DUSP3 Phosphatase Deficiency or Inhibition Limit Platelet Activation and Arterial Thrombosis

Musumeci: DUSP3, a new player in arterial thrombosis

Supplementary material

Methods and reagents

Antibodies, reagents, and recombinant PTPs

Fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin, and phycoerythrin (PE)-conjugated anti-active integrin $\alpha_{IIb}\beta_3$ (JON/A) antibodies were from Emfret Analytics (Würzburg, Germany). Anti-Fyn, anti-phosphotyrosine antibody (4G10) and anti-FcRγ subunit and mouse anti-rabbit light chain specific-HRP were from Millipore (Billecrica, MA). Antibodies against Syk, Syk p-Tyr 525/526, Syk p-Tyr 323, Lyn p-Tyr-507, ERK (p44/42), p-ERK1/2 (Thr202/Tyr204), p38 MAPK, and p-p38 (Thr180/Tyr182) were from Cell Signaling (Danvers, MA). Anti-Src p-Tyr-416/418, anti-Src p-Tyr-529, and anti-Src pan antibodies were from Fisher Scientific (Erembodegem, Belgium). Anti-Fyn p-Tyr-530 was from Abcam (Cambridge, UK). Anti-Lyn and anti-DUSP3/VHR used for mice samples (sc-8889) were from Santa-Cruz (Santa Cruz, CA). Anti-DUSP3/VHR antibody used for human samples (Clone 24/VHR), FITC-conjugated anti-CD3, PE-conjugated anti-B220, APC-Cy7-conjugated anti-Ly6G, PerCP-Cy5-conjugated anti-NK1.1 and Alexa-647 conjugated anti-rat antibodies were from BD Biosciences (Erembodegem, Belgium). Anti-CLEC2 antibody (clone 17D9) was from Serotec (Puchheim, Germany). Anti-vWF antibody was from Dako (Heverlee, Belgium). Goat anti-mouse kappa HRP-conjugated was from Southern Biotech (Birmingham, AL).

D-Phe-Pro-Ala-chloromethylketone (PPACK) was from Calbiochem (San Diego, CA). Fibrillar-type I equine tendon collagen was from Nycomed (Zurich, Switzerland). Bovine thrombin, ADP, and U46619 were from Sigma-Aldrich (Diegem, Belgium). Cross-linked collagen-related peptide (CRP) was provided by Prof. R.W. Farndale's laboratory. Rhodocytin was purified from *C. rhodostoma* venom as described previously. [1] Annexin A5 labeled with Oregon Green OG488 and Fura-2 were from Molecular Probes (Leiden, the Netherlands). Convulxin was obtained from Kordia (Leiden, the Netherlands).

Para-nitrophenyl phosphate (pNPP), 3-O-methylfluorescein phosphate (OMFP), and dithiothreitol (DTT), and sodium orthovanadate (Na_3VO_4) were purchased from Sigma-Aldrich. Biomol Green reagent was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Compounds for follow-up studies were purchased from Specs or ChemBridge. All compounds had a purity of >95% (verified by LC/MS and 1H-NMR). Compounds chosen for cell-based assays were additionally repurified to >99% purity, and activity of the repurified substance was confirmed. All other chemicals and reagents were of the highest grade commercially available. Recombinant DUSP3, DUSP6, DUSP22, HePTP, LYP, PTP-SL, and STEP were expressed in *E. coli* and purified as described previously. [2-4] Recombinant CD45, TCPTP, LAR, and PTP1B were from Biomol Research Laboratories, Inc (Plymouth Meeting, PA, USA).

Platelet RNA sampling and Microarray

Platelet rich plasma (PRP) was prepared from citrate anticoagulated-blood. Depletion of CD45+ leukocytes was performed before total RNA extraction from freshly purified platelets using RNeasy Mini Kit on a QIAcube (Qiagen, Venlo, The Netherlands) and stored at -80°C until used. RNA was quantified by absorbance measurement, and 200 ng of RNA were engaged in reverse transcription with oligo-dT primers (Superscript III RT, Invitrogen), prior to biotin labeling and amplification using the TargetAmp Nano-g Biotin-aRNA Labeling Kit for the Illumina System (Epicentre). Biotin-labeled aRNA were purified using the RNeasy MinElute Cleanup Kit (Qiagen) and 400 ng were hybridized on Human HT-12 v4 arrays (Illumina) following the recommendations of the manufacturer. Arrays were scanned on an iScan microarray scanner (Illumina). Internal controls of the arrays were analyzed for quality control. Cell-specific expression markers were analyzed in all samples, ruling out contamination of platelet RNA with leukocyte RNA. Indeed, in this assay, comparison of the different DSPs mRNA levels is not possible. The raw fluorescence intensities for the probes

corresponding to the atypical DSPs have been corrected for the fluorescence background signal for each sample on the array by subtracting the fluorescence intensity of the negative control probes on the array. The data have then been normalized by dividing the intensity for each probe in each sample by the mean fluorescence intensity of 7 housekeeping genes (EEF1A1, UBC, ACTB, RPS9, GAPDH, TUBB2A and TXN) in the same sample. The data are presented as a ratio of the fluorescence intensity for the probe of interest and the mean fluorescence intensity for the housekeeping genes of each sample. A negative value corresponds to a lack of detection of the expression of the gene (expression level below the background).

Chemical Library Screening for DUSP3 inhibitors

DUSP3 HTS was performed within the MLPCN network, PubChem AID 1654. A total of 291,018 compounds (comprising the full MLPCN library at the time of screening) were screened at a concentration of 13.3 μM. A colorimetric phosphatase assay was set up in 1536-well format, using the general phosphatase substrate pNPP.[5, 6] The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. A detailed protocol of the HTS assay was published previously.[7]

Single-concentration confirmatory assays for DUSP3 hits using OMFP.

Phosphatase activity was measured in triplicate in a 1536-well format assay system, using the fluoresceine-based phosphatase substrate OMFP. The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. For a detailed protocol please see ref. 7.

Selectivity profiling assays.

Selectivity of compounds for inhibiting DUSP3 was tested against 10 additional PTPs using a 96-well format dose-response assay system with OMFP as substrate.[2] Enzyme concentrations were as follows: DUSP3, 2 nM; DUSP6, 10 nM; DUSP22, 10 nM; PTP-SL, 5 nM; HePTP, 5 nM; LYP, 5 nM; TCPTP, 2 nM; CD45, 2 nM; LAR, 1U/mL; STEP, 5 nM;

and PTP1B, 5 nM. OMFP was used at concentrations equal to the corresponding Km values: DUSP3, 13 μM; DUSP6, 50 μM; DUSP22, 2.2 μM; PTP-SL, 28 μM; HePTP, 117 μM; LYP, 185 μM; TCPTP, 56 μM; CD45, 347 μM; LAR, 78 μM; STEP, 32 μM; and PTP1B, 99 μM. The initial rate was determined using a FLx800 micro plate reader (Bio-Tek Instruments, Inc.), an excitation wave length of 485 nm and measuring the emission of the fluorescent reaction product 3-O-methylfluorescein at 525 nm. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without addition of enzyme. IC_{50} values for each enzyme were determined as described previously.[2]

References

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Tautz. Inhibition of the Hematopoietic Protein Tyrosine Phosphatase by Phenoxyacetic Acids. *ACS Med Chem Lett.* 2011;2:113-118.

Figure Legends

Figure S1. *Surface expression of major platelet receptors on Dusp3-deficient platelets*. Resting WT and *Dusp3*-KO platelets were stained with (**A**) anti-GPVI-FITC, (**B**) anti-CD41-FITC, (**C**) anti-CD42C/GPIb-FITC, and (**D**) anti-CD42D/GPV-FITC and analyzed by flow cytometry. Anti-mouse IgG-FITC (grey line) was used as a negative control antibody for the staining. Representative histograms with the mean fluorescence intensity (MFI) for WT (dark line) and *Dusp3*-KO (dashed line) are shown for each staining.

Figure S2. *CLEC-2 surface expression in platelets and mononucleated cells.* (**A**) Resting WP from WT and *Dusp3*-KO platelets were stained with anti-CLEC-2 antibody followed by a secondary staining using Alexa-647 conjugated anti-rat antibody and FITC-conjugated anti-CD41. A rat-anti-mouse antibody was used as a negative control (grey line). (**B**) Resting spleenocytes from WT and *Dusp3*-KO mice were stained using PE-conjugated anti-B220, FITC-conjugated anti-CD3, APC-Cy7-conjugated anti-Ly6G, PerCP-Cy5-conjugated anti-NK1.1 and anti-CLEC-2 followed by Alexa-647 conjugated anti-rat antibody. $CD3^+$ (T lymphocytes), $B220^+$ (B Lymphocytes), $Ly6G^+$ (Neutrophils) and $NK1.1^+$ (NK cells) were separately gated out of total live cells and analyzed for the expression of CLEC-2. Representative histograms with the % of Max of the mean fluorescence intensity for WT (dark line) and *Dusp3*-KO (grey line) are shown for each staining.

Figure S3. *MAPKs activation in Dusp3-KO platelets*. Total cell lysates (TCLs) were prepared from CRP (0.3 μg/mL) activated WT or *Dusp3*-KO mouse platelets. Cells were non-activated or activated for 30, 90, and 300 s (for MAPKs) or 30, 60, and 90 s (for SFKs) with CRP. Equal amounts of protein were resolved by SDS-PAGE, and western blot analysis was performed using: Anti-phospho-ERK1/2 (Thr202/Tyr204), anti-JNK1/2 (Thr183/Tyr185), and anti-phospho-p38 (Thr180/Tyr182). Anti-ERK1/2, anti-JNK1/2, and anti-p38 were used as loading controls.

Figure S4. *Quantification of Syk, FcRγ, and PLCγ tyrosine phosphorylation and recruitment of Syk to FcRγ.* Densitometric analysis of results presented in Figure 4 for tyrosine phosphorylation of immunoprecipitated Syk (Figure 4D and 4E) in CRP-activated conditions (**A**) and in rhodocytin stimulated conditions (**B**). Quantification of Syk phosphorylation on Tyr-323 and Tyr-525/526 (shown in Figure 4F and 4G) in CRP (**C-D**) or rhodocytin (**E-F**) activated platelets. Normalization was performed using total Syk. (**G**) Quantification of tyrosine phosphorylation (4G10) western blots on FcRγ immunoprecipitates. (**H**) Quantification of Syk recruitement to FcγR. (**I-J**) Statistical analysis of tyrosine phosphorylation (4G10) of PLCγ2 immunoprecipitates from equal amounts of TCLs from CRP (**I**) or rhodocytin (**J**) activated platelets. Data were analyzed using Anova Bonteferroni multiple comparison test and are presented as mean ± SEM. Statistical analyses are shown for three independent experiments, each experiment was performed using pooled platelets from three mice.

Figure S5. *SFK activation in Dusp3-KO platelets*. Total cell lysates (TCLs) were prepared from CRP (0.3 μg/mL) or rhodocytin (10nM) activated WT or *Dusp3*-KO mouse platelets. Cells were non-activated or activated for 30, 60, and 90 s with CRP (**A**) or with rhodocytin (**B**). Equal amounts of protein were resolved by SDS-PAGE, and western blot analysis was performed using: Anti-phospho-Src (Tyr416), anti-phospho-Src (Tyr529), anti-phospho-Fyn (Tyr530), or anti-phospho-Lyn (Tyr507). Anti-Src and anti-Fyn were used as loading controls. Data were analyzed using Anova Bonteferroni multiple comparison test and are presented as mean ± SEM. Results are representative of three independent experiments.

Figure S6. *Thapsigargin induced store mediated Ca^{2+} entry and GPCR agonist-triggered intracellular Ca^{2+} increase in Dusp3-KO platelets*. (**A**) Fura-2 loaded platelets were stimulated with thapsigargin (200 nM) before adding 500 mM $CaCl_2$. (**B-D**) Platelets were stimulated with thrombin (IIA, 10 nM) (B), ADP (20 mM) (C), or TXA_2 mimetic U46619 (1

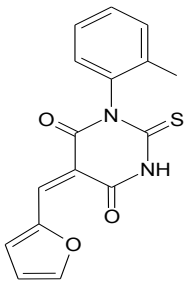
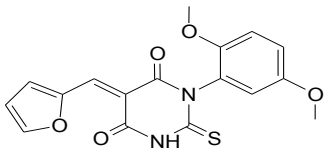
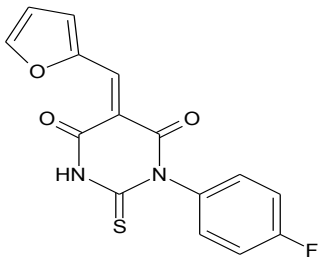
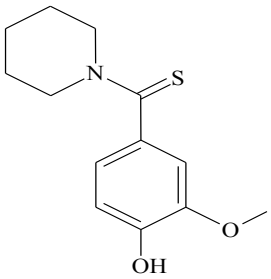
mM) (D). Traces are representative of three independent experiments.

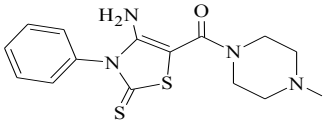
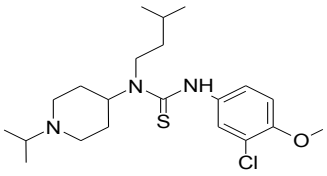
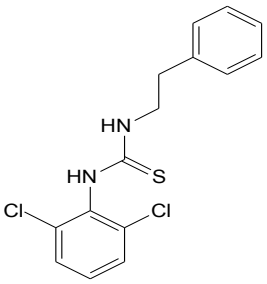
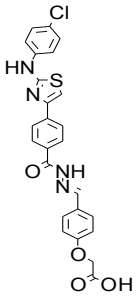
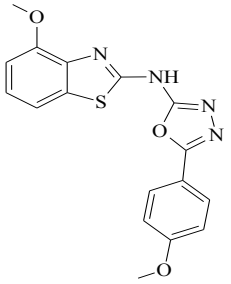
Figure S7. *Platelet aggregate formation on whole blood on vWF coated surface.* Anticoagulated blood from WT or *Dusp3*-KO mice was perfused over vWF-coated coverslip (1.4 μg) through a parallel-plate transparent flow chamber at a wall-shear rate of 1000 s^{-1} for 4 min. Representative phase-contrast images of fixed platelets (**A**) and percentages of surface coverage by platelets (**B**) are shown. Results were analyzed using unpaired Student t-test. Data represent mean ± SEM of three independent experiments; ns=non significant.

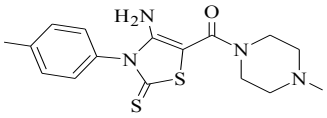
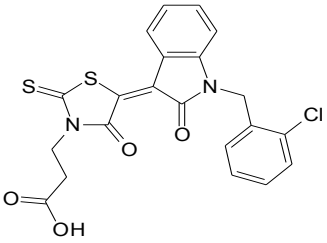
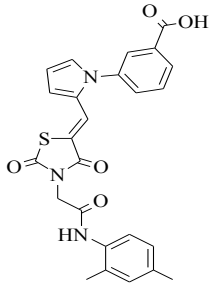
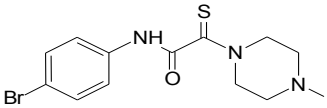
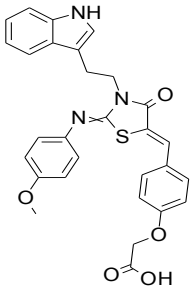
Table S1. Hematological parameters of WT and DUSP3-KO mice.

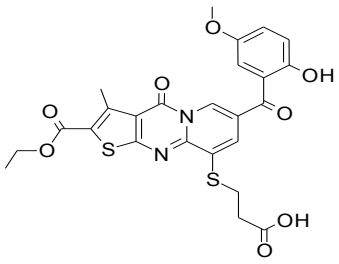
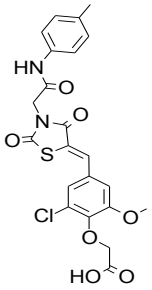
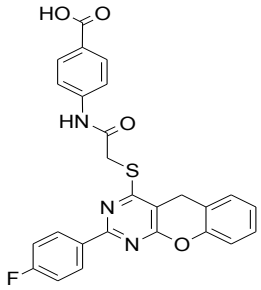
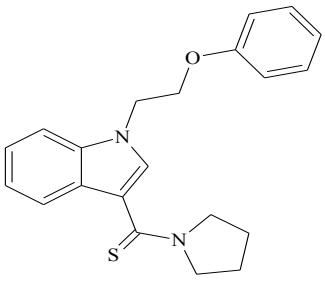
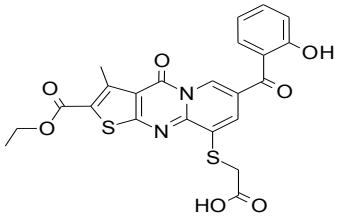
	WT (n=19)	**DUSP3-KO (n=16)**	**P Value**
WBC ($x10^3$/mL)	4.353±0.3183	4.234±0.3369	0.7998 (ns)
Lymphocytes ($x10^3$/mL)	3.339±0.2416	3.299±0.2678	0.9134 (ns)
Neutrophils ($x10^3$/mL)	0.6053±0.0811	0.5575±0.0699	0.6663 (ns)
Monocytes ($x10^3$/mL)	0.1841±0.01791	0.1296±0.0130	0.0232 (*)
Eosinophils ($x10^3$/mL)	0.01864±0.008395	0.02956±0.01254	0.4567 (ns)
Basophils ($x10^3$/mL)	0.2019±0.01793	0.1999±0.01662	0.9356 (ns)
Platelets ($x10^3$/mL)	835 ± 27	905 ± 23	0.0577 (ns)
Hgb (g/dL)	10.73±0.2125	11.24±0.1594	0.0729 (ns)
HCT (%)	34.93±0.5836	36.49±0.5274	0.0584 (ns)
MPV	5.475±0,046	5.099±0,06	<0.0001 (***)

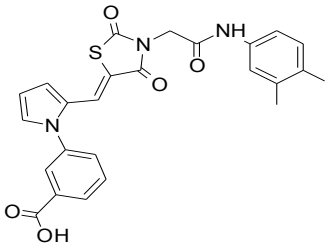
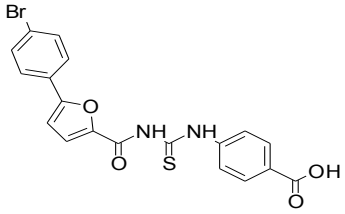
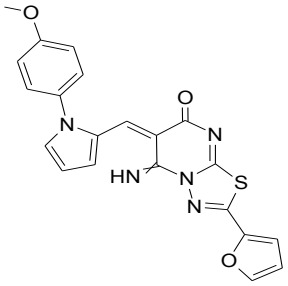
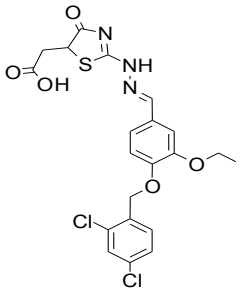
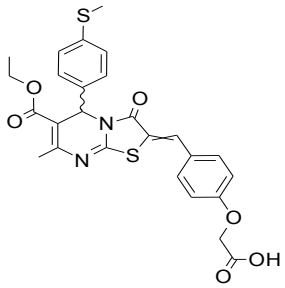
Table S2. Potency and selectivity of 35 selected DUSP3 inhibitors. IC_{50} values are in μM; phosphatase substrates used are given in parentheses (OMFP or pNPP).

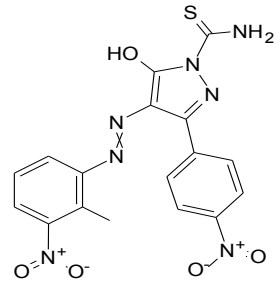
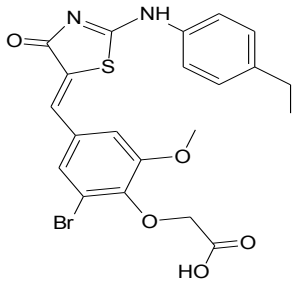
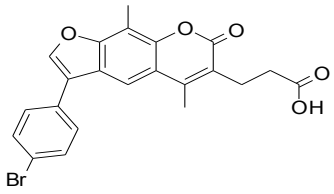
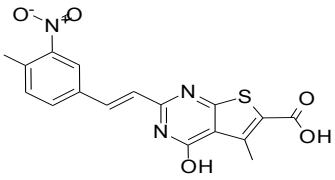
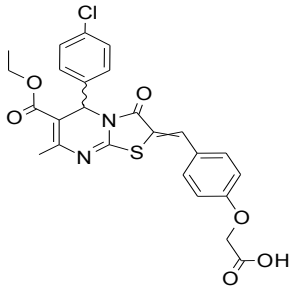
Compound ID	Structure	DUSP3 (OMFP)	DUSP3 (pNPP)	DUSP6 (OMFP)	HePTP (OMFP)	LYP (OMFP)	STEP (OMFP)
MLS-0326173		<1.23	<1.23	<1.23	n/d	n/d	n/d
MLS-0322508		<1.23	<1.23	<1.23	n/d	n/d	n/d
MLS-0347633		<1.23	<1.23	1.36	n/d	n/d	n/d
MLS-0103602		<1.23	3.34	>100	<1.23	n/d	5.11

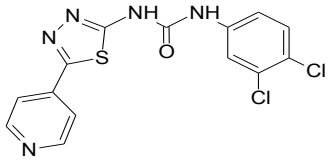
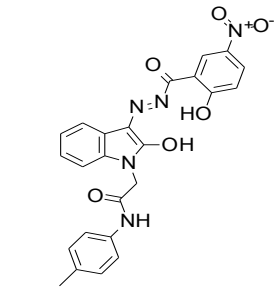
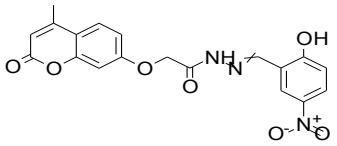
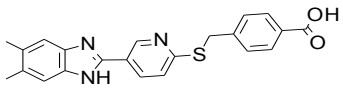
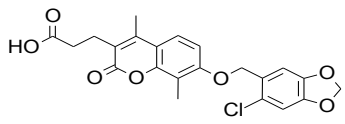
MLS-0044298		1.27	3.53	>100	<1.23	<1.23	n/d
MLS-0330044		2.09	4.01	>100	<1.23	21	3.86
MLS-0307023		2.44	4.31	>100	<1.23	>100	14.5
MLS-0312565		2.52	1.3	27.3	<1.23	2.13	n/d
MLS-0049585		2.68	3.02	>100	8.33	42	>100

MLS-0029484		3.18	5.73	>100	<1.23	n/d	n/d
MLS-0146252		3.2	<1.23	30.9	<1.23	2.88	n/d
MLS-0100722		3.25	1.89	7.9	n/d	n/d	n/d
MLS-0273250		4.76	8.52	>100	n/d	9.73	6.57
MLS-0274366		4.8	2.43	>100	2.52	<1.23	n/d

MLS-0266887		4.87	<1.23	69.8	<1.23	n/d	n/d
MLS-0350105		5.01	<1.23	40.9	<1.23	1.51	n/d
MLS-0249773		5.02	4.04	10.1	n/d	n/d	n/d
MLS-0109562		5.35	8.8	>100	<1.23	n/d	17
MLS-0266851		5.44	3.69	68.6	<1.23	<1.23	2.18

MLS-0276952		6.14	4.36	17.2	n/d	n/d	n/d
MLS-0383777		6.72	1.88	62.7	<1.23	n/d	n/d
MLS-0250197		7.26	2.27	9.04	n/d	n/d	n/d
MLS-0276195		8.44	5.27	25.6	n/d	22.9	4.09
MLS-0350978		9.18	6.94	32.3	<1.23	10.6	n/d

MLS-0265159		10.5	1.71	7.01	n/d	n/d	n/d
MLS-0343544		10.5	3.93	58.2	<1.23	10.3	n/d
MLS-0319547		11.1	11.3	69.2	n/d	n/d	n/d
MLS-0335987		11.1	1.62	8.48	9.05	16.8	n/d
MLS-0351016		11.3	8.8	74.6	<1.23	7.93	n/d

MLS-0216485		11.6	1.56	16.2	n/d	n/d	n/d
MLS-0304619		12.9	6.94	31	n/d	n/d	n/d
MLS-0202850		13.2	2.6	24	n/d	n/d	n/d
MLS-0284989		13.9	5.73	25.8	n/d	n/d	n/d
MLS-0308908		17.4	2.99	78.7	n/d	9	3.97

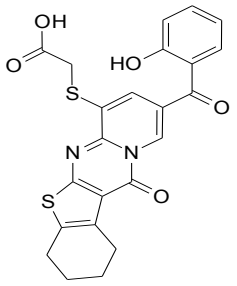
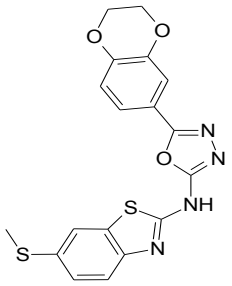
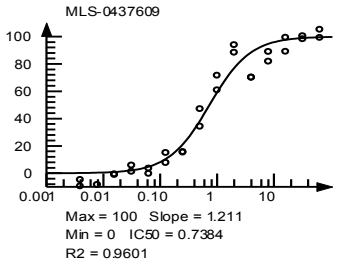
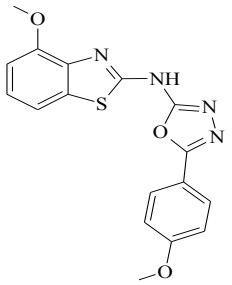
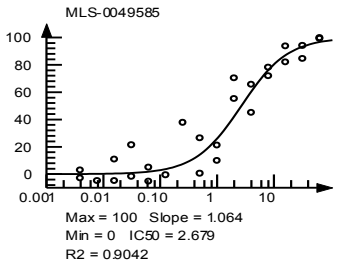
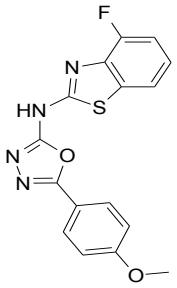
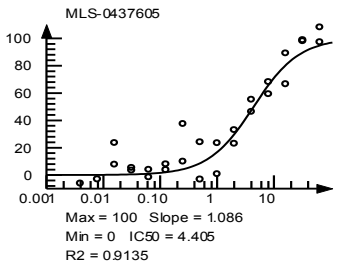
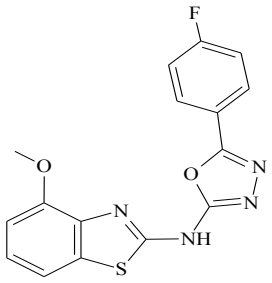
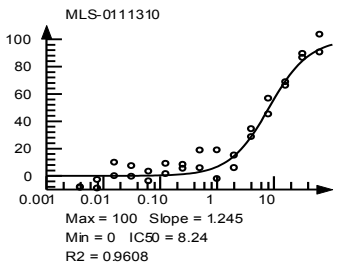
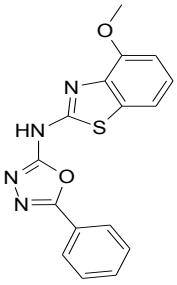
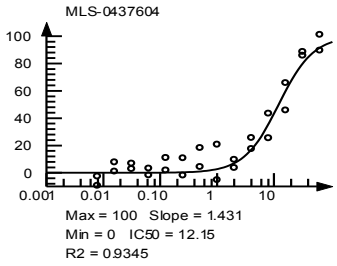
MLS-0312434		19	7.42	>100	<1.23	n/d	n/d

Table S3. SAR studies for compound MLS-0049585.

Compound ID	Structure	Result Graph	IC_{50}, μM	Std Error
MLS-0437609			0.74	0.08
MLS-0049585			2.68	0.47
MLS-0437605			4.40	0.69
MLS-0111310			8.24	0.74
MLS-0437604			12.15	1.25

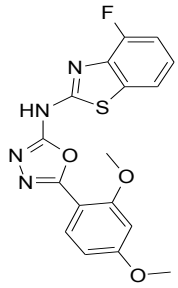
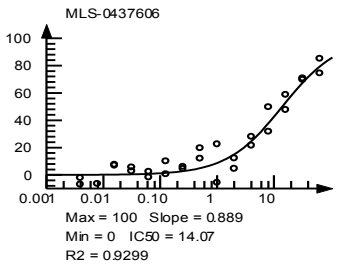
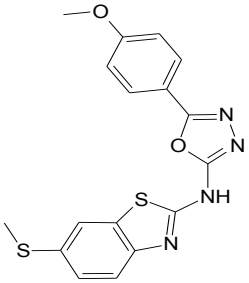
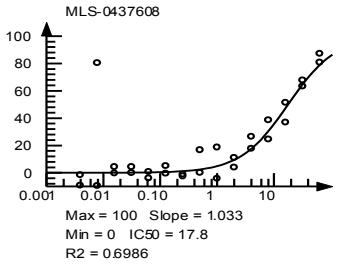
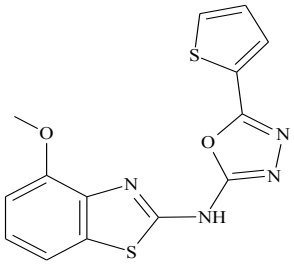
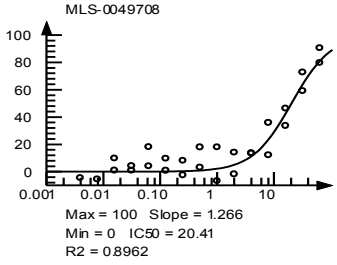
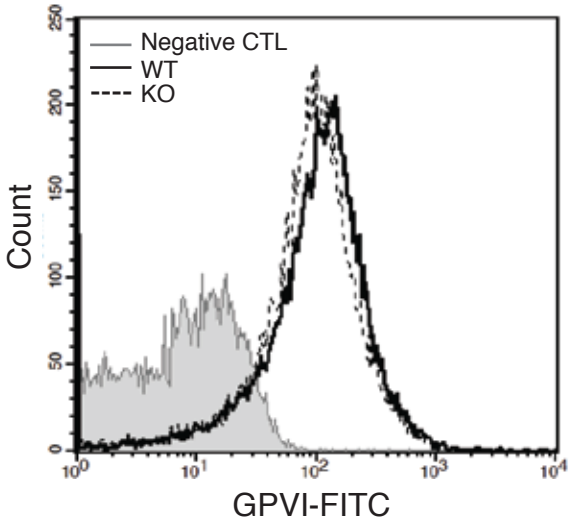
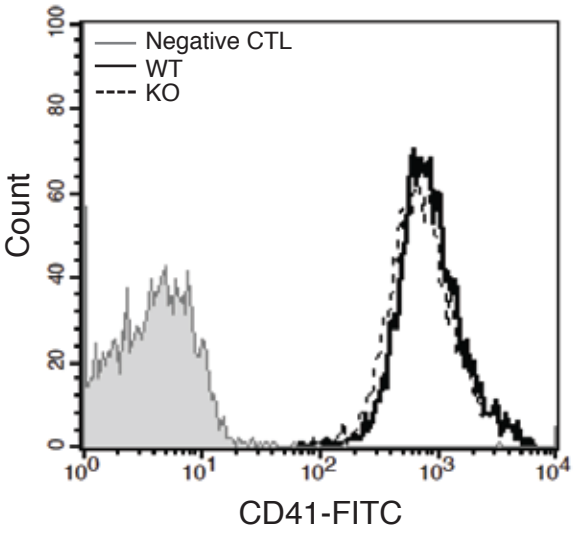
MLS-0437606			14.06	1.67
MLS-0437608			17.80	4.33
MLS-0049708			20.41	2.38

Figure S1

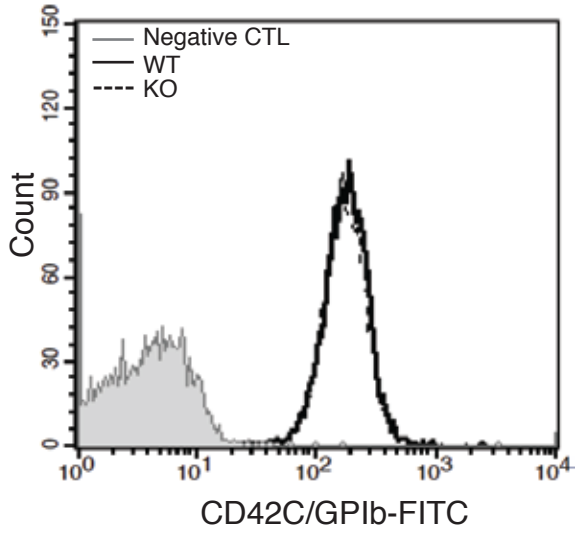
A.



B.



C.



D.

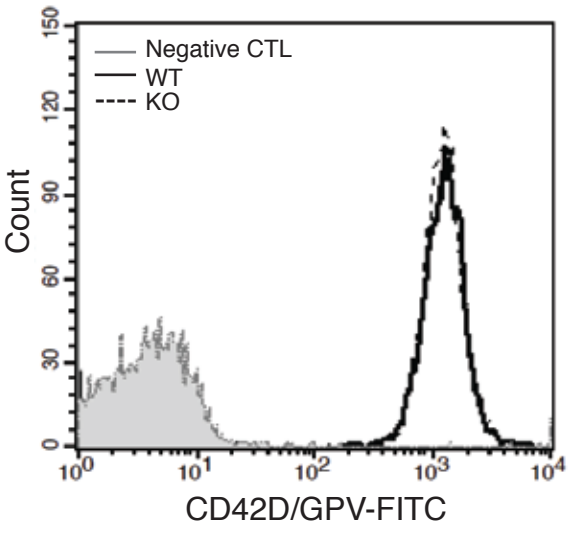
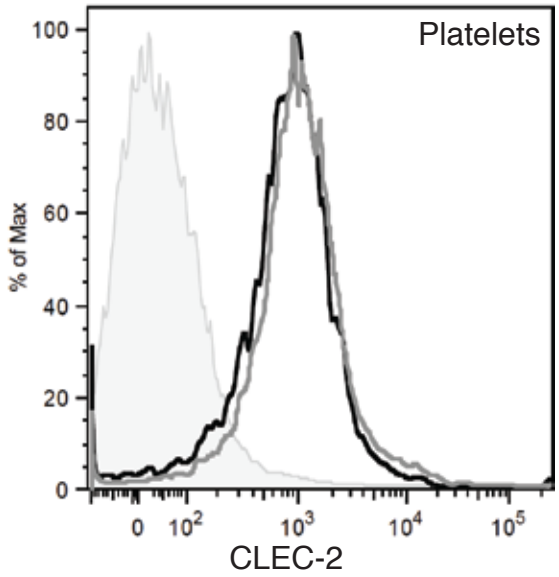
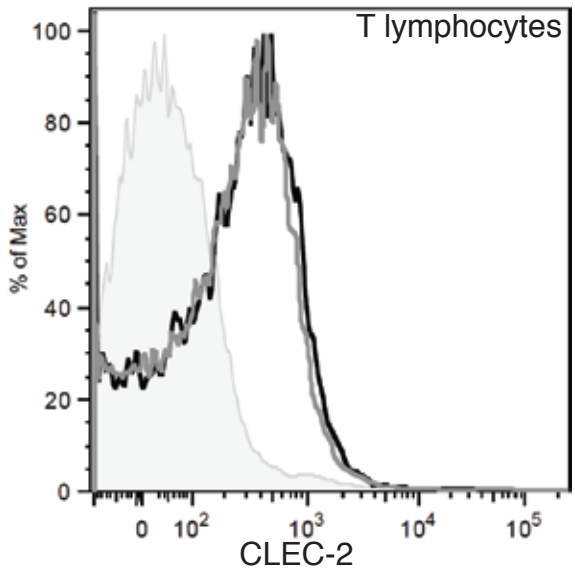


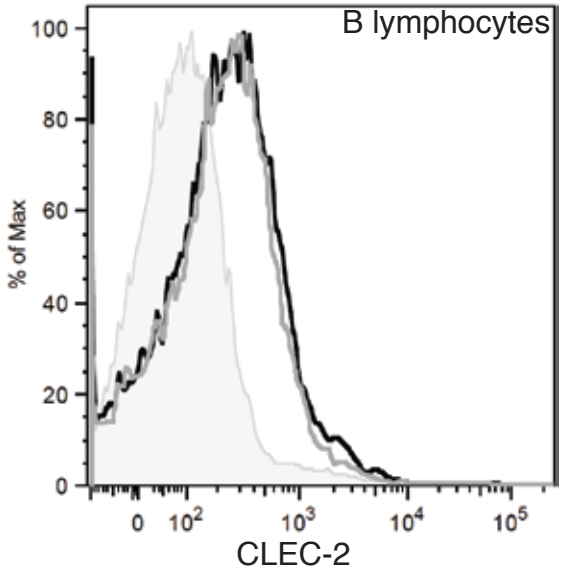
Figure S2

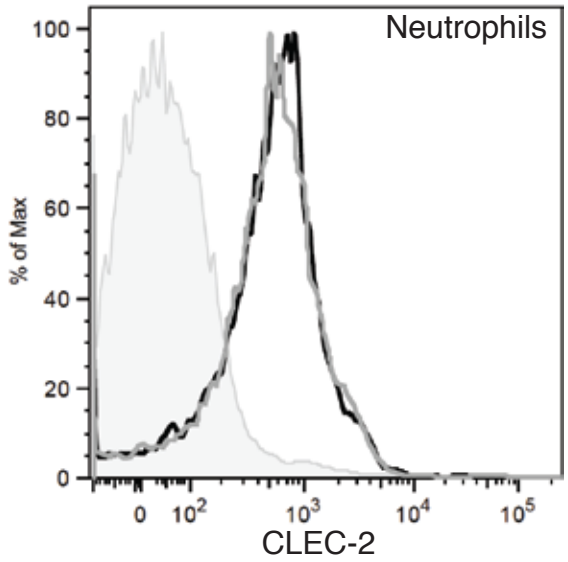
A.


Platelets
% of Max
CLEC-2

B.


T lymphocytes
% of Max
CLEC-2


B lymphocytes
% of Max
CLEC-2


Neutrophils
% of Max
CLEC-2

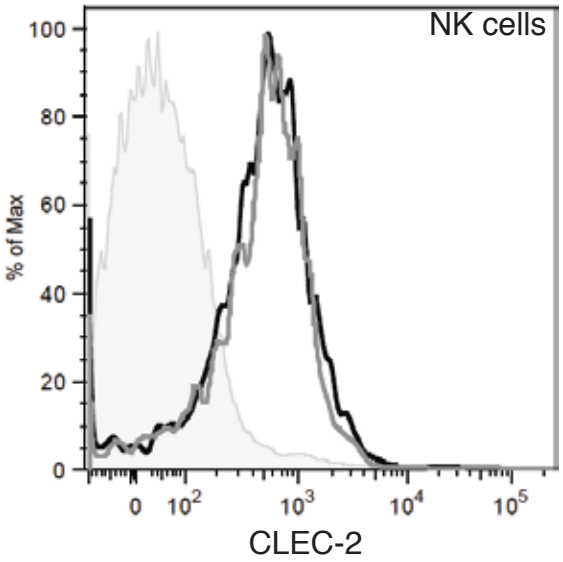

NK cells
% of Max
CLEC-2

Figure S3

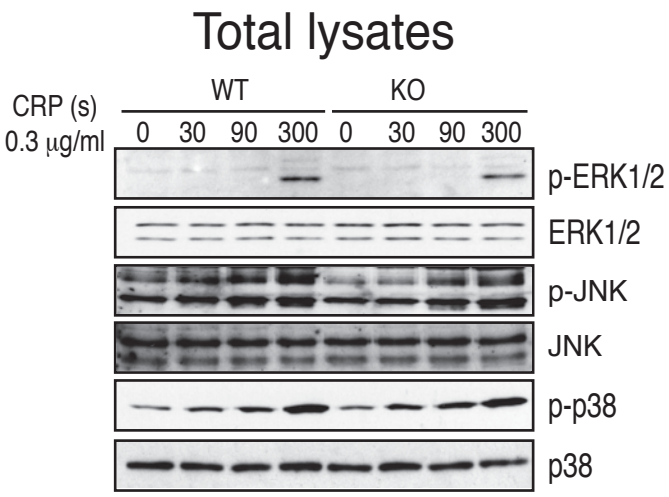

Total lysates
CRP (s)
0.3 μg/ml
WT
KO
0
30
90
300
0
30
90
300
p-ERK1/2
ERK1/2
p-JNK
JNK
p-p38
p38

Figure S4

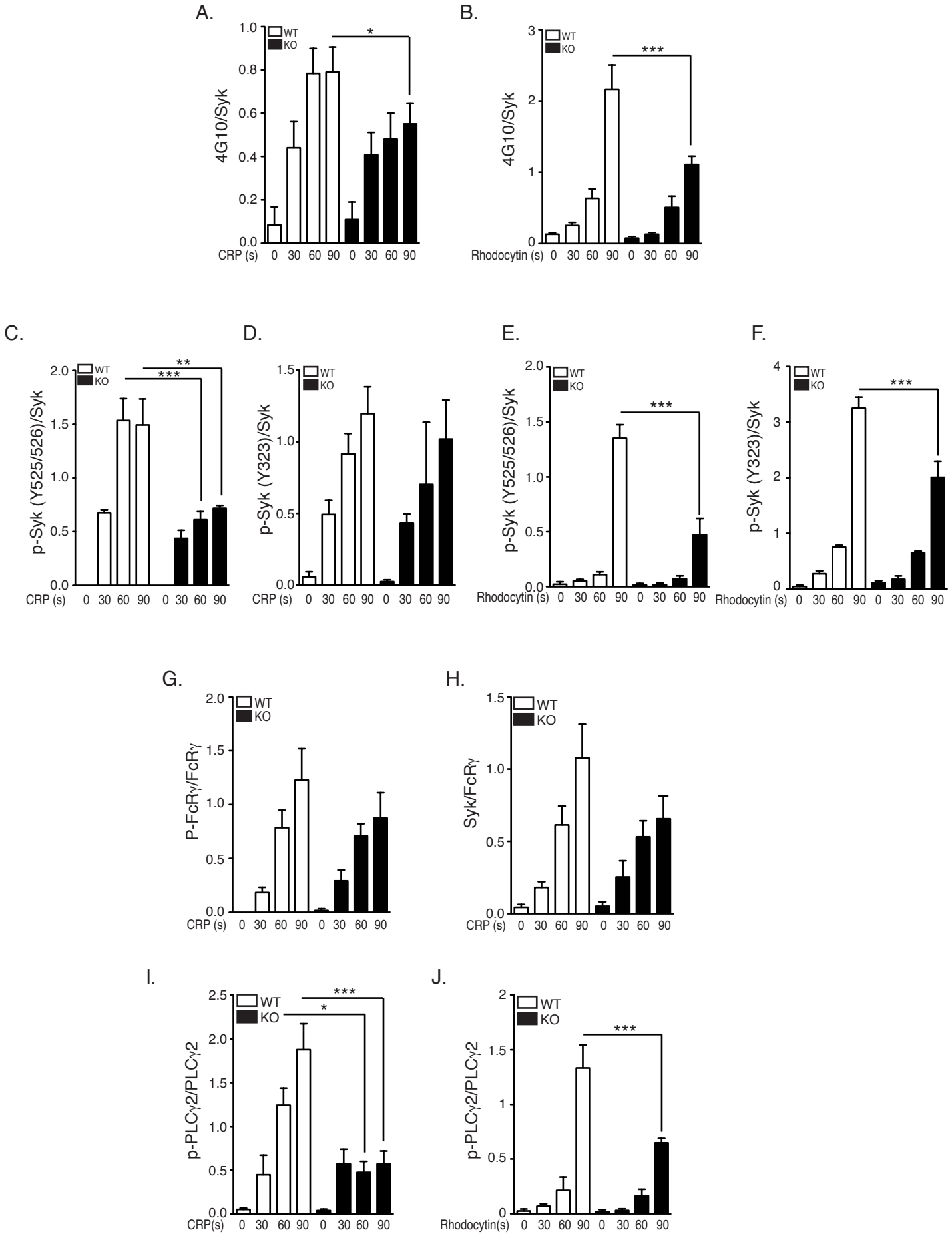

A.
4G10/Syk
WT
KO
*
CRP (s) 0 30 60 90 0 30 60 90
B.
4G10/Syk
WT
KO
***
Rhodocytin (s) 0 30 60 90 0 30 60 90
C.
p-Syk (Y525/526)/Syk
WT
KO
**
***
CRP (s) 0 30 60 90 0 30 60 90
D.
p-Syk (Y323)/Syk
WT
KO
CRP (s) 0 30 60 90 0 30 60 90
E.
p-Syk (Y525/526)/Syk
WT
KO
***
Rhodocytin (s) 0 30 60 90 0 30 60 90
F.
p-Syk (Y323)/Syk
WT
KO
***
Rhodocytin (s) 0 30 60 90 0 30 60 90
G.
P-FcRγ/FcRγ
WT
KO
CRP (s) 0 30 60 90 0 30 60 90
H.
Syk/FcRγ
WT
KO
CRP (s) 0 30 60 90 0 30 60 90
I.
p-PLCγ2/PLCγ2
WT
KO
***
*
CRP(s) 0 30 60 90 0 30 60 90
J.
p-PLCγ2/PLCγ2
WT
KO
***
Rhodocytin(s) 0 30 60 90 0 30 60 90

Figure S5

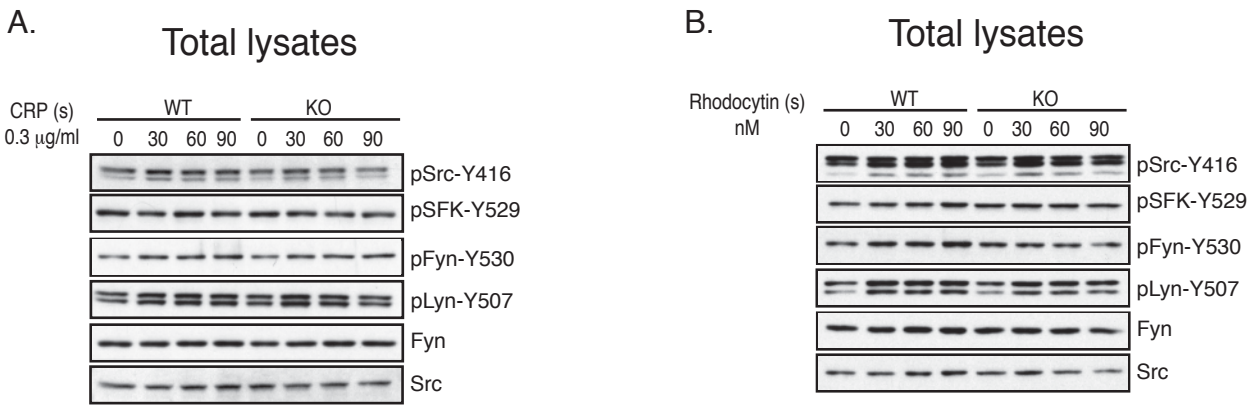
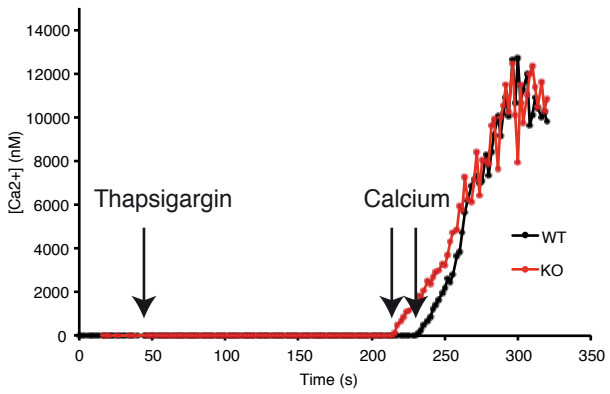

A.
Total lysates
CRP (s)
0.3 μg/ml
WT
KO
0 30 60 90 0 30 60 90
pSrc-Y416
pSFK-Y529
pFyn-Y530
pLyn-Y507
Fyn
Src
B.
Total lysates
Rhodocytin (s)
nM
WT
KO
0 30 60 90 0 30 60 90
pSrc-Y416
pSFK-Y529
pFyn-Y530
pLyn-Y507
Fyn
Src

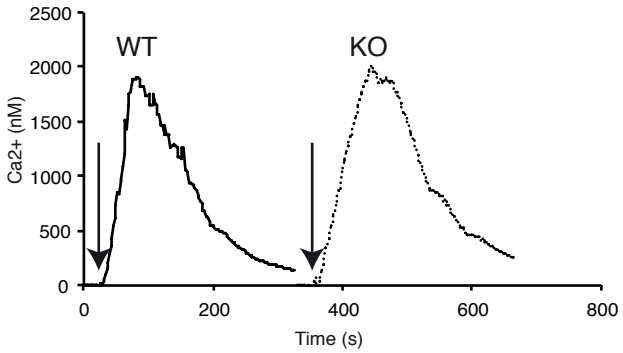
Figure S6

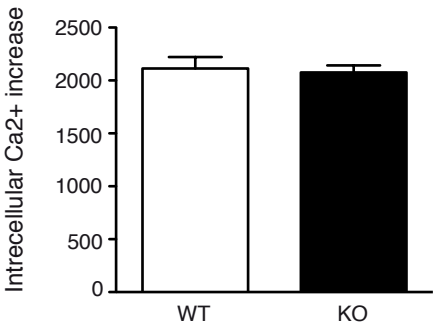
A.



B.

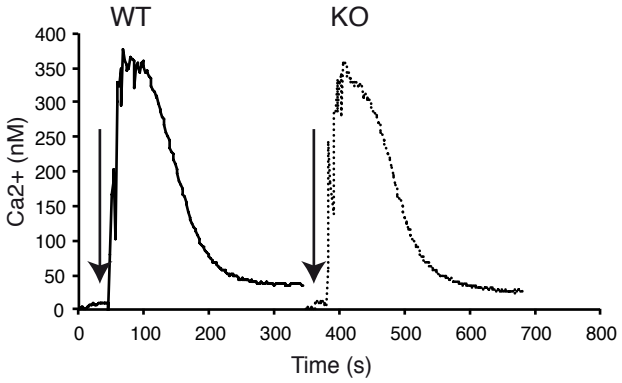
IIA

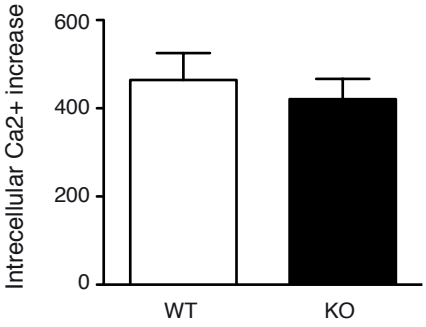




C.

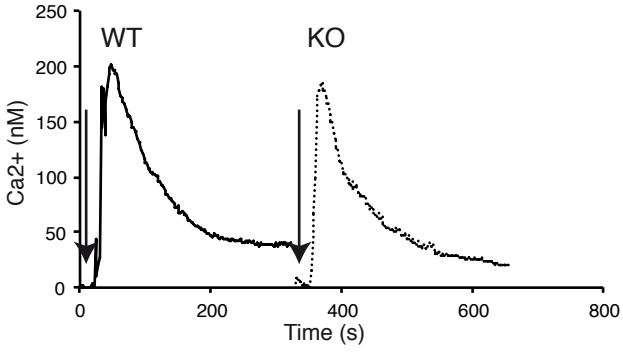
ADP





D.

TXA2



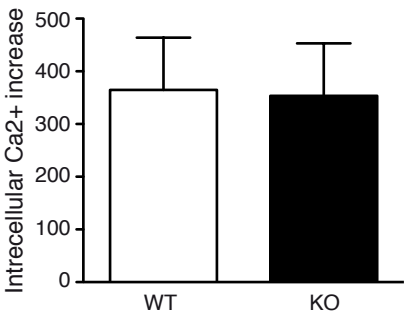
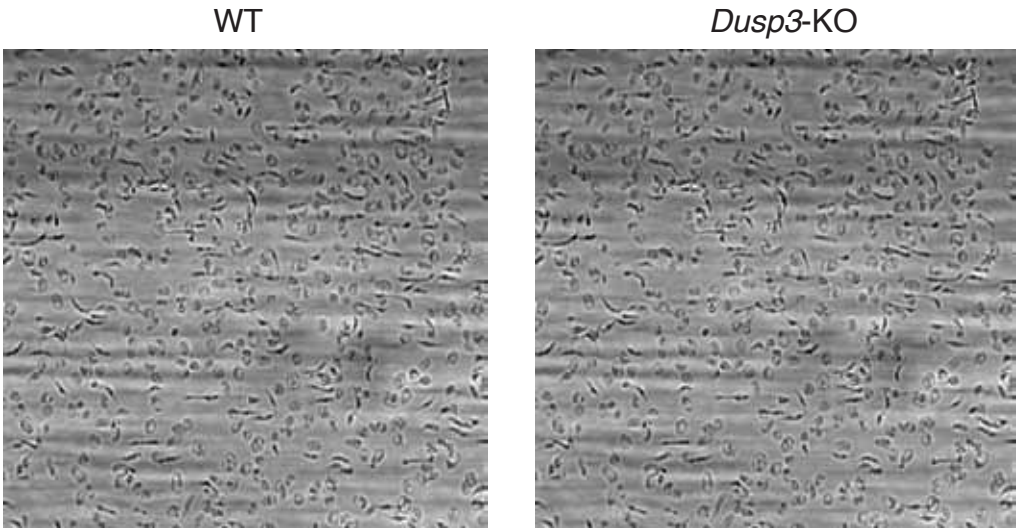
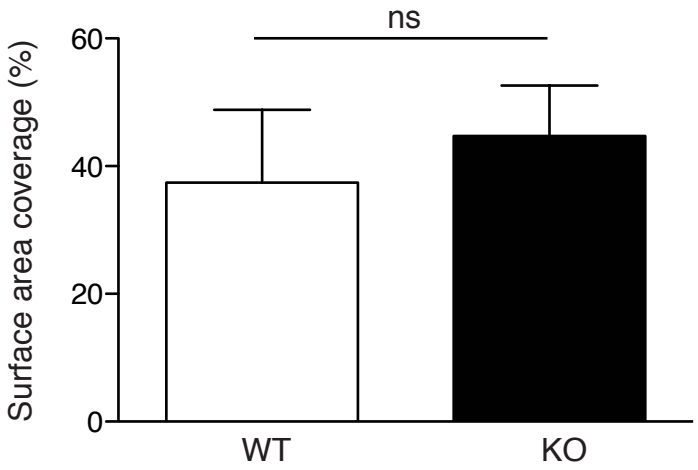


Figure S7

A.


WT
Dusp3-KO
vWF coated surface

B.


ns
Surface area coverage (%)
60
40
20
0
WT
KO