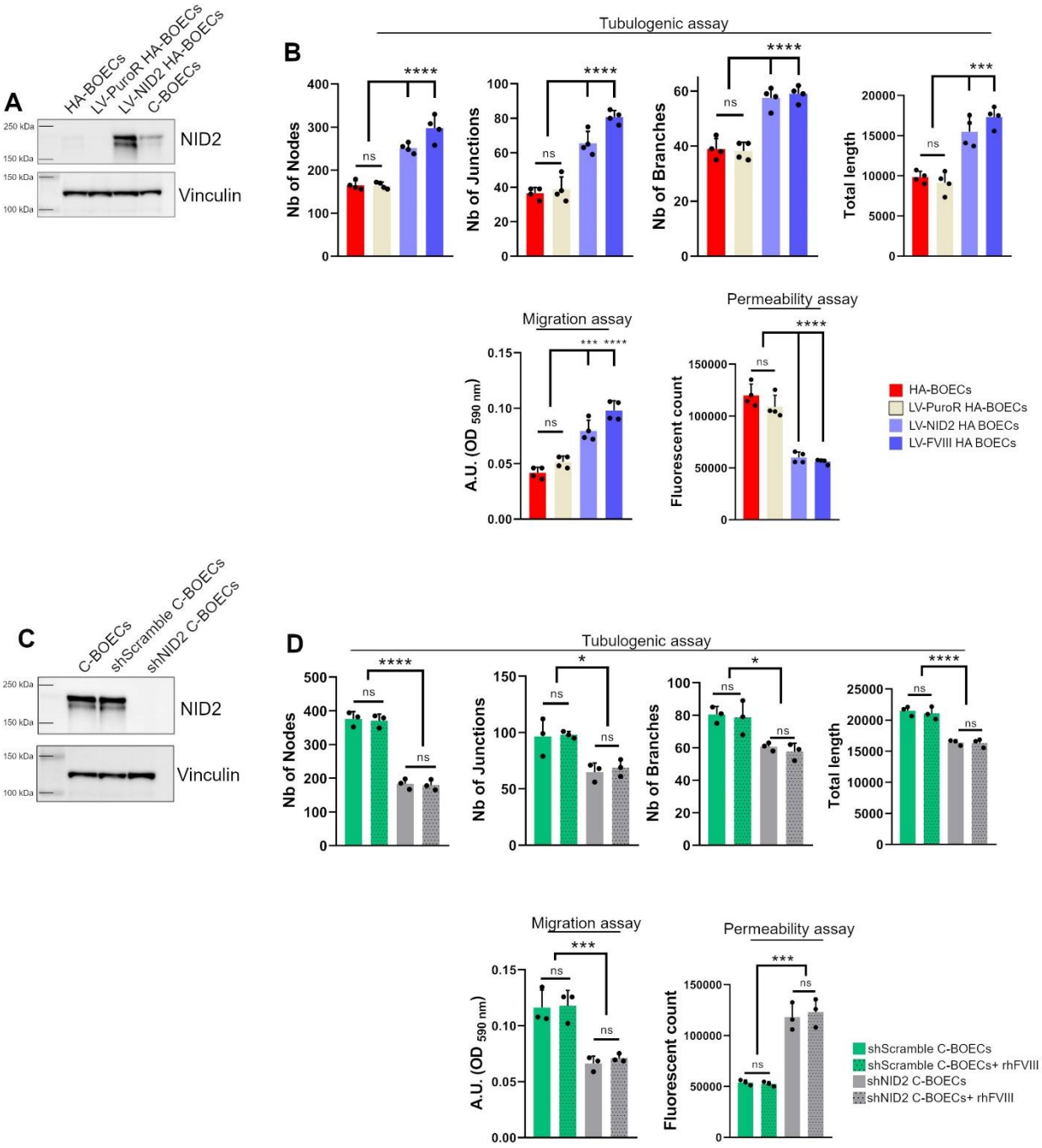
Supplementary Figure 1. Acute treatment of HA-BOECs with FVIII enhances EC functionality. A) Representative image showing the result of the tubulogenic assay on C- (n=3) and HA-BOECs (n=3) treated with 5, 1, 0.5, 0.2 and 0.1 IU/ml of B-domain-deleted rhFVIII. $5x10^4$ BOECs in culture medium with or without rhFVIII, were placed on top of the Matrigel® and incubated overnight. Scale bar = 500 µm. **B)** Quantitative analysis of the number of nodes, junctions, branches, and total length of tubule networks of cells described in **A** using ImageJ Angiogenesis software. **C)** Indirect measurement of cell migration through crystal violet

staining elution of cells described in **A**. BOECs were plated into the upper compartment of 8-μm pore size Transwell at a density of 10^5 cells in serum-free medium with or without B-domain-deleted rhFVIII, while the lower compartment was filled with complete medium and incubated overnight. After incubation, migrated cells were stained with crystal violet that was eluted with acetic acid and quantified using Victor Spectrophotometer at 590 nm. **D)** Quantification of permeability based on the extravasation of FITC-dextran through an intact monolayer of cells described in **A**. Permeability was measured across a monolayer of BOECs ($8x10^4$ cells/well) cultured on 0.1% gelatin coated Transwell (3 μm pore) with or without B-domain-deleted rhFVIII that was added every day for 1 week. At the end of the culture, 5 μg/ml of FITC-conjugated 40-kDa dextran was added to the upper chamber and the fluorescence of the lower chamber was measured in the medium using Victor Spectrophotometer (490 nm excitation/520 nm emission). Fluorescence readings were normalized to dextran permeability in transwell inserts without cells. **E)** Western blot analysis of WCL from HA- and C-BOECs treated for 15 min with or without 1 IU/ml of B-domain-deleted rhFVIII and stained with antibodies against pFAK, total FAK, pSrc, total Src, pAKT, total AKT, pmTOR, total mTOR, pp38, total p38. Tubulin was used as a loading control. Data in **B**, **C** and, **D** are expressed as mean ± SD. All experiments were performed once for each subject. Statistical analysis was performed by one-way ANOVA test (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).



Supplementary Figure 2. GSEA and Gene Ontology analysis of FVIII-modulated genes. A) Gene set enrichment analysis (GSEA) plots depicting pathways that are downregulated in HA *vs* C-BOECs and rescued in LV-FVIII HA *vs* HA-BOECs. Key pathways include vascular development, cell migration, regulation of cell adhesion, extracellular matrix (ECM) organization and integrin cell surface interactions. Genes were considered as significantly differentially expressed (DEGs) when having |logFC| >1 and raw p value < 0.01 in

each reported comparison, as advised by SEQC consortium After the identification of a dataset of differentially expressed genes (DEGs), Enrichr online tool was used to identify pathways and gene ontology (GO) terms enriched using DEGs as input. A term is defined as significantly enriched if the reported adjusted p value is < 0.01. Gene set enrichment analysis (GSEA) was performed using a Broad Institute java package version 3.0 (classic mode) and MSigDB. **B)** On the left panel, the heatmap shows the top commonly enriched gene ontology (GO) process terms among the differentially expressed genes (DEGs) identified by RNAseq. These DEGs are compared across two categories: "Down in HA *vs* Healthy" and "Up in HA LV-FVIII *vs* HA". Hierarchical clustering was applied using the log10-adjusted *p*-values from the enrichment analysis. On the right panel, the STRING protein-protein interaction (PPI) network illustrates the interactions among these DEGs. Nodes within the network are color-coded to correspond with the commonly enriched GO annotations highlighted on the right.



Supplementary Figure 3. NID2 expression regulates *in vitro* ECs activity. A) Western blot comparison of NID2 levels in LV-NID2 HA-BOECs *vs* HA-BOECs, with LV.PuroR HA-BOECs and C-BOECs used as controls. **B)** Quantification of number of nodes, junctions, branches, and total tubule network length in HA-BOECs and LV-PuroR, LV-NID2, and LV-FVIII HA-BOECs. LV-PuroR was used as control for LV transduction. Indirect measurement of cell migration in the BOEC groups through crystal violet staining elution. Permeability assay results, based on the extravasation of FITC-dextran through an intact monolayer in the BOEC groups, as described in **B**. **C)** Western blot analysis of NID2 expression in shNID2 C-BOECs compared to non-transduced or shScramble-transduced C-BOECs. **D)** Quantification of number of nodes, junctions, branches, and total tubule network length in shScramble C-BOECs and shNID2 C-BOECs, with or

without 1 IU/ml rhFVIII. Indirect cell migration assay results by crystal violet staining elution in the BOEC groups, as described in **B**. Permeability assay results, based on the extravasation of FITC-dextran through an intact monolayer in the BOEC groups, as described in **B**. Data in **B** and **D** are expressed as mean ± SD. All the experiments were replicated three or four times. Statistical analysis was performed by one-way ANOVA test (****$p < 0.0001$; ***$p < 0.001$ **$p < 0.01$; *$p < 0.05$).