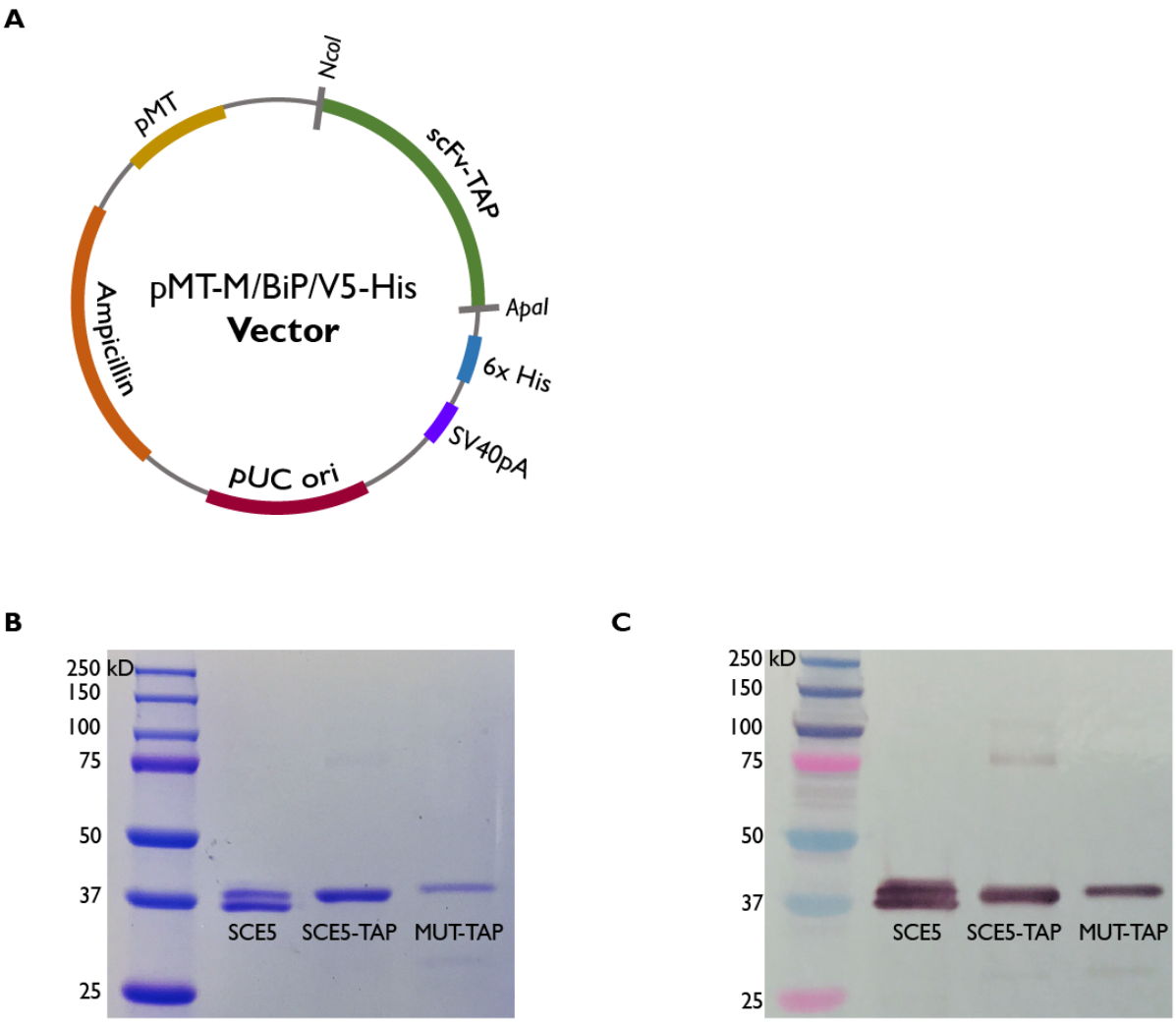
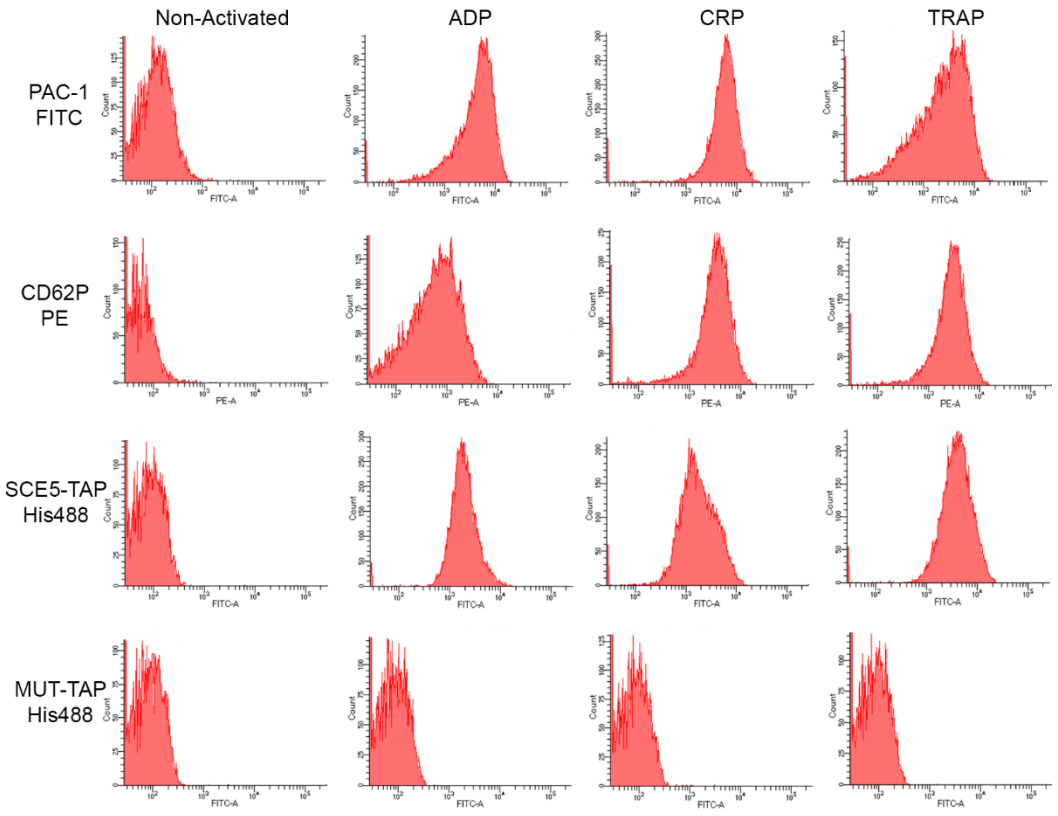
SUPPLEMENTAL MATERIAL

Platelet-Targeted Dual Pathway Antithrombotic Inhibits Thrombosis with Preserved Hemostasis

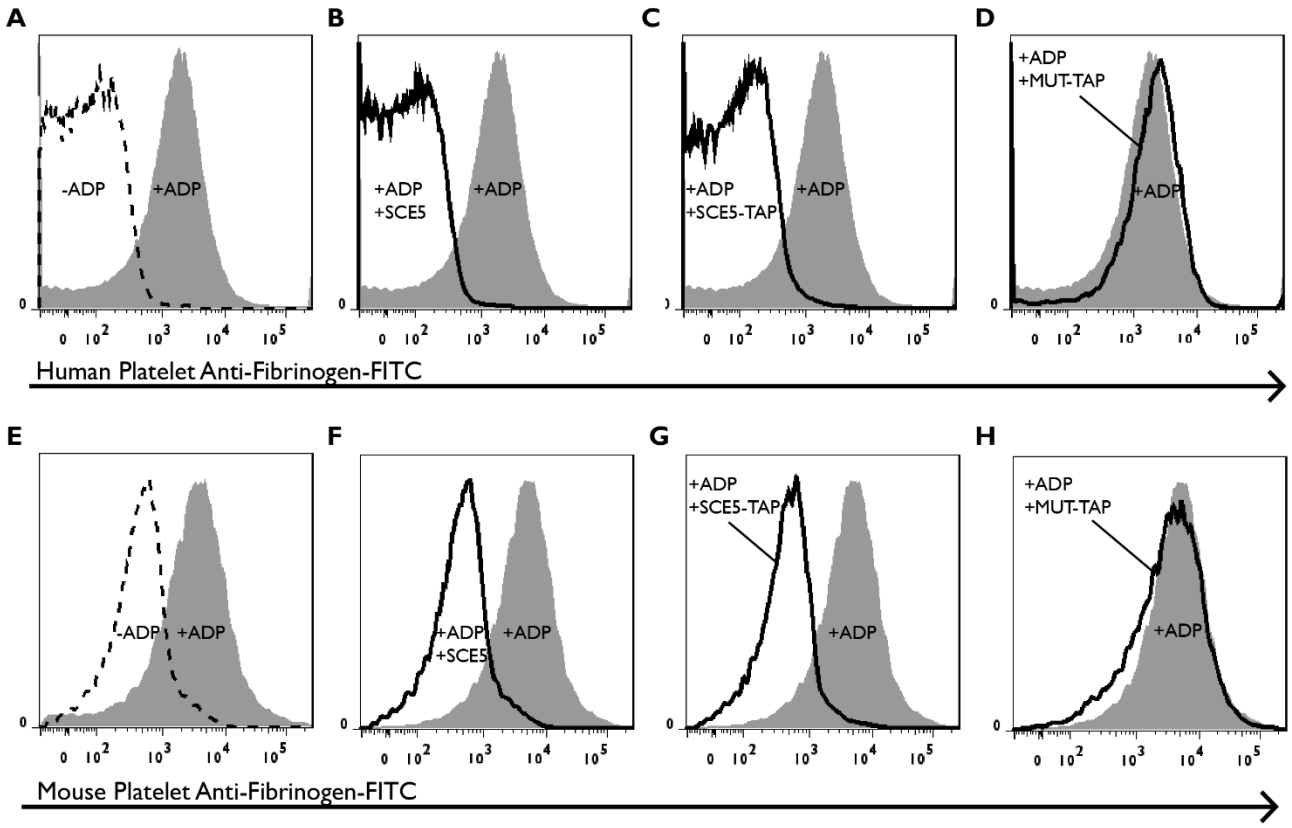
Authors: Donny Hanjaya-Putra, PhD, Carolyn Haller, PhD, Xiaowei Wang, PhD, Erbin Dai, MD, Bock Lim, PhD, Liying Liu, MD, Patrick Jaminet, MD, Joy Yao, Amy Searle, Thomas Bonnard, PhD, Christoph E. Hagemeyer, PhD, Karlheinz Peter, MD, Elliot L. Chaikof, MD, PhD



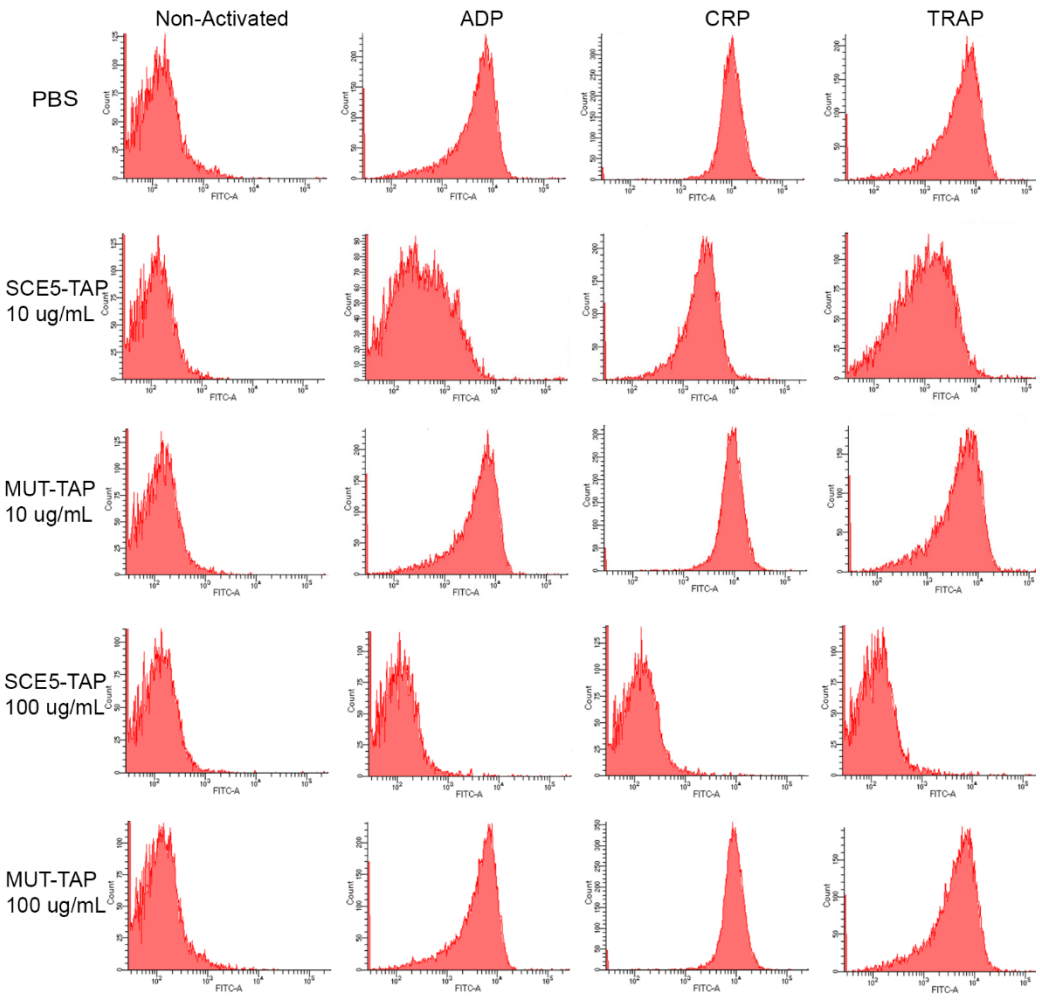
Supplement Figure 1. **Expression and purification of scFvs**. (**A**) Plasmid map of scFv-TAP constructs in pMT. (**B**) scFvs were purified by metal affinity chromatography and characterized via SDS-PAGE and Coomassie total protein stain. (**C**) Western blot (anti-His HRP) analysis of scFv SCE5, SCE5-TAP, and MUT-TAP.



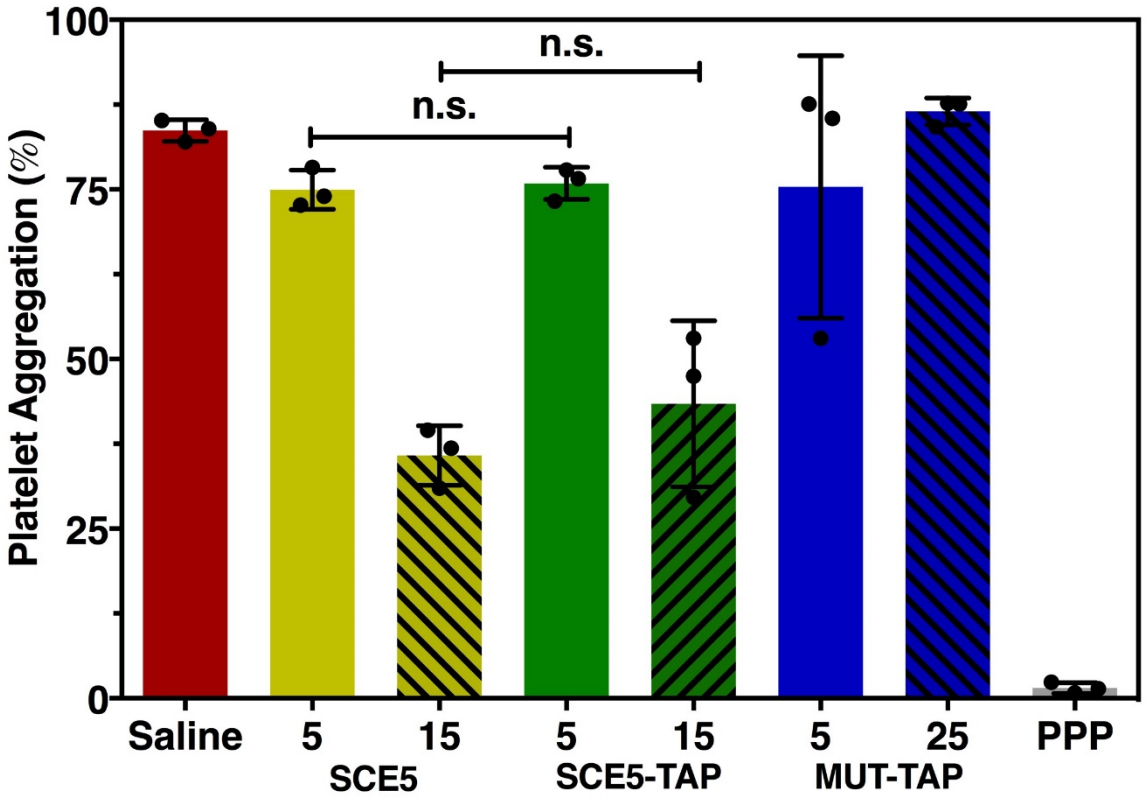
Supplement Figure 2. **SCE5-TAP binds ADP-, CRP-, and TRAP-activated platelets**. Representative flow cytometry histograms demonstrating ADP, CRP, and TRAP agonist platelet activation and correlative binding of SCE5-TAP. Platelets were activated with 20 μM ADP, 5 μg/mL CRP, or 30 μM TRAP and examined for activation of GPIIb/IIIa (PAC-1-FITC, top row) or P-selectin (CD62P-PE, second row). Binding of SCE5-TAP and MUT-TAP was characterized with anti-His-AlexaFluor488. Activation specific binding of SCE5-TAP was observed for all agonists (third row), no binding was observed for MUT-TAP (fourth row).



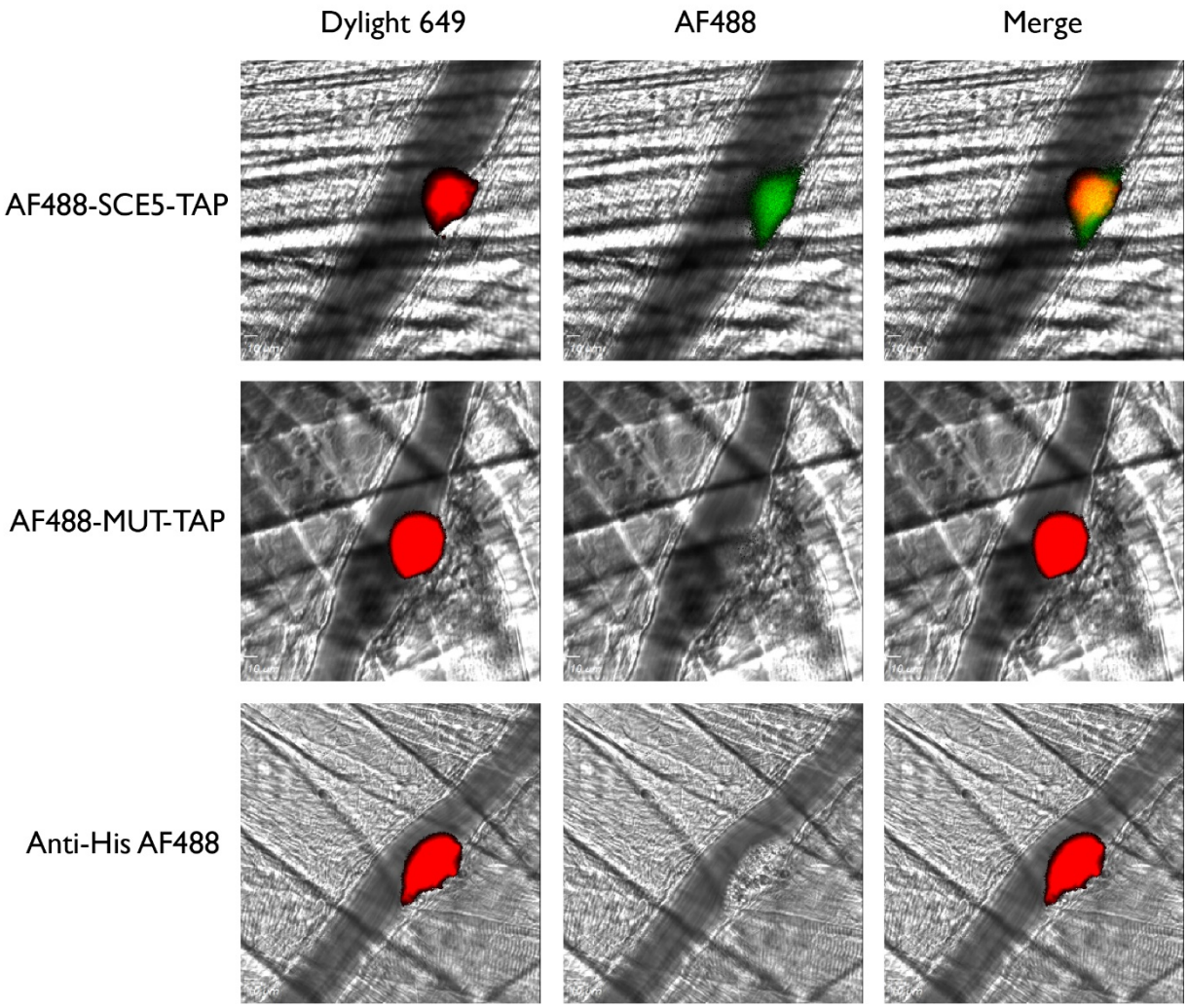
Supplement Figure 3. **Inhibition of fibrinogen binding to ADP-activated platelets**. Fibrinogen binding to 20 µM ADP-activated (**A**) human and (**E**) mouse platelets is given by grey histogram as detected using FITC anti-fibrinogen antibody. The dotted open histogram indicates non-activated platelets stained with FITC anti-fibrinogen antibody. Inhibition of fibrinogen binding with (**B,F**) SCE5, (**C,G**) SCE5-TAP, and (**D,H**) MUT-TAP (15 µg/mL) is given by white open histograms. Fibrinogen binding to activated platelets is inhibited by SCE5 and SCE5-TAP, but not MUT-TAP.



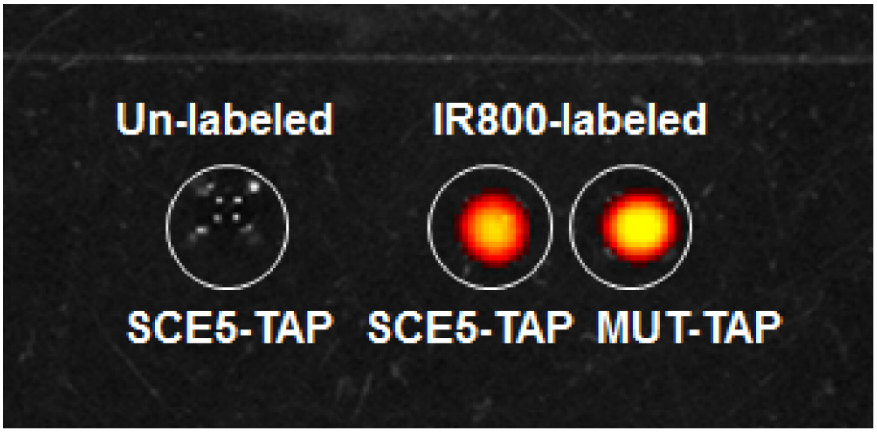
Supplement Figure 4. **SCE5-TAP inhibits fibrinogen binding to ADP-, CRP-, and TRAP-activated platelets**. Representative flow cytometry histograms demonstrating ADP, CRP, and TRAP agonist platelet activation and correlative binding of Fibrinogen-Alexafluor488. Platelets were activated with 20 μM ADP, 5 μg/mL CRP, or 30 μM TRAP for activation of GPIIb/IIIa, Fibrinogen-Alexafluor488 binding can be observed in activated platelets incubated with PBS (top row). Low dose SCE5-TAP (10 ug/mL, second row) demonstrates moderate inhibition of fibrinogen binding, while high dose (100 ug/mL, fourth row) completely inhibits binding. No inhibition was observed with MUT-TAP at 10-100 ug/mL.



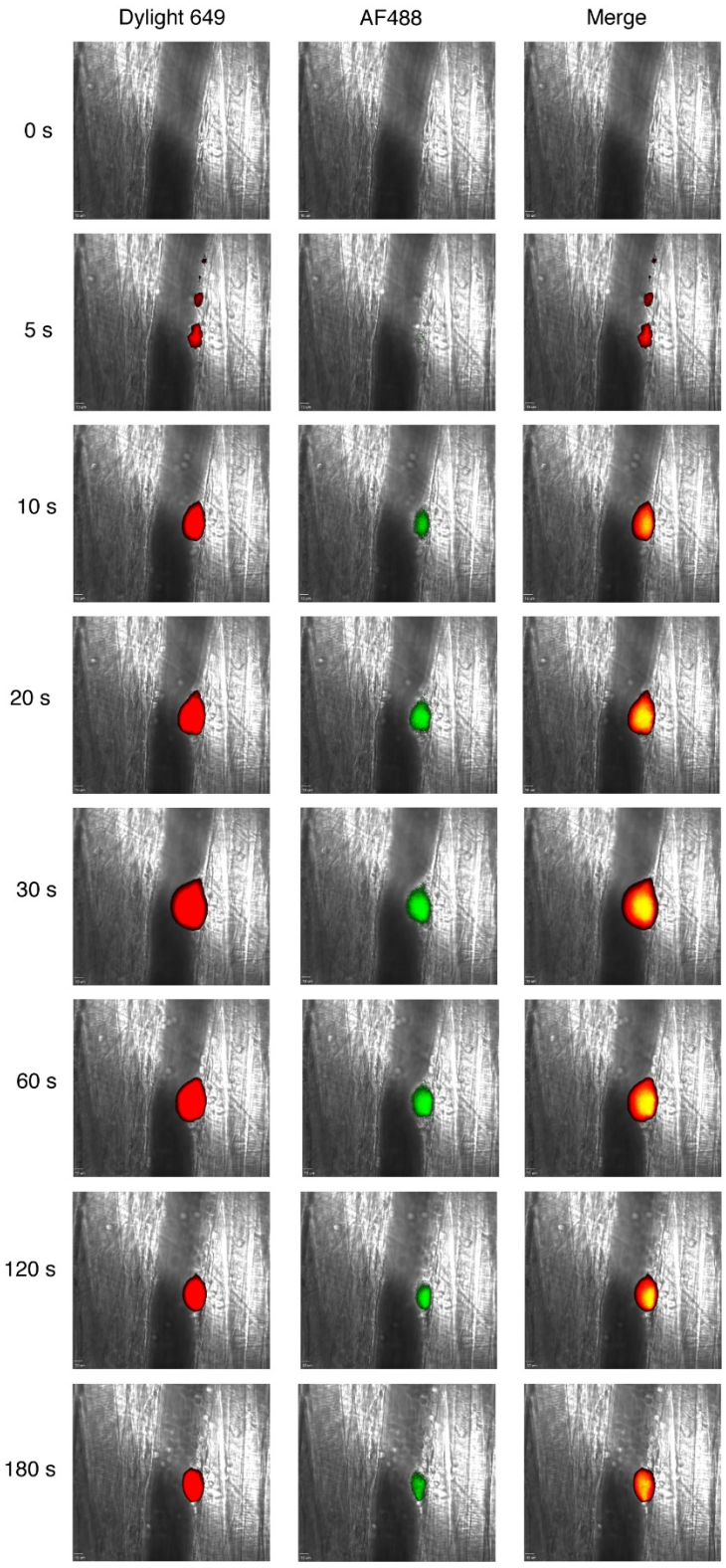
Supplement Figure 5. **Inhibition of platelet aggregation to ADP**. 100 µL PRP was incubated with 5 and 15 µg/mL SCE5 or SCE5-TAP. MUT-TAP was examined at 5 and 25 µg/mL. Plotted data represent aggregation recorded at 10 min after addition of 10 µM ADP. 15 µg/mL SCE5 and SEC5-TAP equally inhibit aggregation suggesting no impact of the C-terminal TAP fusion on anti-aggregatory activity of SCE5. Data represent mean ± SD, n=3/group, n.s. $p>0.05$.



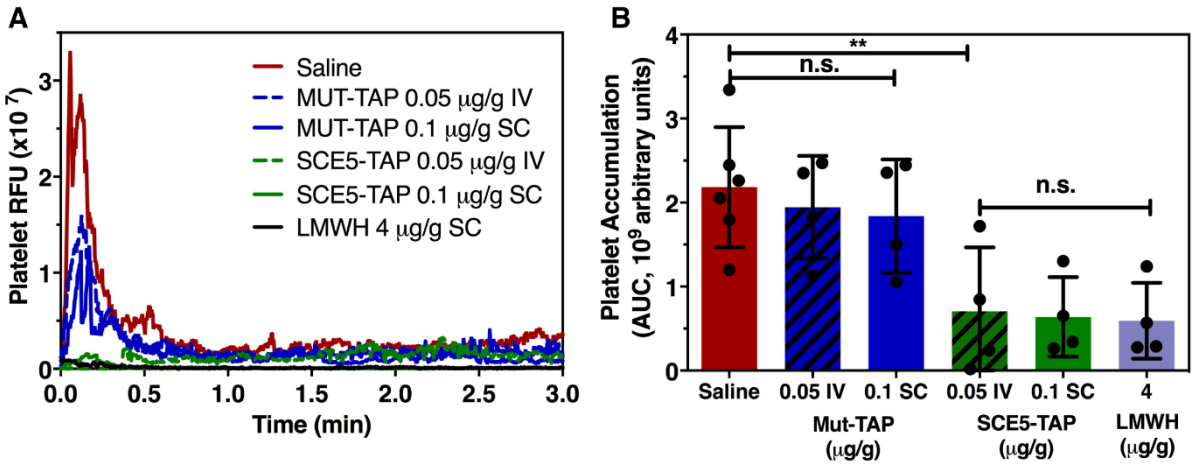
Supplement Figure 6. **SCE5-TAP targets arterial platelet thrombus in vivo.** Platelet specific Dylight 649 labeled-anti-CD42b was infused along with either AF488-labeled SCE5-TAP (top row) AF488-labeled MUT-TAP (middle row) or AF488 control (bottom row) prior to laser injury of cremaster arterioles. Representative images illustrate that SCE5-TAP targets to the platelet thrombus. Co-localization of MUT-TAP or AF488 control was not observed.



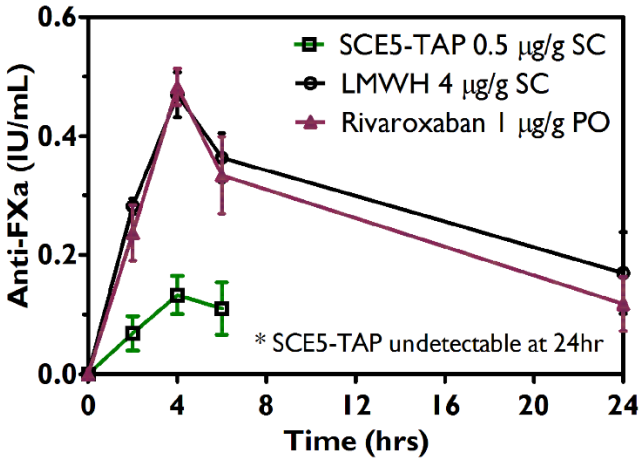
Supplement Figure 7. **IR800 labeling for IVIS characterization.** SCE5-TAP and MUT-TAP were labeled with NHS-IR800 dye and confirmed via IVIS scan.



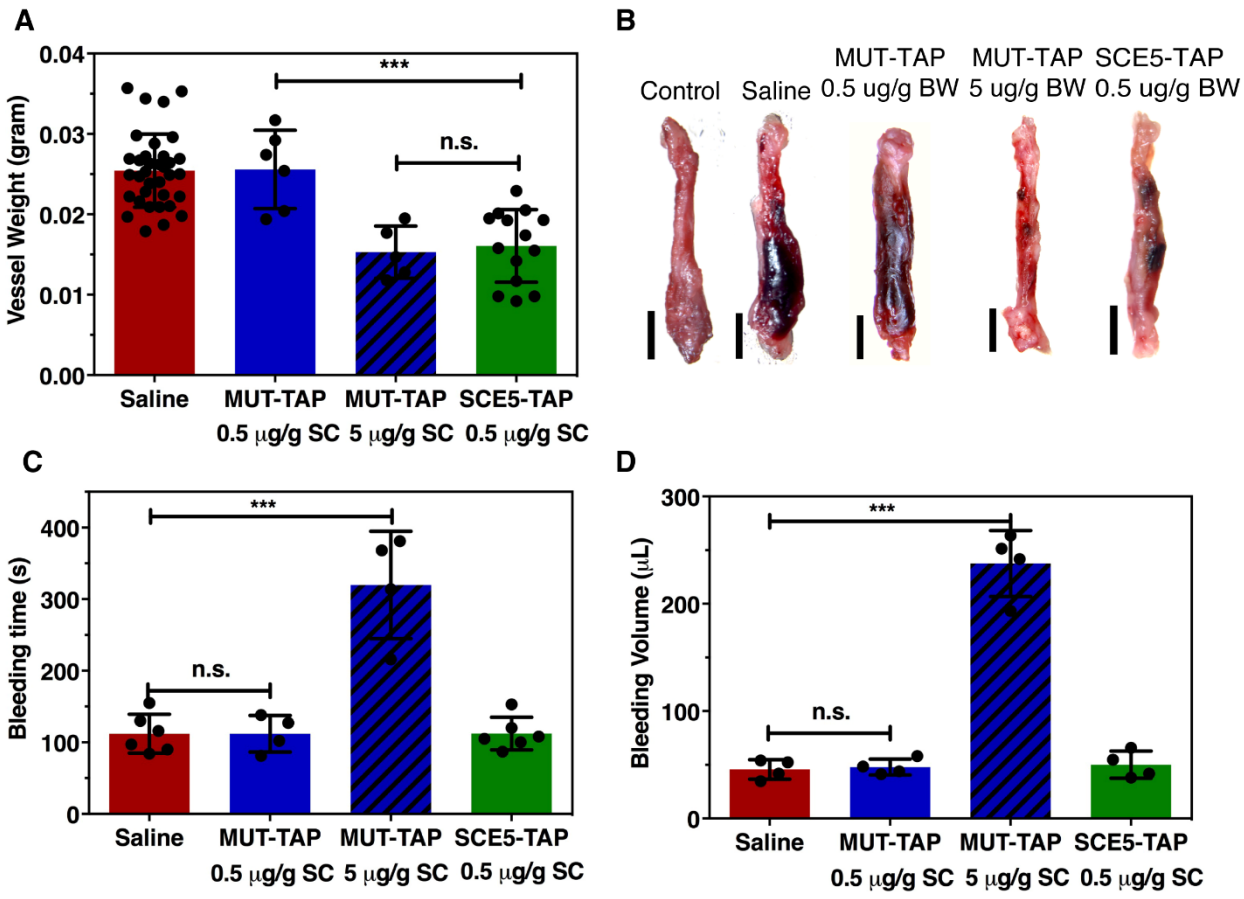
Supplement Figure 8. **Delayed targeting of SCE5-TAP to arterial platelet thrombus in vivo.** Platelet specific Dylight 649 labeled-anti-CD42b (left column) was infused along with AF488-labeled SCE5-TAP (middle column) prior to laser injury of cremaster arterioles. Representative images illustrate that Dylight 649 platelets enrich to the platelet thrombus immediately. Co-localization of SCE5-TAP occurs on an established layer of sealing platelets.



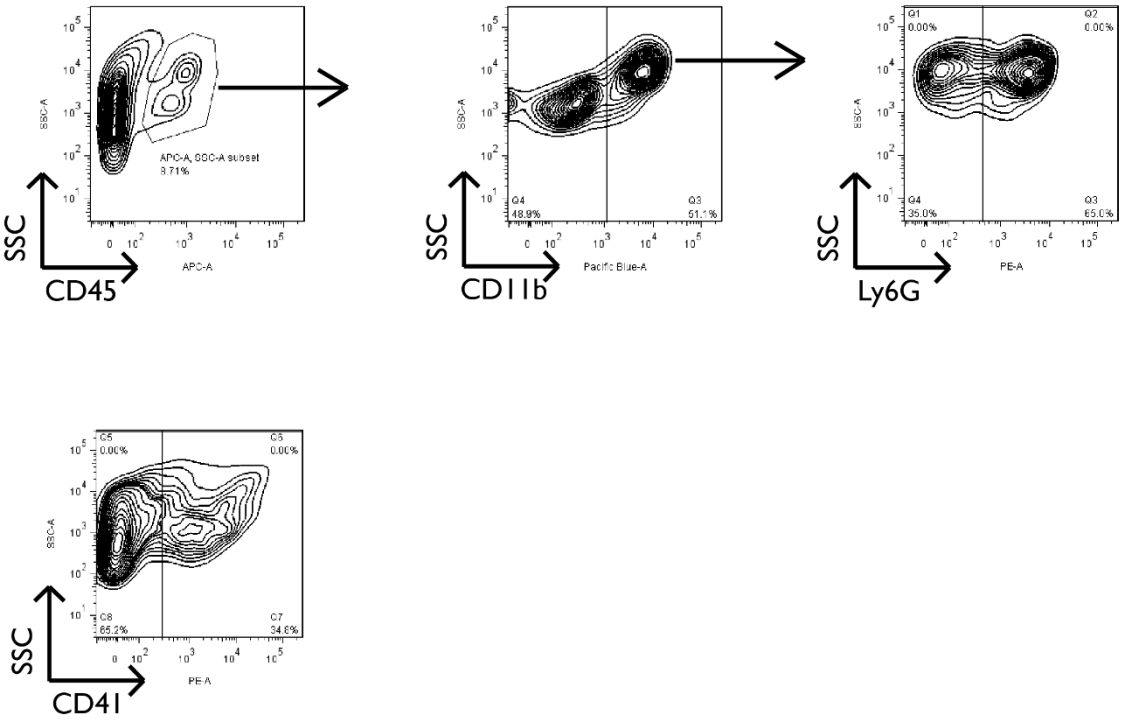
Supplement Figure 9. SCE5-TAP targets venous platelets and inhibits thrombosis in vivo, IV verses SC administration. SCE5-TAP efficiently reduces venous thrombus with 0.05 μg/g IV delivery (patterned green, thrombus inhibition characterized within 1 h of IV administration) or 0.1 μg/g SC delivery (solid green, thrombus inhibition characterized at 4 h post-SC administration), results compared to equivalent doses of MUT-TAP (IV and SC) or LMWM (Enoxaparin, 4 μg/g SC, characterized at 4 h post-SC administration). Results are plotted as median integrated platelet fluorescence (**A**) or platelet accumulation quantified as AUC (**B**). Data represent mean ± SD, n = 25-30 vessels per group in n=4 mice, n.s. $p>0.05$, ** $p \leq 0.01$ (ANOVA and Bonferroni's multiple comparison test).



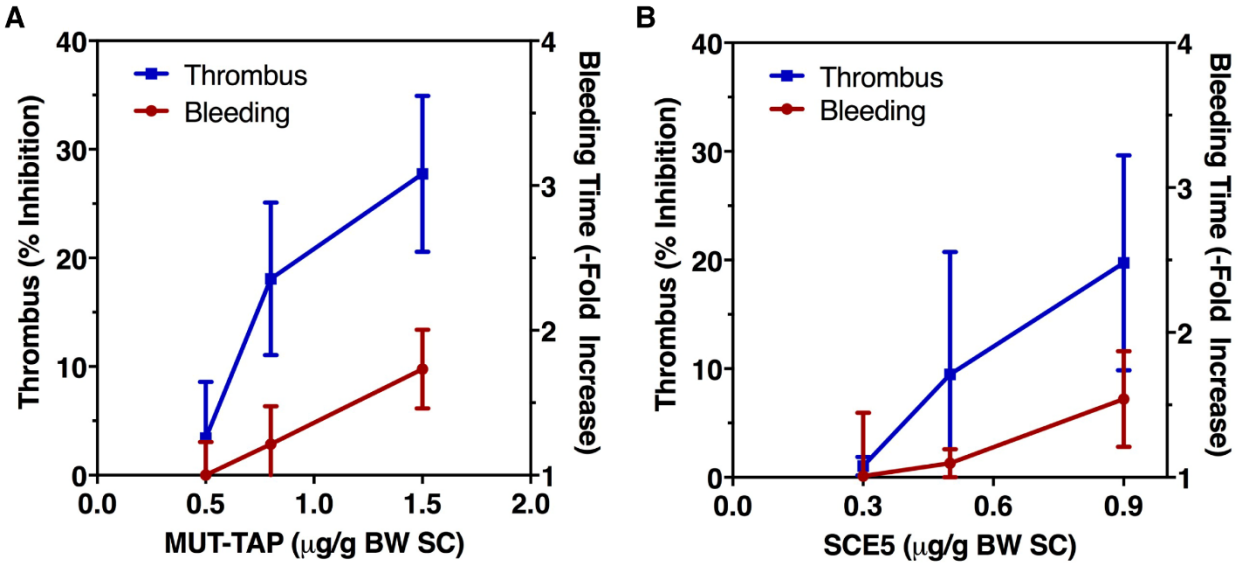
Supplement Figure 10. Peak systemic anti-FXa activity. SCE5-TAP (0.5 µg/g SC), LMWH (4 µg/g SC), or Rivaroxaban (1 µg/g PO) were administered and plasma anti-FXa activity was monitored at 2h, 4h, 6h, and 24h, n=4/group. Anti-FXa activity was below the lower limit of detection (0.03 IU/mL) at 24 h post-SCE5-TAP administration. All agents displayed peak anti-FXa activity 4h post-administration. SCE5-TAP demonstrated equivalent antithrombotic activity with minimal impact on systemic anti-FXa activity.



Supplement Figure 11. High dose of non-targeted MUT-TAP inhibits IVC thrombus with increased bleeding risk. An electrolytic inferior vena cava model (EIM) was used to generate consistent venous thrombi in the presence of continuous blood flow. SCE5-TAP (0.5 μg/g SC), MUT-TAP (0.5 μg/g SC), or MUT-TAP (5 μg/g SC) were administered 4 h prior to electrolytic injury and at 24 h post-injury, IVC was harvested at 48 h for thrombus characterization. (**A-B**) Uniform length of IVC was harvested and immediately weighed to compare vessel wall + thrombus weight, control enrollment is uninjured IVC with no thrombus. High dose of non-targeted MUT-TAP (5 μg/g SC) displays thrombus inhibition equivalent to low dose of targeted SCE5-TAP (0.5 μg/g SC), n = 5-10 mice/group. Systemic bleeding risk was characterized through tail transection bleeding time (**C**), and tail transection blood loss (**D**), n = 5-10 mice/group. Data represent mean ± SD, n.s. $p>0.05$, ***$p \leq 0.001$ (ANOVA and Bonferroni's multiple comparison test).



Supplement Figure 12. Flow cytometry gating strategy. 48 h post-EIM, uniform length IVC wall + thrombus were harvested and digested to single cell suspension for flow cytometry analysis of total leukocytes ($CD45^+$) and platelets ($CD41^+$). Single cell suspension was triple stained with CD45, CD11b, and Ly6G to select for neutrophils and monocytes population. $CD45^+$ and $CD11b^+$ cell population is gated for neutrophils ($Ly6G^+$) and monocytes ($Ly6G^-$). The graphs show representative gating strategy from n=5 mice/group.



Supplement Figure 13. Dose-dependent inhibition of IVC thrombosis versus tail transection bleeding time for equimolar control enrollment of MUT-TAP and SCE5. Antithrombotic efficacy (% inhibition) is calculated based on percent reduction of thrombus weight in treatment groups vs. saline control 48 h after electrolytic injury of the IVC. Bleeding time is reported as fold-increase in tail transection bleeding time over saline control, 4 h after administration of the test agent. (**A**) MUT-TAP (0.5-1.5 μg/g). (**B**) SCE5 (0.3-0.9 μg/g). Data represent mean ± SD, n = 3 per treatment dose for thrombus inhibition studies and n = 3 per treatment dose for bleeding time studies. Enrollment, which was designed to inform dose escalation studies reported in Figure 6D, does suggest independent contribution of both SCE5 and TAP toward inhibition of IVC thrombus formation, however isolated components do not achieve the antithrombotic potency observed for the SCE5-TAP fusion construct.