Supplemental Material

Sprouty4 regulates endothelial cell migration via modulating Integrin β3 stability through c-Src

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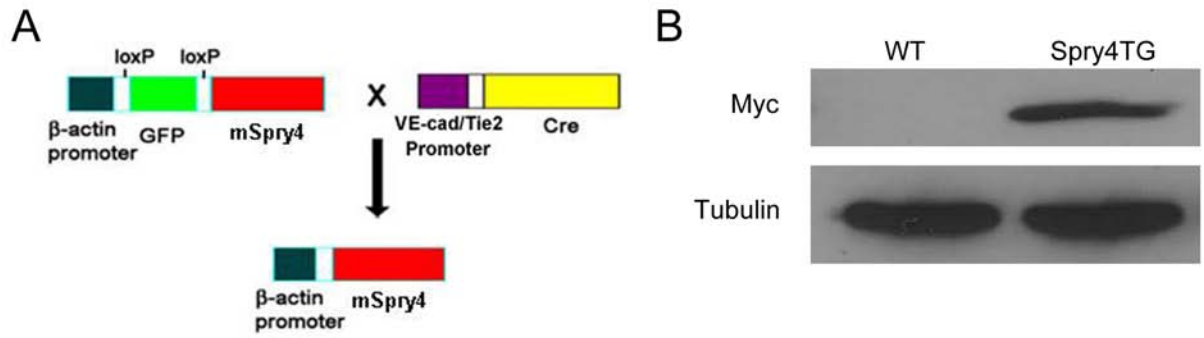
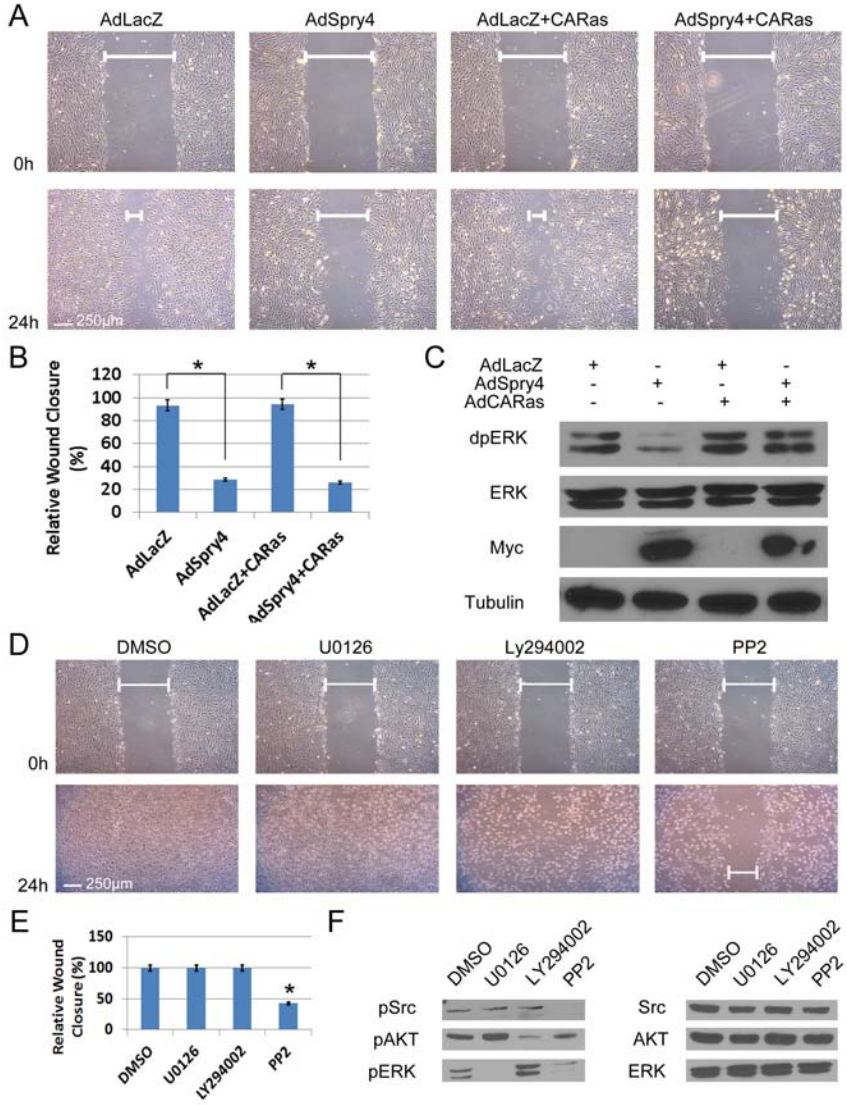
Supplemental Figure 1



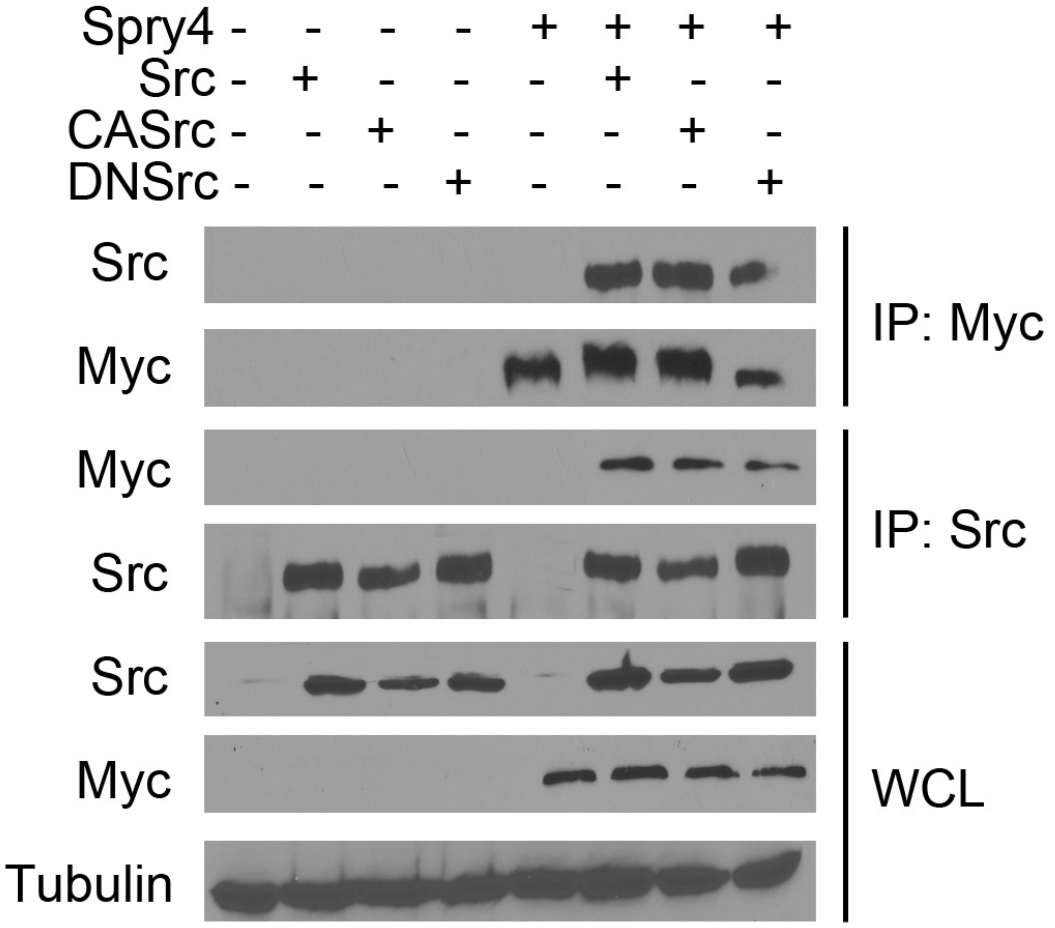
Suppl. Fig. 1 Spry4 transgenic mouse model. (**A)** Scheme of the Spry4 transgenic mice is shown. Conditional overexpression of Spry4 in endothelial cells was achieved by mating female *CAGGFP-Spry4* mice to male *VE-cad-Cre* or *Tie2-Cre* transgenic mice. (**B)** Murine primary endothelial cells were isolated from the lungs of WT and *CAGGFP-Spry4;VE-cad-Cre* (Spry4TG) mice. Representative immunoblots are shown. Equal amount of cell lysate were loaded for both samples.

Supplemental Figure 2



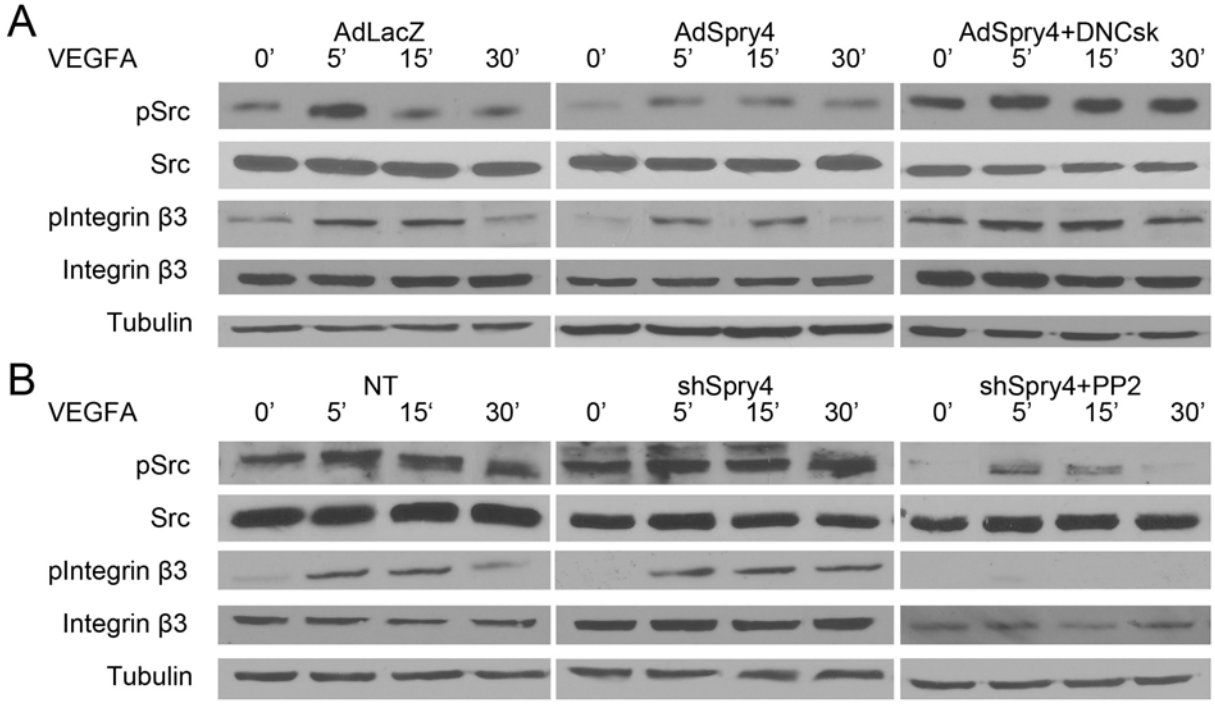
Suppl. Fig. 2 Inhibition of HUVEC migration by Spry4 is Ras-independent and c-Src-dependent. (**A)** CARas does not rescue Spry4-mediated inhibition of HUVEC migration on VTN. HUVECs grown on VTN were transduced with AdLacZ or AdSpry4 with or without AdCARas. After pre-treated with mitomycin C (10 μg/ml) for 20 min, confluent monolayers were scratched and photographed at the indicated times. (**B)** Quantification of relative wound closure; n = 6; data are means ± S.D. * $p < 0.05$. (**C)** Cell lysates of the transduced HUVECs were analyzed by immunoblotting to confirm the rescue of ERK phosphorylation by CARas. (**D)** The effect of chemical inhibitors (5 μM) on HUVEC migration was analyzed by wound healing assays. Confluent HUVECs grown on VTN were pre-treated with mitomycin C (10 μg/ml) for 20 min, scratched and photographed at the indicated times. (**E)** Quantification of relative wound closure; n = 6; data are means ± S.D. * $p < 0.05$. (**F)** Cell lysates of the inhibitor-treated HUVECs were analyzed by immunoblotting to show the inhibition of specific pathways.

Supplemental Figure 3



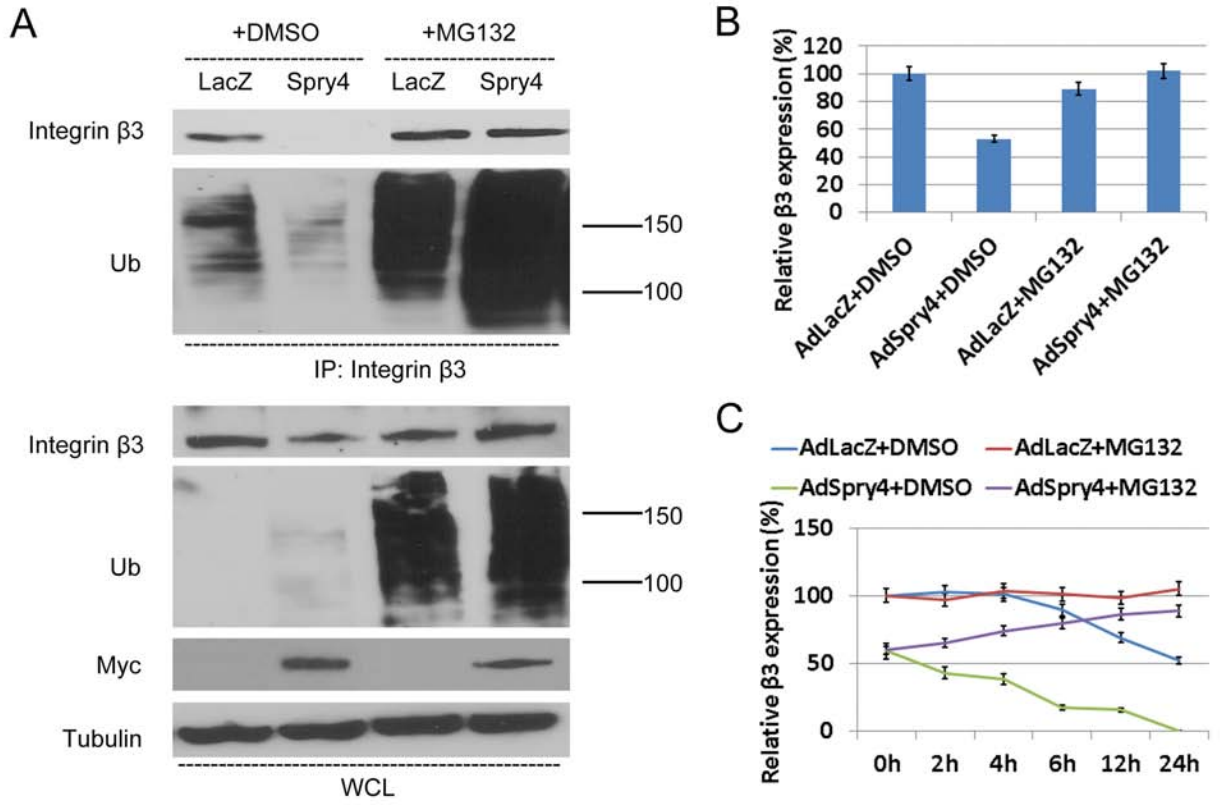
Suppl. Fig. 3 Spry4 co-immunoprecipitates with c-Src in 293T cells. Plasmids encoding Spry4 and WT or mutant c-Src were transfected into 293T cells, followed by co-immunoprecipitation analysis, which shows that Spry4 interacts with wild-type, constitutively active (CA), and dominant negative (DN) c-Src.

Supplemental Figure 4



Suppl. Fig. 4 Spry4 regulates tyrosine phosphorylation of integrin β3 via c-Src. (**A)** Spry4 overexpression inhibits VEGF-induced c-Src and integrin β3 tyrosine phosphorylation in HUVECs grown on VTN, which is rescued by DNSrc. Transduced HUVECs were grown on VTN and starved in serum-free medium for 24 h after viral transduction and then stimulated with VEGF-A (20 ng/ml). Cell lysates were prepared at different time points as indicated and analyzed by immunoblotting. (**B)** Spry4 knockdown increases VEGF-induced c-Src and integrin β3 tyrosine phosphorylation in HUVECs grown on VTN, which is repressed by the SFK inhibitor PP2 (5 μM).

Supplemental Figure 5



Suppl. Fig. 5 Spry4 regulates the stability of integrin β3 in HAECs. (**A)** Spry4 overexpression decreases integrin β3 protein levels in HAECs, whereas MG132 reverses this effect. When proteasome degradation is blocked by MG132, Spry4 overexpression increases ubiquitination levels of integrin β 3 proteins. (**B)** Quantification of relative integrin β3 expression normalized to tubulin and relative to the LacZ+DMSO control; n = 3; data are means ± S.D. * $p < 0.05$. (**C)** HAECs were transduced with AdLacZ or AdSpry4. After cycloheximide (10 μg/ml) treatment, DMSO or MG132 were added into culture medium. Cell lysates were prepared at different time points as indicated. Quantification of relative integrin β3 protein levels normalized to tubulin and relative to the lacZ+DMSO control at 0 h; n = 3; data are means ± S.D.