SUPPLEMENTAL DATA

Packaging of supplemented urokinase into alpha-granules of *in vitro*-grown megakaryocytes for targeted nascent clot lysis

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Supplement Methods

Western blot of α-granule proteins

$CD34^+$-MKs differentiated for 10 days were incubated with purified plasmin-free plasminogen at 20 µg/ml for 24 hours, washed and then incubated for an additional 18 hours with recombinant human scuPA or uPAT (200 nM each)[1] in the absence or presence of human FV (400 nM)[2]. The cells were then lysed in 1×radioimmunoprecipitation assay (RIPA) buffer with added 1× proteinase inhibitor mixture (Sigma) and 1× phosphatase inhibitors mixture 2 (Sigma). Protein concentrations in cell lysates were measured using the Bradford Protein Assay. Lysates were subjected to SDS-PAGE and Western blotting using the following antibodies: rabbit anti-human uPA polyclonal antibodies; rabbit anti-human VWF polyclonal antibodies; mouse anti-human FV monoclonal antibody; and HRP-conjugated anti-β-actin rabbit polyclonal antibody. Secondary horse radish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit polyclonal antibodies were used for detection, and protein bands were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate. Luminescent signals on the blots were scanned and quantified using a Li-COR C-Digit Blot scanner (LI-COR Biosciences)/Image Studio™ software (LI-COR Biosciences).

Agonist-induced activation assays of megakaryocytes and platelets

$CD34^+$-derived human megakaryocytes differentiated on days 11 were loaded with uPAT (400 nM) for 24 hours, washed in phosphate-buffered saline (PBS, Invitrogen) and then re-suspended as previously described to study agonist responsiveness[3]. The thrombin receptor–activating peptide 6 (TRAP6, Sigma Cat # T1573) (50 µg/ml) was added to each sample and incubated at room temperature for 15 minutes. After washing the sample with PBS, allophycocyanin (APC)-labeled polyclonal rabbit anti-CD41 (BD Pharmingen, 1:100) and BV421-labeled polyclonal rabbit anti-CD62P (BD Pharmingen, 1:100) were added, and activation of human megakaryocyte was determined by surface expression of P-selectin. Flow cytometry analysis was done and analyzed using FlowJo software version 10.6 (BD Biosciences). Activation of human platelets released in vivo from $CD34^+$-derived human NT-MKs vs scuPA-MKs infused in NSG mice was measured 6 hours after infusion of MKs. P-selectin exposure after activation of the non-treated (NT)-human (h) PLTs and uPAT-hPLTs by (TRAP6, 50 µg/mL) was measured in whole mouse blood by Flow cytometry using FITC anti-human CD41 and APC anti–P-selectin monoclonal antibody.

Immunofluorescent staining of megakaryocytes

To stain endogenous or endocytosed unlabeled proteins, megakaryocytes, immobilized on slides, were fixed in 4% paraformaldehyde (PFA) for 15 minutes, permeabilized in PBS/0.1% Triton X-100 (Tx-100), and 1% bovine serum albumin (BSA) blocking solution was added for 1 hour. Von Willebrand Factor (VWF) and/or anti-interferon-induced transmembrane protein (IFITM)-3, uPA and PF4 were detected using rabbit anti-VWF, anti-IFITM-3 polyclonal antibodies, or mouse anti-uPA and anti-PF4 monoclonal antibodies, respectively, rabbit and mouse control antibody, followed by staining with the secondary anti-mouse or anti-rabbit Alexa 488- or Alexa 555-conjugated antibodies.

Confocal studies of uPA-MKs

Megakaryocytes were examined using a confocal laser-scanning microscope (Zeiss LSM 710; Carl Zeiss), equipped with a Plan Apo 40× water-immersion objective lens (NA 1.2). Z-stacking (also known as focus stacking) was used to combine multiple images taken at different focal distances to provide a composite image with a greater depth of field (e.g., the thickness of the plane of focus). The Z-stack distance between the slices was set as 0.3 μm, with a 1024 × 1024 pixel resolution for each slice. Three-dimensional reconstruction and maximal projection were performed using JmageJ software. In most experiments, to quantify co-localization of the signals in different fluorescence channels, the Pearson's Manders overlaps coefficients were calculated using ImageJ software to determine the proportion of signals from scuPA that coincided with signals from uPAT, or signals from scuPA or uPAT that coincided with signals from target markers. Values close to 1 indicate that a large proportion of uPAs signals overlapped with signals from the target markers[4].

Carotid artery thrombosis model

Carotid artery thrombosis was induced by photochemical injury, as described by us[5]. Adult male and female mice were anesthetized with phenobarbital (80 mg/kg), and the right carotid artery and left jugular vein were exposed by blunt dissection. A 3 mW, 540-nm laser beam (green) was applied to the artery from a distance of 5 cm. Rose bengal dye (50 mg/kg body weight) was injected into the left jugular vein and blood flow in the artery was recorded using a small animal blood flow meter (model T106; Transonic Systems) over the ensuing 40 minutes. Time to initial formation of a stable completely occlusive thrombus (occlusion > 10 minutes) was used as the endpoint.

Agonist responsiveness of the released human platelets was done as described for *in vitro*–generated human megakaryocytes, with the exception that it was performed in 100 µl of murine blood obtained by retro-orbital puncture.

Measurement of amount of endocytosed scuPA in megakaryocytes

0.2-1x10^6 MKs, pre-loaded with scuPA (600 nM in medium for 24 hours), as above, were lysed in the corresponding volume (100-500 µl) of the lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 mM PMSF) (Cell Signaling Technology). Protein concentration in the cell lysate was measured using Bradford Protein Assay (Bio-Rad), and scuPA protein concentration in the untreated and scuPA-treated megakaryocyte lysates was measured using the huPA Quantikine® ELISA kit (R&D Systems) per manufacturer.

Supplement References

1. Fuentes RE, Zaitsev S, Ahn HS, et al. A chimeric platelet-targeted urokinase prodrug selectively blocks new thrombus formation. *J Clin Invest*. 2016;126(2):483-494.
2. Ayombil F, Petrillo T, Kim H, Camire RM. Regulation of factor V by the anticoagulant protease activated protein C: Influence of the B-domain and TFPIalpha. *J Biol Chem*. 2022;298(11):102558.
3. Jarocha D, Vo KK, Lyde RB, Hayes V, Camire RM, Poncz M. Enhancing functional platelet release in vivo from in vitro-grown megakaryocytes using small molecule inhibitors. *Blood Adv*. 2018;2(6):597-606.
4. Manders EM, Stap J, Brakenhoff GJ, van Driel R, Aten JA. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. *J Cell Sci*. 1992;103 (Pt 3):857-862.
5. Rauova L, Hirsch JD, Greene TK, et al. Monocyte-bound PF4 in the pathogenesis of heparin-induced thrombocytopenia. *Blood*. 2010;116(23):5021-5031.

Major Resources Tables

Antibodies

Summary of primary antibodies used for immunofluorescence (IF) and immunoblot analysis (WB)

Type	Antigen	Source	Product #/Clone
Rabbit			
	VWF	Dako	A0082
	mouse uPA	Meridian	K63679R
	β-actin HRP	CST	# 5125
	LRP-1	Novus Biologicals	SA0290
	IFITM-3	ProteinTech	CL594-11714
	Total rabbit IgGs	Jackson ImmunoResearch	
Mouse			
	human uPA	IMTEK	
	PLG	SCBT	
	Factor V	Hematologic Technologies	Clone AHV-5146
	P-selectin APC	BD Pharmingen	#550888
	hCD41a APC	BD Pharmingen	#559777
	hCD41a FITC	BD Pharmingen	# 555466
	mCD41 BUV395	BD Pharmingen	#565980
	hCD91(LRP1) BV421	BD Biosciences	#743856
	BV421 Mouse IgG1, k isotype Control	BD Horizon	#562438
	Human PF4	In-house	RTO
	Total mouse IgGs	Jackson ImmunoResearch	# 015-000-003
goat			
	Anti mouse HRP Anti-rabbit HRP	Jackson ImmunoResearch Jackson ImmunoResearch	
recombinant			
	Reo-Pro (Absiximab)	Centocor B.V.	CAS #: 143653-53-6

Recombinant Proteins

Protein	Type	Conjugate	Source
scuPA	recombinant	None Alexa488 Alexa555 Alexa568	In-house
uPAT	recombinant	Alexa488	In-house
Factor V	recombinant	None	In-house

		Alexa568	
ncPLG	recombinant	Alexa647	In-house
PLG	purified	None	Enzyme Research Labs
Fibrinogen	purified	Alexa488	Hyphen/Aniara
FcRAP	recombinant	None Alexa555	In-house

Video 1. Uptake of scuPA *vs*. uPAT by CD34$^+$ megakaryocytes.

Uptake of Alexa Fluor 568-scuPA (red) and Alexa Fluor 488-uPAT (green) by CD34$^+$-MKs. Cells were incubated, fixed and imaged via confocal fluorescence microscopy as described in Figure 2A. Images were collected at multiple focal planes (Z-stacked) with the 0.3 μm interval. This video has 2 parts: First part shows rotation of the virtual 3-D images of the cell around X and Y axes. Second part shows Z-stacked images combined into a brief "real-time" video, using ImageJ software, that allows to explore subcellular distribution of the internalized scuPA and uPAT proteins at every plane of focus.

Video 2. Uptake of scuPA *vs*. FV by CD34$^+$ megakaryocytes.

Uptake of Alexa Fluor 488-uPAT (green) and Alexa Fluor 568-FV (red) by CD34$^+$-MKs. Cells were incubated, fixed and imaged via confocal fluorescence microscopy as described in Figure 2C. Images were collected at multiple focal planes (Z-stacked) with the 0.3 μm interval. This video has 2 parts: First part shows rotation of the virtual 3-D images of the cell around X and Y axes. Second part shows Z-stacked images combined into a brief "real-time" video that allows to explore subcellular distribution of the internalized uPAT and FV proteins at every plane of focus.

Video 3. Uptake of scuPA *vs*. fibrinogen by CD34$^+$ megakaryocytes.

Uptake of scuPA Alexa Fluor 568-scuPA (red) and Alexa Fluor 488-Fibrinogen (green) by CD34$^+$-MKs. Cells were incubated, fixed and imaged via confocal fluorescence microscopy as described in Figure 4A. Images were collected at multiple focal planes (Z-stacked) with the 0.3 μm interval. This video shows Z-stacked images combined into a brief "real-time" video that allows to explore subcellular distribution of the internalized scuPA and Fibrinogen proteins at every plane of focus.

Video 4. Uptake of scuPA *vs*. IFITM3 distribution by CD34$^+$ megakaryocytes.

Cells were incubated, fixed, stained for scuPA (green) and IFITM3 (red) and imaged via confocal fluorescence microscopy as described in Figure 4D. Images were collected at multiple focal planes (Z-stacked) with the 0.3 μm interval. This video shows Z-stacked images combined into a brief "real-time" video that allows to explore subcellular distribution of the internalized scuPA and endogenous IFITM proteins at every plane of focus.

Video 5. Uptake of uPAT *vs*. VWF distribution by CD34$^+$ megakaryocytes.

Cells were incubated, fixed, stained for uPAT (green) and VWF (red) and imaged via confocal fluorescence microscopy as described in Figure 4E and S3. Images were collected at multiple

focal planes (Z-stacked) with the 0.3 μm interval. This video shows Z-stacked images combined into a brief "real-time" video that allows to explore subcellular distribution of the internalized scuPA and endogenous VWF proteins at every plane of focus.

Supplement Figures

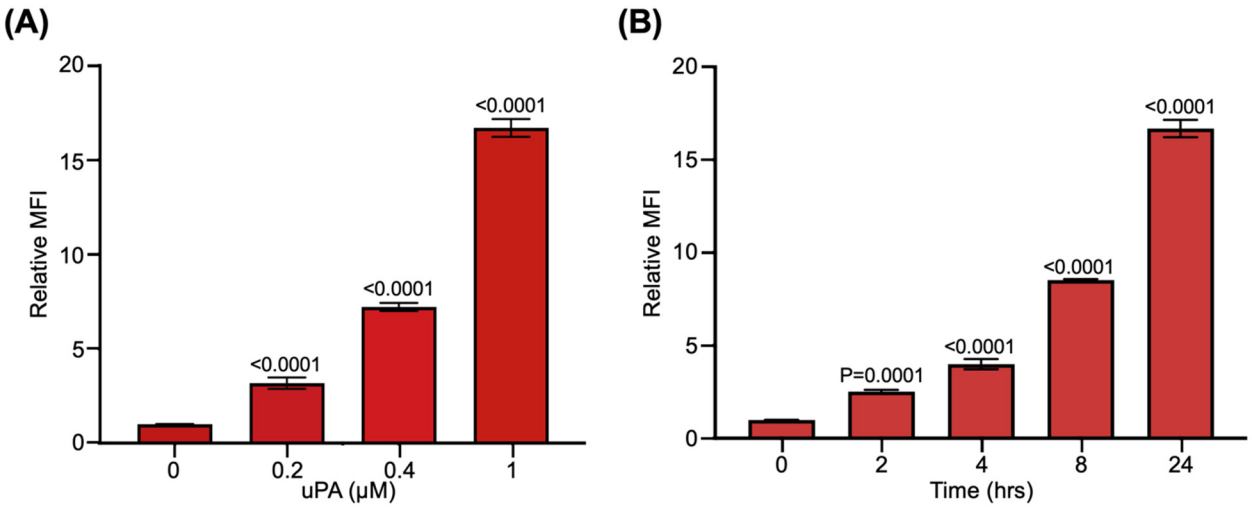

Figure S1. Dose-dependent (A) and time course (B) uptake of Alexa^{488}-uPAT (400 nM) uptake by CD34^{+}-MKs.

Ordinate denote MFI measured by flow cytometry. Mean ± 1 SD are shown for N = 4 independent studies.

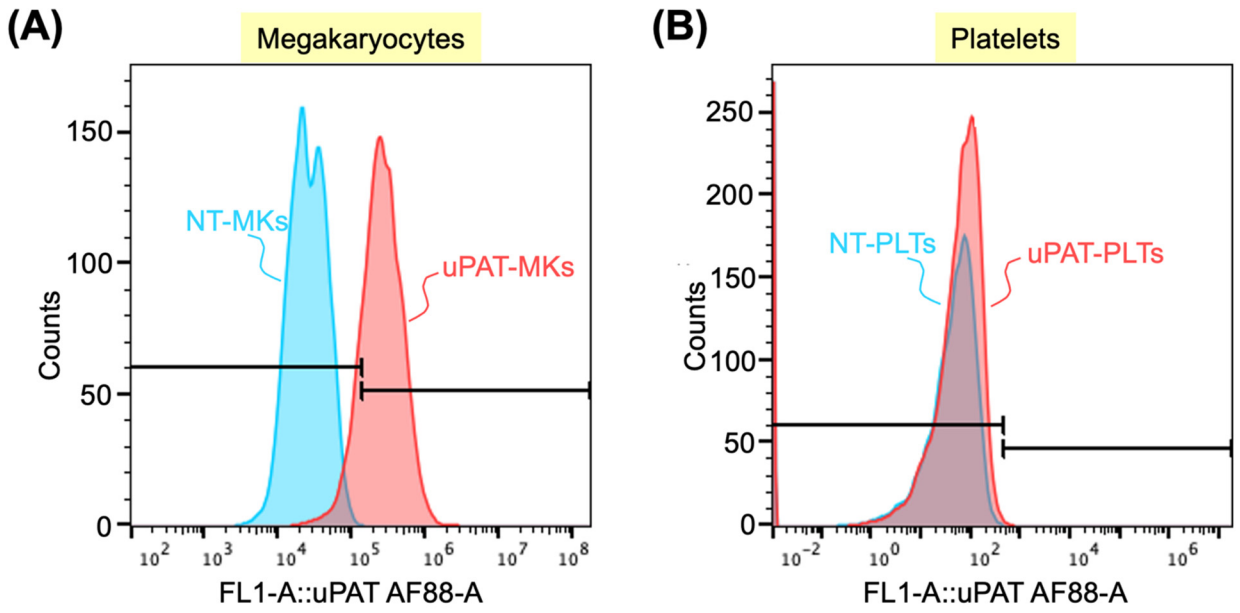

Figure S2. Flow cytometry analysis of endocytosed uPAT by human megakaryocytes *vs*. platelets.

Flow cytometry analysis of endocytosed Alexa^{488}-uPAT (400 nM) by d10 CD34^{+}-MKs (**A**) vs. isolated platelets (**B**). Ordinate denote MFI measured by flow cytometry.

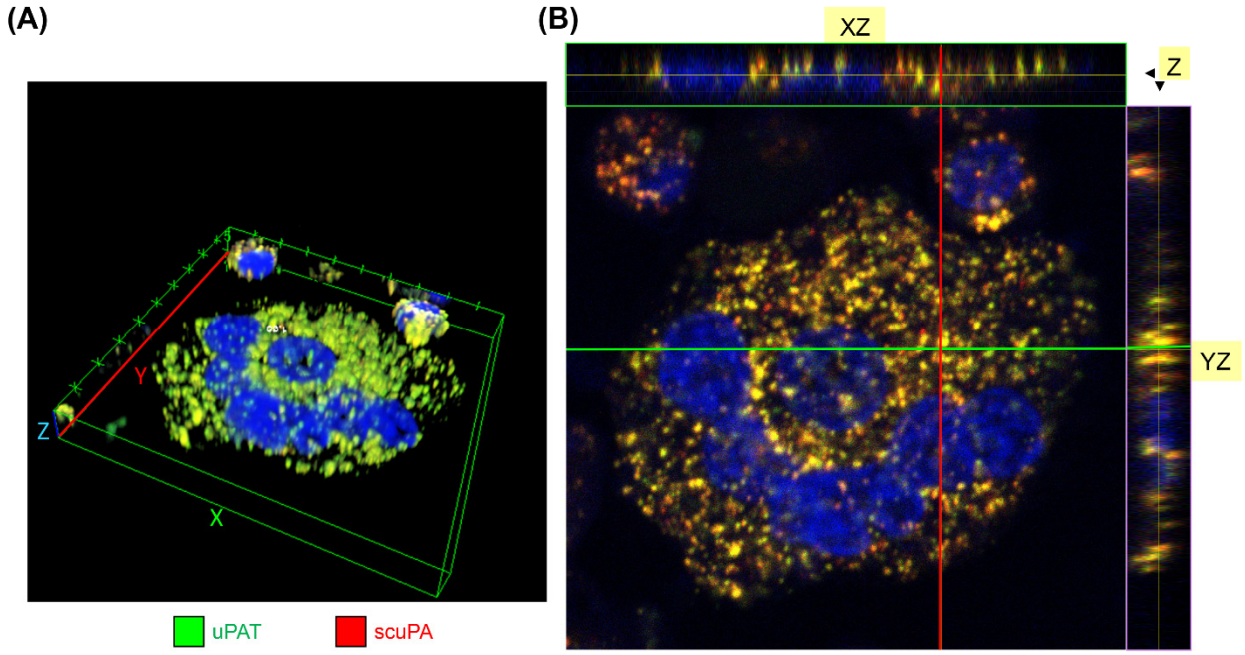

Figure S3. Confocal analysis of uPAT *vs*. scuPA uptake by megakaryocytes.

(**A**) 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded simultaneously with Alexa-568 scuPA (red) and Alexa-488 uPAT (green) for 24 hours, acquired for Figure 2A at different focal depths with Z interval 0.3 µm. (**B**) Orthogonal projections of the virtual 3D image of the cell sectioned along the XZ (green) and YZ planes (red) at fixed Z-position (shown as a yellow lane at the top (XZ) and side (YZ) projection images).

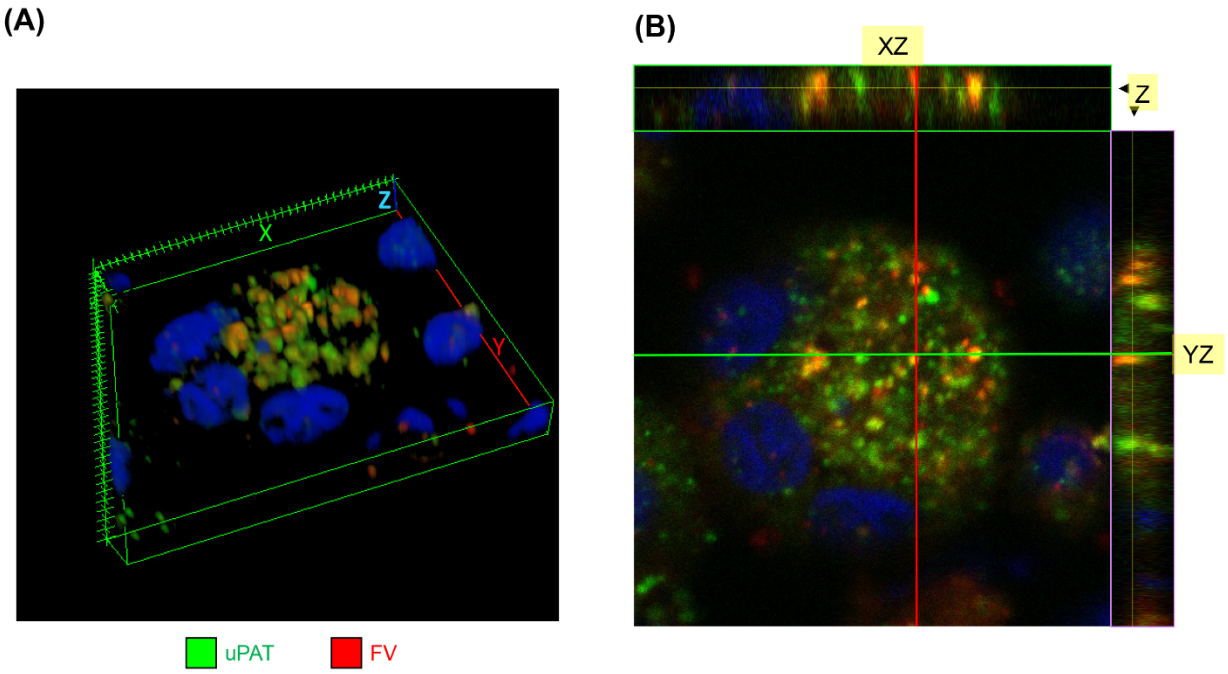

Figure S4. Confocal analysis of uPAT *vs*. FV uptake by megakaryocytes.

(**A**) 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded with Alexa-488 uPA-T (green) and Alexa-568 FV (red), as described in Figure 2C's legend and acquired for Figure 2C at different focal depths with Z interval 0.3 µm (**B**). Orthogonal projections of the virtual 3D image of the cell sectioned along the XZ (green) and YZ planes (red) at fixed Z-position (shown as a yellow lane at the top (XZ) and side (YZ) projection images).

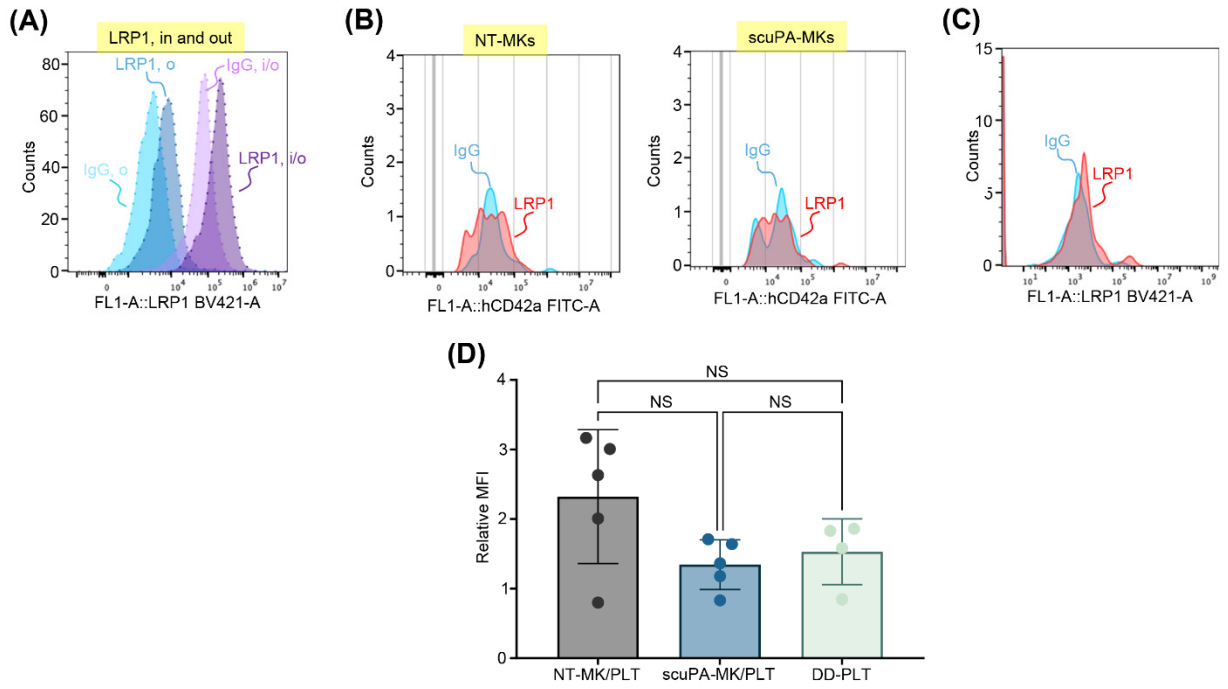

Figure S5. Flow cytometric LRP1 studies of megakaryocytes *vs*. platelets.

(**A**) Flow cytometry analysis of LRP1 on non-permeabilized (out, blue histograms) and permeabilized (in and out, purple histograms) d12-CD34$^+$-MKs using BV421-conjugated anti-LRP1 (dark blue or purple histograms) vs BV421-IgG control (light blue or purple histograms). Y axes denote MFI measured by flow cytometry. (**B**) Representative flow cytometry analysis of LRP1 in whole blood on hCD42a$^+$/hCD42b$^+$ platelets generated *in vivo* from the control (NT, left panel) and scuPA-MKs (right panel) 6 hours post injection in NSG mice, using BV421-conjugated anti-LRP1 (red histogram) and BV421-IgG (blue histogram). (**C**) Representative flow cytometry analysis of LRP1 in whole blood on human donor-derived hCD42a$^+$/hCD42b$^+$ platelets 15 minutes post injection in NSG mice, using BV421-conjugated anti-LRP1 (red histogram) and BV421-IgG (blue histogram). (**D**) Quantification summary of the data obtained in “B” and “C” from N = 5 (B) and N=4 (C) independent experiments.

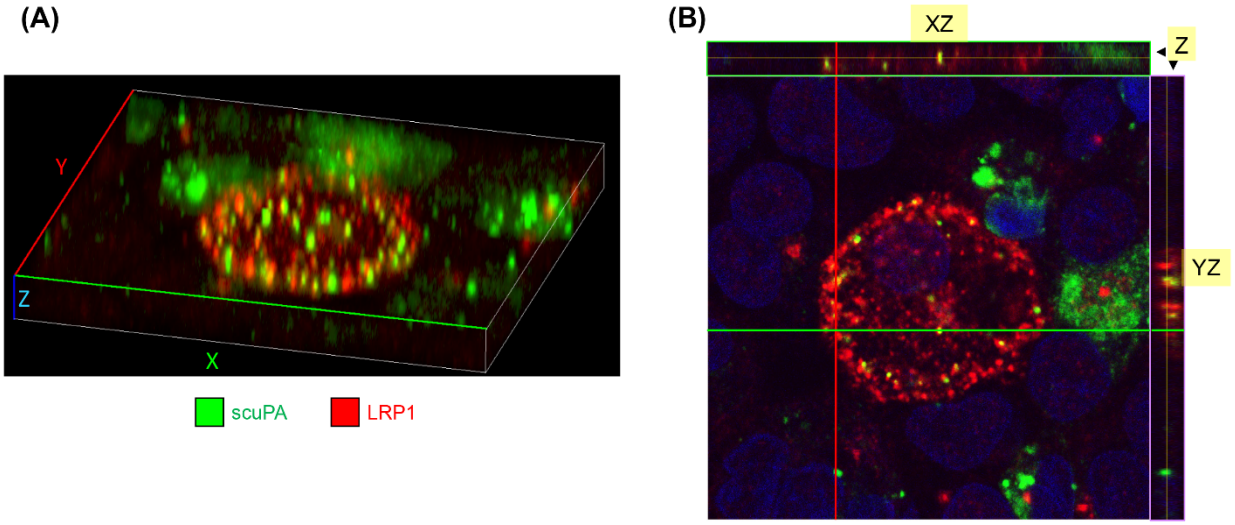

Figure S6. Confocal analysis of scuPA uptake *vs*. LRP1 by megakaryocytes.

(**A**) 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded with Alexa-488 scuPA (green) for 24 hours and stained for LRP1 (red) , as described in Figure 3B's legend and acquired for Figure 3B at different focal depths with Z interval 0.3 µm. (**B**). Orthogonal projections of the virtual 3D image of the cell sectioned along the XZ (green) and YZ planes (red) at fixed Z-position (shown as a yellow lane at the top (XZ) and side (YZ) projection images).

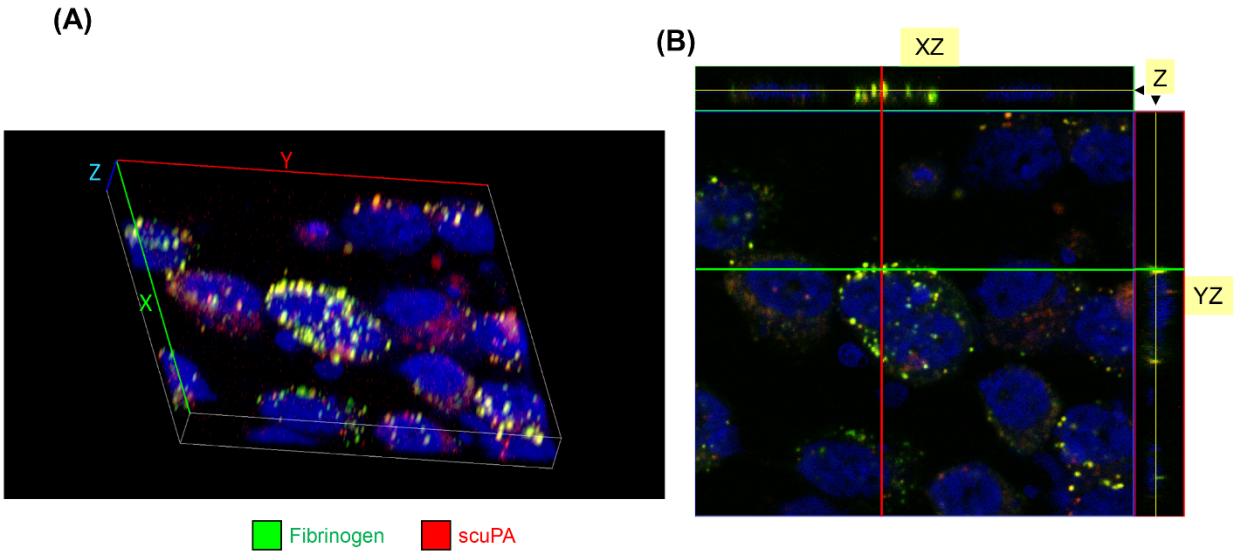

Figure S7. Confocal analysis of scuPA *vs*. fibrinogen uptake by megakaryocytes.

(**A**) 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded with Alexa-488 fibrinogen (green) and Alexa-568 scuPA (red) for 24 hours, acquired for Figure 4A at different focal depths with Z interval 0.3 µm. (**B**) Orthogonal projections of the virtual 3D image of the cell sectioned along the XZ (green) and YZ planes (red) at fixed Z-position (shown as a yellow lane at the top (XZ) and side (YZ) projection images).

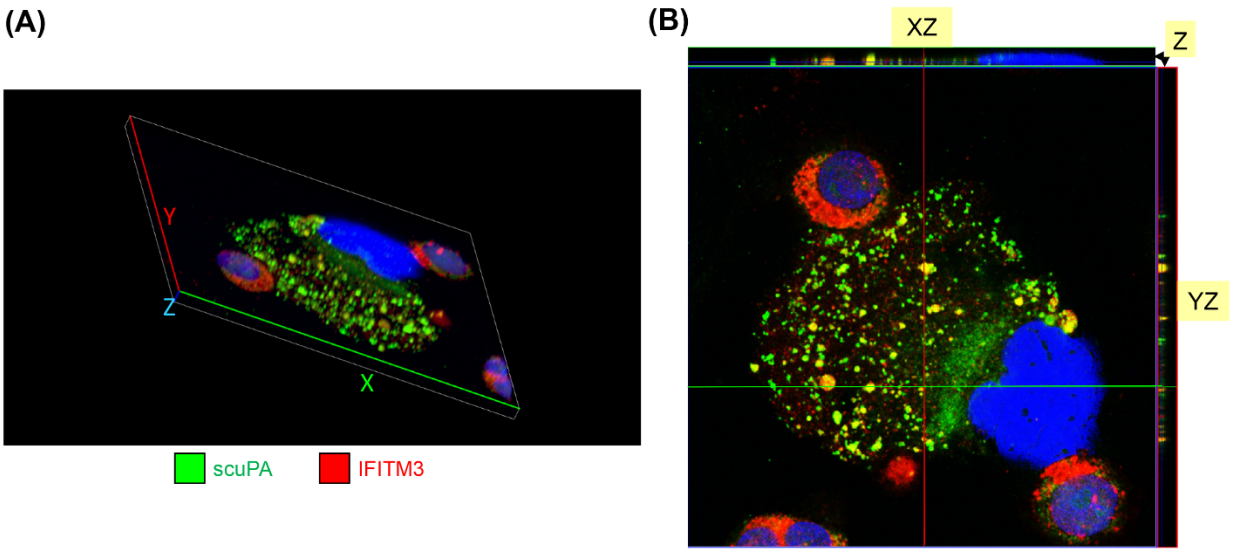

Figure S8. Confocal analysis of scuPA uptake *vs*. IFITM3 in megakaryocytes.

(**A**) 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded with Alexa-488 scuPA (green) for 24 hours and stained for IFITM3 (red) , as described in the legend to Figure 4B and acquired for Figure 4B at different focal depths with Z interval 0.3 µm. (**B**) Orthogonal projections of the virtual 3D image of the cell sectioned along the XZ (green) and YZ planes (red) at fixed Z-position (shown as a yellow lane at the top (XZ) and side (YZ) projection images).

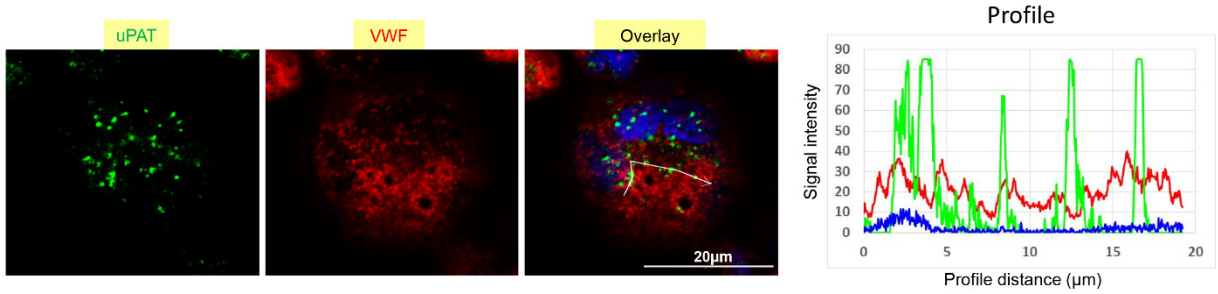

Figure S9. Confocal analysis of uPAT uptake *vs*. VWF by megakaryocytes.

Confocal images of d11-CD34^{+}-MKs preincubated with uPAT (600 nM) for 24 hours starting at d10, fixed with 4% PFA in PBS, permeabilized with 0.1% Triton X-100 and stained with mouse anti-uPA MAbs (IMTEK) and anti-VWF Ab (Dako) followed by Alexa 488 goat anti-mouse Ab (green) and Alexa 647 goat anti-rabbit Ab (red) and DAPI nuclear stain (blue). Scale Bar = 20 µm. White segmented line in overlay image represents profile along which the intensity of the fluorescence signal in each channel was measured using the ImageJ software. The plotted profile is presented in right panel. Abscissa indicates the length of the profile in µm. Ordinate indicates relative fluorescence intensity.

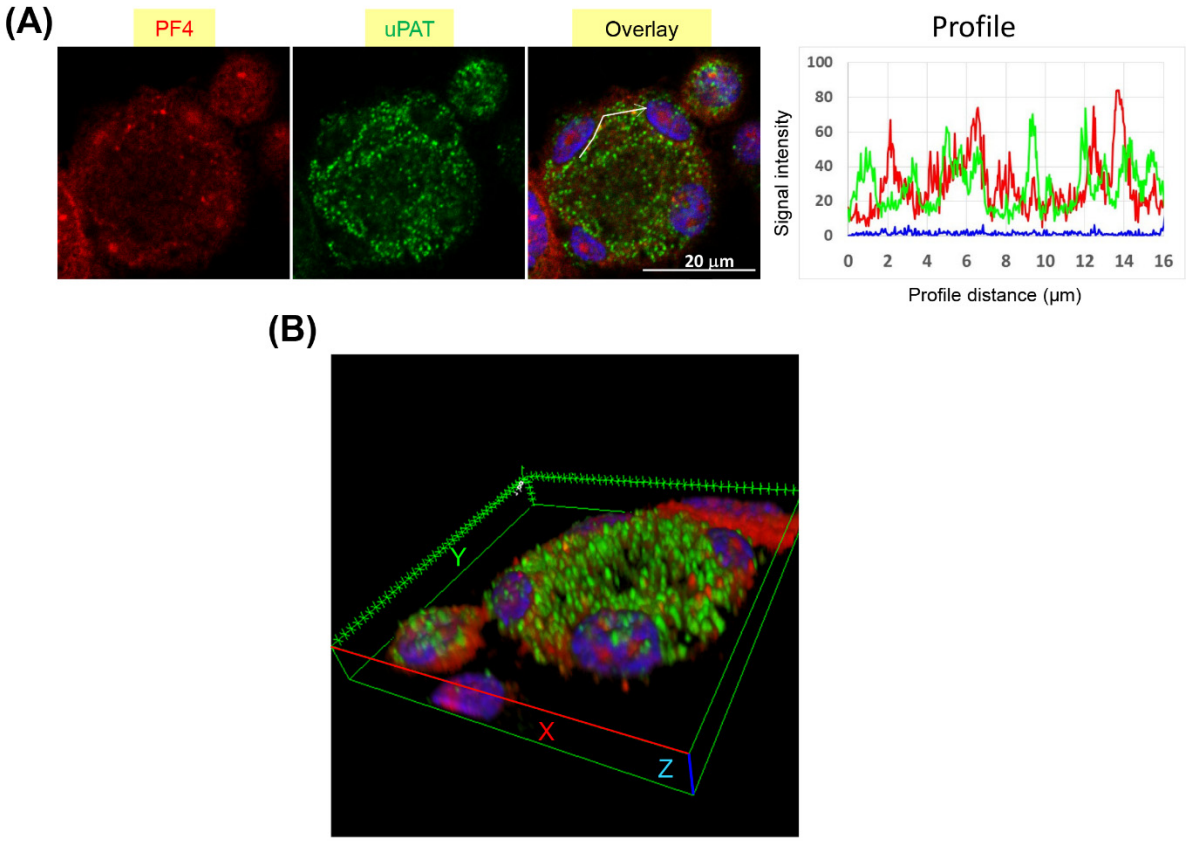

Figure S10. Confocal analysis of uPAT uptake *vs*. PF4 by megakaryocytes.

(**A**) Confocal images of d11-CD34^{+}-MKs preincubated with uPAT (600 nM) for 24 hours starting at d10, fixed with 4% PFA in PBS, permeabilized with 0.1% Triton X-100 and stained with mouse Alexa 488-conjugated anti-uPA MAbs (IMTEK)) and Alexa 568-conjugated anti-PF4 antibody and DAPI nuclear stain (blue). Scale Bar = 20 µm. White segmented line in overlay image represents profile along which the intensity of the fluorescence signal in each channel was measured using the ImageJ software. The plotted profile is presented in right panel. Abscissa indicates the length of the profile in µm, ordinate indicates relative fluorescence intensity. (B) 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded with Alexa-488 scuPA (green) for 24 hours and stained for PF4 (red), and acquired at different focal depths with Z interval 0.3 µm.

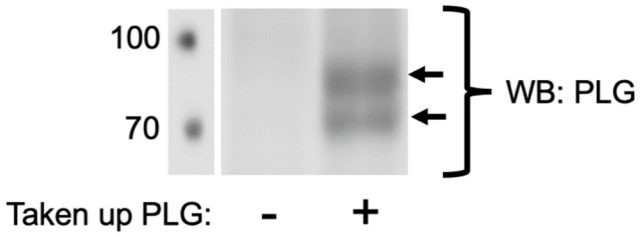

Figure S11. WB analysis of PLG by megakaryocytes.

WB analysis of lysates prepared from d11-CD34^{+}-Mks with or without loading with enzymatically active PLG (20 μg/ml) on d10 for 18 hours. WB membranes were probed with mouse monoclonal antibody recognizing PLG and HRP-conjugated anti-β-actin rabbit polyclonal antibody .

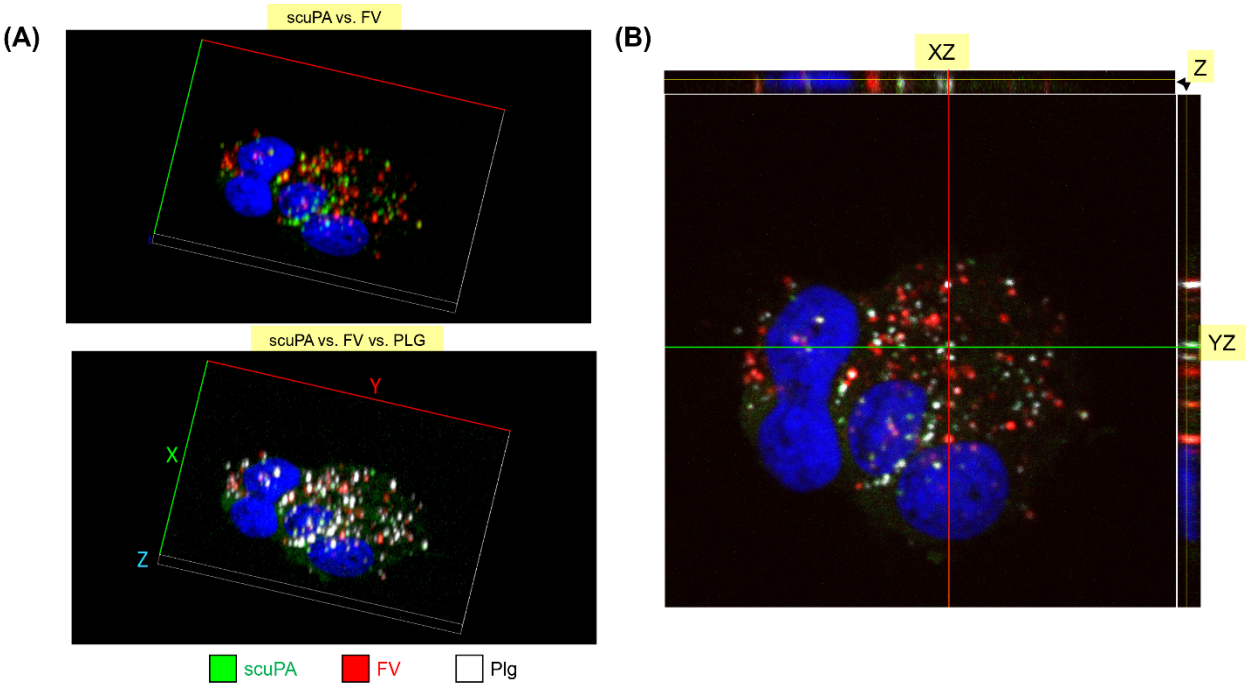

Figure S12. Confocal analysis of uPAT vs. FV *vs*. PLG uptake by megakaryocytes. (A) On the top, 3D reconstitution of the serial images (Z-stack) of d10-CD34^{+}-MKs, loaded with Alexa-488 uPAT (green) and Alexa-568 FV (red), as described in the legend to Figure 5A**,** and acquired for Figure 5A at different focal depths with Z interval 0.3 µm. On the bottom, same 3D reconstitution of the serial images (Z-stack) of d10 CD34^{+}-MKs, loaded with Alexa-488 uPAT (green), Alexa-568 FV (red) and Alexa 647-PLG (pseudo-colored in white), as described in the legend to Figure 5A**,** and acquired for Figure 5A at different focal depths with Z interval 0.3 µm. **(B)** Orthogonal projections of the virtual 3D image of the cell sectioned along the XZ (green) and YZ planes (red) at fixed z-position (shown as a yellow lane at the top (XZ) and side (YZ) projection images).