SUPPLEMENTARY APPENDIX

Loss of ATE1-mediated arginylation leads to impaired platelet myosin phosphorylation, clot retraction, and *in vivo* thrombosis formation

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Supplemental Table 1

Accession	Description	Arginylated site	Peptide	XCorr	DeltaCN	MPlusH	CalcMPlusH	DeltaMass	SpScore	p-value	Confidence	FileName
NP_075631.2	actin related protein 2/3 complex, subunit 1B	S343*	C.(170.1168)SQFCTTGMDGGMSIWDVK.S	4.6734	0.4732	2191.979	2189.9778	-2.7	7.966751	0.413215085	100	080910WTPMA-11.367.367.2
NP_598917.1	actinin, alpha 1	I835	K.(156.1011)ILAGDKNYITEDELR.R	4.558	0.2841	1906.007	1906.0028	1.9	4.7120957	0.968489683	100	platelet-5.3905.3905.2
NP_031466.2	arachidonate 12-lipoxygenase	L311	K.(156.1011)LLPMAIQIQPPNPSSPAPTLFLPSDPPLAWLLAK.I	5.914	0.4923	3793.123	3790.1128	0	8.241585	0.313875145	100	platelet-16.12430.12430.3
NP_862897.1	fibrinogen beta chain	H449	K.(156.1011)HGTDDGVVWMNWK.G	3.8005	0.2859	1701.796	1700.7961	-2	6.0415154	0.67659321	100	080910WTPMA-12.6464.6464.2
NP_034357.2	filamin, alpha	P2143*	A.(170.1168)PSVANIGSHCDLSLKIPEISIQDMTAQVTSPSGK.T	4.4954	0.3566	3751.932	3750.9153	3.6	6.351275	0.408654758	99.8	platelet-11.7291.7291.4
		Y2493	K.(156.1011)YGGPYHIGGSPFK.A	3.2011	0.2789	1537.79	1535.7754	4.9	4.946237	0.136666137	100	platelet-14.3736.3736.2
		F2303	K.(156.1011)FNEEHIPDSPFVVPVASPSGDAR.R	5.1615	0.3957	2624.299	2623.29	2	6.256327	0.990884745	100	platelet-6.6267.6267.3
NP_032063.2	fos-like antigen 2	Y26	S.(156.1011)YSSGGGGQQKFRVDMPGSGSAFIPTINAITTSQDLQWMVQP.T	4.9101	0.3059	4516.206	4513.2026	-1.6	4.6043887	0.06846069	100	platelet-3.15819.15819.4
NP_031464.1	fructose-bisphosphate aldolase A	F244	K.(156.1011)FSNEEIAMATVTALR.R	6.2268	0.4164	1809.93	1808.9324	-2.9	7.0414934	0.350893061	100	080910WTPMA-10.5603.5603.2
NP_032138.3	guanosine diphosphate (GDP) dissociation inhibitor 2	V30	K.(156.1011)VLHMDQNPYYGGESASITPLEDLYK.R	7.1309	0.5348	2997.454	2996.4458	1.5	8.937426	0.807840624	100	platelet-6.6825.6825.3
NP_071705.2	heat shock protein 5	N83	K.(156.1011)NQLTSNPENTVFDAK.R	3.3106	0.4132	1833.903	1833.9089	-3.5	6.63242	0.201712464	100	080910WTPMA-6.3865.3865.2
NP_032244.2	hemoglobin alpha 1 chain	V63	K.(156.1011)VADALANAAGHLDDLPGALSALSDLHAHK.L	4.6374	0.3083	3020.58	3019.5708	1.9	5.5295057	0.968489683	100	platelet-10.8457.8457.3
NP_444322.1	lysyl-tRNA synthetase isoform 2	I369	K.(156.1011)ITYHPDGPEGQAYEVDFTPPFR.R	6.1081	0.5286	2692.294	2692.279	5.4	8.577643	0.081341053	100	platelet-8.5406.5406.3
NP_899099.2	mKIAA0540 protein	P352*	P.(170.1168)PEGDSDLATWLLTEPDVQK.V	4.8655	0.3309	2288.155	2284.1455	-1.8	6.266108	0.762265694	99.9	080910WTPMA-2.12692.12692.2
NP_071855.2	myosin, heavy polypeptide 9, non-muscle isoform 1	L1844	K.(156.1011)LKDVLLQVEDER.R	2.3485	0.2307	1613.903	1612.9016	-1.2	4.2675695	0.967039588	98.8	080910WTPMA-11.3375.3375.2
NP_034990.1	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	V64	K.(156.1011)VLDFEHFLPMLQTVAK.N	4.214	0.2669	2046.117	2044.1049	2.8	5.586929	0.675329762	100	platelet-10.9286.9286.3
XP_001480531.1	PREDICTED: similar to GTP-binding protein (smg p21B)	E45*	V.(170.1168)EVDAQQCMLEILDTAGTEQFTAMR.D	6.0396	0.3557	2930.375	2927.3696	-1.5	6.508986	0.076554821	99.8	platelet-8.11171.11171.3
NP_035202.1	profilin 1	T39	K.(156.1011)TFVSITPAEVGVLVGK.D	4.4067	0.3889	1775.037	1773.0269	1.9	6.8232403	0.968489683	100	platelet-7.6656.6656.3
NP_033051.2	RAS p21 protein activator 3	Q782	K.(156.1011)QVIAGVGTLEQEHAQYR.R	6.9754	0.5147	2055.077	2055.073	1.9	8.978428	0.968489683	100	platelet-8.2743.2743.3
NP_789807.1	Rho GTPase activating protein 18	N632*	G.(170.1168)NIGERCLDDDTHMKDLYQLNPNAEWVIK.S	5.9448	0.1416	3559.735	3557.7263	0.7	5.4416122	0.515284027	99.8	platelet-5.7800.7800.3
NP_443205.1	striatin, calmodulin binding protein 3	A781	K.(156.1011)AYIASAGADALAK.V	3.4799	0.3743	1377.744	1377.7485	-3.5	6.4240017	0.201712464	100	080910WTPMA-9.409.409.2
NP_035710.2	thrombospondin 1	G574*	C.(170.1168)GACPPGYSGNGIQCK.D	4.1003	0.4045	1737.802	1735.8003	-2.8	6.832743	0.381272777	99.9	080910WTPMA-6.12653.12653.2
NP_035707.1	transforming growth factor, beta 1	L342*	A.(170.1168)LYNQHNPGASASPCCVPQALEPLPIVYYVGR.K	5.1112	0.3979	3644.837	3640.8152	2.2	6.256313	0.90980918	99.8	platelet-7.9658.9658.3
NP_659054.1	transmembrane protein 40	E2*	M.(170.1168)EASGSSSQSQDSGGVHR.E	2.654	0.2975	1847.847	1845.8434	-1.8	4.9107823	0.762265694	99.6	080910WTPMA-2.5851.5851.2
		G111	G.(156.1011)GAAGEMVPTGESGLR.R	3.8962	0.3918	1588.793	1587.7908	-0.7	6.8197827	0.743028266	99.9	080910WTPMA-6.3325.3325.2
NP_058659.1	tropomodulin 3	I173	K.(156.1011)ILPVFDEPPNPTNVEESLK.R	6.3407	0.4739	2297.212	2294.2026	-0.2	8.954529	0.538971428	100	080910WTPMA-8.8264.8264.2
NP_789830.1	ubiquitin associated and SH3 domain containing, B	S601*	C.(170.1168)SCEELGETGIWQLTDPPILPLTHGPTGGFNWR.E	4.3971	0.3945	3751.857	3748.854	-1.8	6.7648215	0.054377593	99.9	platelet-13.14671.14671.3
NP_035838.3	von Willebrand factor	V1409	K.(156.1011)VIVIPVGIGPHASLK.Q	3.5052	0.3164	1656.041	1656.0319	5.3	5.3865337	0.090640178	100	platelet-15.5003.5003.3

*Monomethylated Arg

Supplemental Methods (online only)

Reagents and Antibodies

Reagents: human fibrinogen (Enzyme Research Laboratories, #FIB3), prostaglandin E1 (PGE1) (Cayman Chemicals, #13010), collagen (Chrono-log, #385), human α thrombin (kindly provided by Dr. Sriram Krishnaswamy, with the activity of 3000U per mg), protease inhibitor cocktail (Roche, San Francisco, PA), Convulxin (Enzo Life Sciences, Plymouth Meeting, PA), Fluor-phalloidin (Life Technology, Grand Island, NY), rProtein G agarose (life technologies, Cat# 15920-010), Anti-non-muscle Myosin IIA antibody (Abcam, Cat# ab55456), and the reagents for plasma coagulation assays (Trinity Biotech, Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO.)

Antibodies: anti-ATE1 (12, 18) and anti-β-actin (Cell Signaling, Cat. #4870), anti-phospho-myosin light chain ($pSer^{19}$) (Sigma-Aldrich, St. Louis, MO), anti-phospho-myosin light chain ($pThr^{18}$) (Santa Cruz Biotechnology, CA), anti-myosin light chain (Sigma-Aldrich, St. Louis, MO), and anti-myosin IIA (Sigma-Aldrich, St. Louis, MO).

Genotyping

The PCR primers used to genotype for the ATE1 deletion were LoxF 5' TGC CTC CAG CAT TGG ATG AA-3' and LoxR 5'- CCA TGG GTC TCC AAT TTG CA-3', or FrtR2 5'- AGA CAG GGC CTC ATC AAG TA -3'. The PCR primers used to genotype for the PF4-Cre strain were the forward primer, located at the PF4 promoter (5'- CCC

ATA CAG CAC ACC TTT TG-3') and the reverse primer, located at the Cre cDNA sequence (5'- TGC TAC AGT CAG CAG GTT-3').

Platelet Aggregation And Spreading

Platelet aggregometry was performed as previously described (19). For studies of platelet spreading, glass coverslips were coated with either 100 μg/ml fibrinogen or 30 μg/ml collagen type I in tissue culture plates at 4^{o} C overnight. The coverslips were washed three times with PBS and coated with 5% BSA at room temperature for 2 hours following two washes with PBS. Washed platelets were loaded onto the coverslip. The platelets were spread upon collagen or fibrinogen in the presence of either 1 U/ml thrombin or 500 nM PMA. Cell spreading was stopped by adding paraformaldehyde to a final concentration of 3.7% and then, permeabilized with 0.1% Triton X-100. The cells were stained with Alexa Fluor ® phalloidin and probed with antibodies directed against β or γ - actin. The static platelet spreading images were taken by using the Nikon Image System and IP Lab Software. The area of cell spreading and the quantification of total F-actin and β or γ – actin were analyzed with NIH Image J software. Platelet adhesion was performed as described by Eriksson and colleagues (7).

Clot retraction

Platelet rich plasma was prepared by collecting blood with 1:10 3.8% sodium citrate via the vena cava and followed by spinning the blood at 200 g for 7 minutes. The platelets were pelleted by spinning the PRP at 1000 g for 10 minutes, while the

platelet poor plasma supernatant was reserved for resuspending platelets and adjusting platelet density to 3 X 10^8 cells/ml. The platelet suspension was supplemented with 1mM $CaCl_2$ and incubated at 37^o C for 5 minutes in a water bath without shaking. Clot retraction took place by adding 10 U/ml of human thrombin followed by a gentle mix and incubation in a 37^o C water bath. The clot retraction was photographed at several time points during the full observation time. The percentage of clot retraction was quantified based on the 2D-image by using NIH Image J Software.

Carotid Artery Thrombosis Induced By $FeCl_3$

Six to eight week old mice (18-25 g) were used for these studies. This procedure has been previously described (22, 23).

Plasma Coagulation Assays

Mouse citrated platelet poor plasma (PPP) was prepared from blood collected via the vena cava with 3.8% sodium citrate and separated by centrifugation at 800g for 20 minutes at room temperature. Prothrombin time (PT) and activated partial thromboplastin (aPTT) assays were performed using 50 µl PPP diluted in buffer (10mM Tris, 150mM NaCl, 0.01% BSA) at 1:1, mixed with the TriniClot PT excel reagents for the PT assay or with the TriniClot automatic aPTT for the aPTT assay by following the manufacturer's instructions. PT or aPTT time was measured at 37^{o}C with the Diagnostica Stago STart 4 Hemostasis Analyzer.