Loss of DEP-1 (Ptprj) promotes myeloproliferative disease in *FLT3*-ITD acute myeloid leukemia

Anne Kresinsky,[1] Reinhard Bauer,[1] Tina M. Schnöder,[2,3] Tobias Berg,[4] Daria Meyer,[5] Volker Ast,[5] Rainer König,[5] Hubert Serve,[4] Florian H. Heidel,[2,3] Frank- D. Böhmer[1] and Jörg P. Müller[1]

[1]Institute of Molecular Cell Biology, Center for Molecular Biomedicine (CMB), Jena University Hospital; [2]Innere Medizin II, Hämatologie und Onkologie, Jena University Hospital; [3]Leibniz Institute on Aging, Fritz-Lipmann-Institute (FLI), Jena; [4]Department of Medicine II, Hematology/Oncology, Goethe University, Frankfurt and [5]Network modelling, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Jena, Germany

Correspondence: joerg.mueller2@med.uni-jena.de
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Supplement

MATERIALS AND METHODS

Mouse lines

Heterozygous B6 *Ptprj*$^{+/-}$ mice were purchased from Deltagen (San Mateo, CA). FLT3$^{ITD/ITD}$ (B6.129-FLT3^{tm1Dgg} mice) were from Jackson Lab[1]. Homozygous FLT3$^{ITD/ITD}$ mice were crossed with *Ptprj*$^{-/-}$ mice[2]. Primers used to confirm the presence of the FLT3-ITD and *Ptprj* knock-out mutation are given in the supplemental table S1. Experiments were approved by the committee of the Thuringian State Government on Animal Research and performed according to the Protection of Animals Act of the Federal Republic of Germany. The animals were treated in accordance with the declaration of Helsinki and the guiding principles in the care and use of animals.

Complete peripheral blood cell count and cytology

Blood was collected from the retro bulbar venous plexus and differential cell counts determined using a Mindray BC5300Vet Hematology system. Peripheral blood smears and bone marrow or spleen cytospins were visualized using the Pappenheim staining kit from Morphisto GmbH (Frankfurt am Main, Germany).

Fluorescent antibodies and immunomagnetic beads used for FACS analysis and sorting

Antibodies used for cell surface staining were as follows: CD11b (Clone M1/70), CD19 (clone 1D3), CD3e (Clone 145-2C11), CD117 (c-Kit, clone 2B8) Ly-6A/E (Sca-1,Clone D7) purchased from eBioscience (Santa Clara, California). Ly-6G/Ly-6C (Gr-1, Clone RB6-8C5) from Biolegend (San Diego, California). Modified lineage cocktail of CD3e, Ter119, CD11b, Gr-1, B220 (Biotin mouse lineage Panel), CD19 biotin (clone 1D3) obtained from BD Pharmingen (Heidelberg, Germany). Biotinylated antibodies were visualized with Streptavidin-PerCP (BD Pharmingen). MACS column enrichment of Lin$^-$ cells was done using Direct lineage cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufactures protocol. PE-labelled CD135 (clone A2F10.1) was used for detection of FLT3 positive cell population by intracellular staining after fixation and permeabilization using Cytofix/Cytoperm kit from BD Pharming according the manufactures protocol.

Flow cytometric analysis

For flow cytometric analysis, BM suspensions were obtained from tibia and femur. Blood was collected by post-mortem aspiration in the left cardiac ventricle. Blood, BM and spleen cells were isolated from 30 - 35 week old mice carrying the mutations of interest and corresponding wild type (WT) littermates. Cells were washed and stained in PBS buffer supplemented with 2% FCS and incubated with indicated antibodies for 30 min at 4°C. To analyse immune cell differentiation, cells taken from blood, BM or spleen were stained for Gr-1 and CD11b. Double-positive cells were considered as monocytes/granulocytes, double-negative cells were considered as myeloid undifferentiated cells. Subsequent gating of CD19 or CD3e positive cells allowed differentiation into differentiated B cells (CD19$^+$) and T cells (CD3e$^+$). For detection of Lin$^-$ cells and stem cells, a “lineage cocktail” consisting of CD3e, Ter119, CD11b, Gr-1, B220, CD19 and subsequent staining with Streptavidin was used. Lin$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells were detected by staining with CD117 (c-Kit), Ly-6A/E (Sca-1) and CD135. Flow cytometry analysis was performed on a FACS Canto II (Becton Dickinson, Heidelberg, Germany) flow cytometer

using Diva software. Processing of data was done using FCS express software (De Novo; Glendale, USA).

In vitro clonogenic assay for Lin$^-$ cells

Lineage-negative cells from BM or spleen cells purified using a direct lineage-cell depletion kit following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified Lin$^-$ precursors from BM and spleen were plated at a concentration of 250 cells per well in a 24 well plate in M3434 methylcellulose (StemCellTechnologies, Vancouver, BC, USA). Colony counts of granulocyte/ macrophage colonies (CFU-GM), were characterized 7 days after incubation at 37 °C and 5 % CO_2. Afterwards, cells were harvested and used for re-plating and cytospin preparation.

Histopathology

Histopathology studies were carried out for spleen, liver, and kidney from all transgenic mouse lines and non-transgenic littermates. Mice were deeply anesthetized and perfused with 4 % PFA in phosphate buffer by aspiration from the left cardiac ventricle. Organs were fixed for 24 h in 4 % PFA at 4 °C. Tissue samples were embedded in paraffin, and 4 µm tissue sections were dewaxed and stained with hematoxylin and eosin (H & E). Subsequently, samples were dehydrated and coverslipped in Neomount (Merck) Representative images of H & E-stained sections were acquired using a Nikon eclipse Ti-E invert microscope system. Immunohistochemical analysis of myeloperoxidase (MPO, purchased by Dianova, Hamburg, Germany) was performed on dewaxed, rehydrated, 4 µm tissue samples which were boiled in 0.01 M citrate buffer (tri-sodium citrate-dihydrate, pH 6.0) for 11 min for antigen retrieval. Afterwards, the slices were rinsed in phosphate-buffered saline (PBS, pH 7.4) and in PBS supplemented with 0.05 % Tween 20 (PBS-T), treated for 20 min with 3 % H_2O_2 in 50 % Isopropanol for 20 min to eliminate endogenous peroxidases, subsequently incubated in 0.5 mg/ml sodium borohydrate for 20 min and finally blocked in 10 % normal donkey serum in PBS-T for 30 min at 37 °C. Incubation with the primary MPO antibody (1:100) was performed over night at 4 °C. After three washing steps with PBS-T sections were incubated with secondary biotin-labelled donkey-anti-rabbit antibody (1:200, Dianova) and washed with PBS-T. The labelling was developed using a vector ABC kit (Vector laboratories, Burlington, Canada) and Sigmafast tablets (Sigma, Oakvill, Canada). Sections were dehydrated and coverslipped in Fluoromount-G (Southern Biotech, Birmingham, USA).

SDS-PAGE and Westernblot

Immunoblotting was carried out by standard procedures. Purified Lin$^-$ cells were suspended in RIPA lysis buffer composed of 50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 % (v/v) NP-40, 0.5 % (v/v) sodium deoxycholate, 0.1 % (w/v) SDS, 100 mg/ml Pefa-Block, 1 mg/ml pepstatin, 10 mM sodium vanadate and 1 mg/ml leupeptin. Supernatants of a 20-min centrifugation at 13500 x g, 4 °C, were mixed with 5 X protein sample buffer (5 % SDS, 33 % glycerol, 25 % ß-Mercaptoethanol) and heated to 95 °C for 5 min. Protein samples were separated on 10 % polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and immunoblotted with indicated antibodies followed by enhanced chemiluminescence detection (Perkin Elmer Life Sciences; Boston, USA) and quantitative evaluation using a CCD camera-based system (LAS4000, Fuji).

Gene expression data

Transcriptomic data sets used were from the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo). Dataset GSE61804 contained expression data derived from 286 diagnostic samples of peripheral blood

form AML patients[3]. Analysis on samples was restricted to patients with a normal karyotype and further selected for FLT3-ITD mutation positive samples (n=31) and FLT3 wild type patients (n=62). To test whether *PTPRJ* mRNA is differentially expressed between AML patients with and without FLT3-ITD mutation, we performed a two-sided Student's t-test using expression values of probe 210173_at.

Furthermore, the data set GSE1159 from GEO, which contained gene expression data of 293 AML patients[4], was used to investigate the relation of *PTPRJ* mRNA expression and overall survival. Normal karyotype FLT3 ITD positive AML patients (n = 41) and normal karyotype FLT3 wild type AML patients (n = 53) were grouped according to their expression level of PTPRJ (probe ID: 214137_at) into *PTPRJ* high and *PTPRJ* low expression. Cut-off between the 5 % and the 95 % quantile of *PTPRJ* expression values, incremented in steps of 5% was tested to obtain the best separating cut-off value leading to the 75% quantile, resulting in 11 patients with high and 30 patients with low *PTPRJ* expression. The same cutoff was applied to the FLT3 wild type samples involving 13 patients with high and 40 patients with low *PTPRJ* expression. Survival analysis (overall survival) was performed by applying the Survival Analysis package [5] and the statistic software package R (www.r-project.org). To test for statistical significance, we performed a log-rank test.

Data analysis and statistical procedures

The statistical analysis was performed using SigmaPlot Software (SigmaPlot Software, San Jose, USA). Data are reported as mean +/- standard error of the mean (SEM). Comparisons between groups were made with one-way analysis of variance using Bonferroni correction as post-test if appropriate. A p-value of < 0.05 was considered to be statistically significant.

Suppl. Figures

Suppl. Figure S1) Peripheral blood from FLT3$^{ITD/ITD}$ Ptprj$^{-/-}$ mice shows expansion of granulocytes/monocytes. Peripheral blood samples of 30 – 35 week old WT, FLT3$^{ITD/ITD}$; Ptprj$^{-/-}$ and FLT3$^{ITD/ITD}$ Ptprj$^{-/-}$ were analysed using flow cytometry. Graphical presentation of CD11b/Gr-1 expressing cells in peripheral blood.

Suppl. Figure S2) Infiltration of MPO-positive cells in peripheral organs. MPO-DAB stained histopathology showing liver from 30 - 35 weeks old WT, FLT3$^{ITD/ITD}$, *Ptprj*$^{-/-}$ or FLT3$^{ITD/ITD}$ *Ptprj*$^{-/-}$. Magnification 4x (top) and 20x (bottom), Scale bars 100 µm.

Suppl. Figure S3) Aberrant blood phenotype of FLT3$^{ITD/ITD}$ Ptprj$^{-/-}$ mice. (A-D) Differential blood cell counts of FLT3$^{ITD/ITD}$ *Ptprj*$^{-/-}$ mice demonstrate increased white blood cell count (WBC, A) compared to all other genotypes. Absence of *Ptprj* had little effect on decreased count of red blood cells (RBC, B), platelets (C), and haemoglobin (HGB, D) in FLT3$^{ITD/ITD}$ mice compared with wildtype and *Ptprj*$^{-/-}$ mice. Samples of peripheral blood were analysed from 30 – 35 week old WT, FLT3$^{ITD/ITD}$, Ptprj$^{-/-}$ and FLT3$^{ITD/ITD}$ Ptprj$^{-/-}$ mice using a Mindray BC5300Vet Hematology system. (E) Myeloid expansion of FLT3$^{ITD/ITD}$ positive mice. May & Giemsa staining of cytospins of BM and Spleen from 30 - 35 weeks old WT, FLT3$^{ITD/ITD}$, Ptprj$^{-/-}$ and FLT3$^{ITD/ITD}$ Ptprj$^{-/-}$ mice. The images were acquired at room temperature using a Nikon Eclipse Ti-E microscope invert system (Nikon, Tokyo, Japan) with 40x magnification.

Suppl. Figure S4) Aberrant differentiation of committed progenitors in FLT3-ITD mice is not changed upon additional inactivation of *Ptprj*. The Lin^{-} Sca1^{-} c-kit^{+} compartment was analyzed for the abundance of megakaryocyte erythroid progenitors (MEP), granulocyte-macrophage progenitors (GMP) and common myeloid progenitors (CMP) cells. (A) Gating strategy for the identification of MEP, GMP and CMP cells. Percentage of these cells in BM (B) and spleen (C).

Suppl. Figure S5) Relative TKI resistance of FLT3-ITD expressing cells lacking *Ptprj*. *Ptprj* was inactivated in FLT3-ITD expressing 32D cells using CRISPR/Cas9. (A) Immunoblotting demonstrates the absence of Ptprj (B) pY589 site specific phosphorylation of FLT3-ITD of indicated 32D cell lines. Numbers under the phospho-specific blot represent quantification of the phosphor-specific signals of three independent experiments (mean +/- SEM), normalized to the corresponding signals with pan-specific antibodies, and relative to the signal in 32D FLT3-ITD cells, which was set to 1.0. (C) Relative resistance against TKI AC220 (Quizartinib) (left) or PKC412 (Midostaurin) (right) of 32D FLT3-ITD cells (black lines) or 32D FLT3-ITD *Ptprj*$^{-7-}$ cells (red). IC50 values are indicated in the representative graphs.

target	DNA sequence (5` - 3`)
Ptprj REV	GGAGAATGTATACGAAGTGCCTGGG
Neo FWD	GGGCCAGCTCATTCCTCCCACTCAT
Ptprj FWD	AGCTGTCGTCAGCCCAACTAGTGTG
FLT3 ITD FWD	AGGTACGAGAGTCAGCTGCAGAT
FLT3 ITD REV	TGTAAAGATGGAGTAAGTGCGGG

Suppl. Table S1: Primer used for genotyping FLT3 ITD and inactive Ptprj.

	WT (n=21)	FLT3 ITD (n=16)	Ptprj$^{-/-}$ (n=8)	FLT3 ITD Ptprj$^{-/-}$ (n=14)
WBC x10^9/l	7.78 ± 0.50	7.72 ± 1.51	8.21 ± 0.94	19.75 ± 5.38 * #
% Neutrophiles	0.11 ± 0.01	0.35 ± 0.02 ***	0.20 ± 0.04 *	0.46 ± 0.05 *** #
% Lymphocytes	0.82 ± 0.01	0.34 ± 0.05 ***	0.68 ± 0.04 **	0.22 ± 0.05 ***
%Monocytes	0.03 ± 0.01	0.21 ± 0.03 ***	0.08 ± 0.02	0.24 ± 0.04 ***
% Eosinophiles	0.04 ± 0.01	0.08 ± 0.01 **	0.02 ± 0.00	0.06 ± 0.01
Neutrophiles x10^9/l	0.85 ± 0.08	3.04 ± 0.72 **	1.72 ± 0.42	9.60 ± 2.75 ** #
Lymphocytes x10^9/l	6.38 ± 0.42	2.39 ± 0.43 ***	5.64 ± 0.69	3.14 ± 1.09 *
Monocytes x10^9/l	0.24 ± 0.05	1.48 ± 0.31 **	0.66 ± 0.20 *	5.66 ± 2.09 *
Eosinophiles x10^9/l	0.28 ± 0.04	0.78 ± 0.25	0.19 ± 0.04 *	0.88 ± 0.24 *
Basophiles x10^9/l	0.03 ±0.01	0.03 ± 0.01	0.01 ± 0.00	0.47 ± 0.31
RBC x 10^{12}/l	9.07 ± 0.11	6.27 ± 0.28 ***	8.71 ± 0.46	6.91 ± 0.17 ***
HGB	142.29 ± 1.19	111.74 ± 4.30 ***	129.33 ± 7.11	126.07 ± 2.65 *** ##
HCT	0.45 ±0.00	0.35 ± 0.01 ***	0.41 ± .0.2	0.40 ± 0.01 ##
MCV fl	49.18 ± 0.37	55.22 ± 1.12 ***	46.01 ± 1.01 *	58.17 ± 0.85 #
MCH pg	15.71 ± 0.13	17.58 ± 0.34 ***	14.52 ± 0.34 **	18.29 ± 0.25
PLT x10^9/l	692.67 ± 45.6	417.44 ± 43.7 ***	748.92 ± 86.63	451,07 ± 57,66 **
MPV fL	5.18 ± 0.07	6.02 ± 0.19 ***	5.00 ± 0.24	5.73 ± 0.20 *
PDW	15.22 ± 0.08	15.26 ± 0.28	15.04 ± 0.48	15.64 ± 0.14 *
PCT ml/l	3.55 ± 0.22	2.49 ± 0.22 ***	3.77 ± 0.41	2.47 ± 0.26 **

Suppl. Table S2: Differential blood cell analysis. Peripheral blood samples of 30 – 35 week old WT, FLT3$^{ITD/ITD}$; Ptprj$^{-/-}$ and FLT3$^{ITD/ITD}$ Ptprj$^{-/-}$ mice were analysed using Mindray BC5300Vet Haematology system. WBC, white blood cell count; RBC, red blood cell count (RBC), HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; PLT, platelets; MPV, mean platelet volume.; PDW, platelet distribution width; PCT, plateletcrit. Values are shown in means ± SEM. * p<0.05, ** p<0.01, *** p<0.001 compared to WT; # p<0.05, ## p<0.01 compared to FLT3$^{ITD/ITD}$ mice

Suppl. REFERENCES

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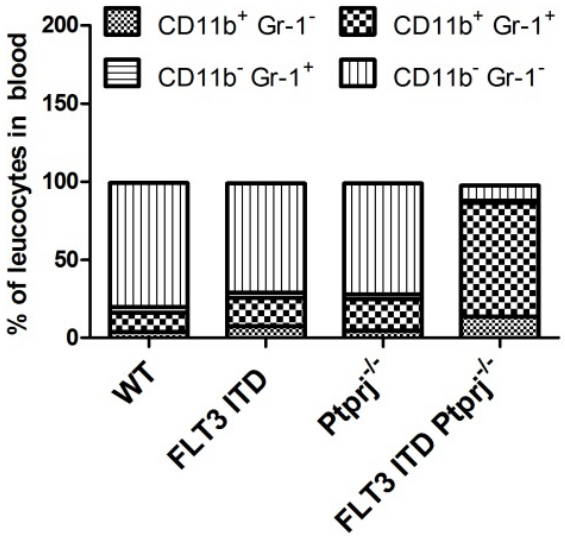

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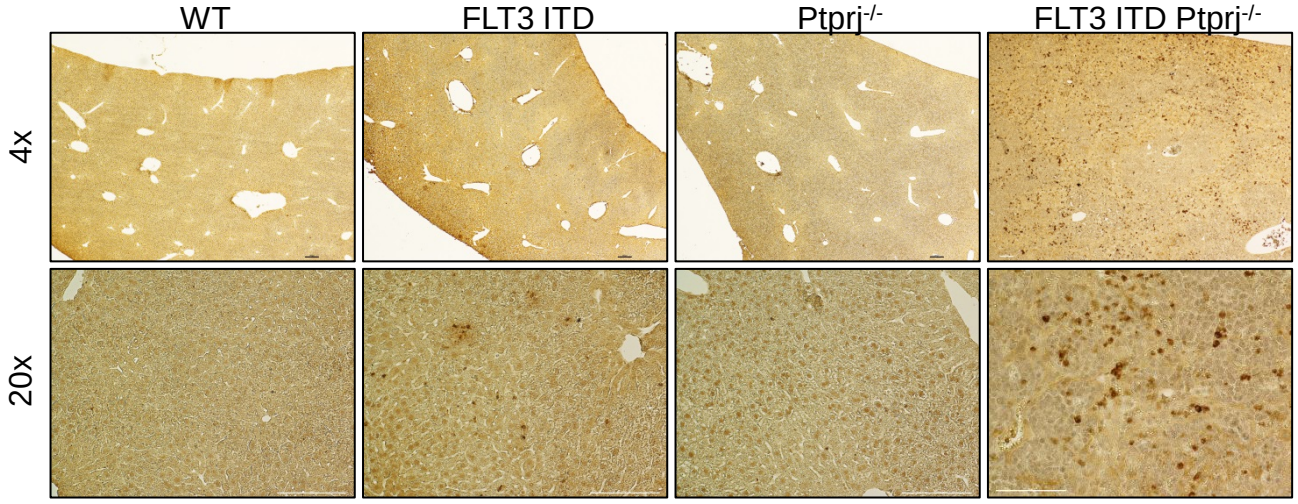

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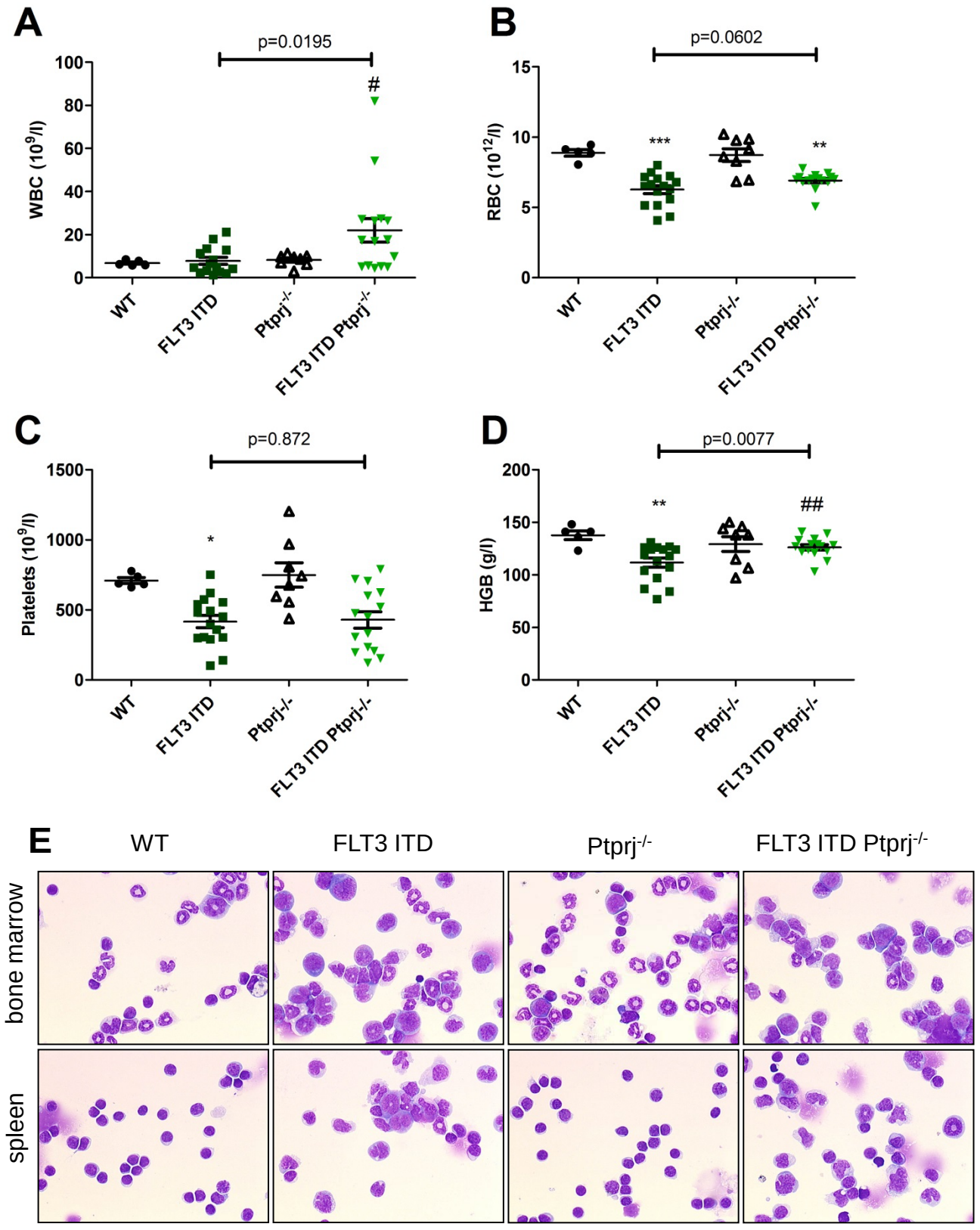

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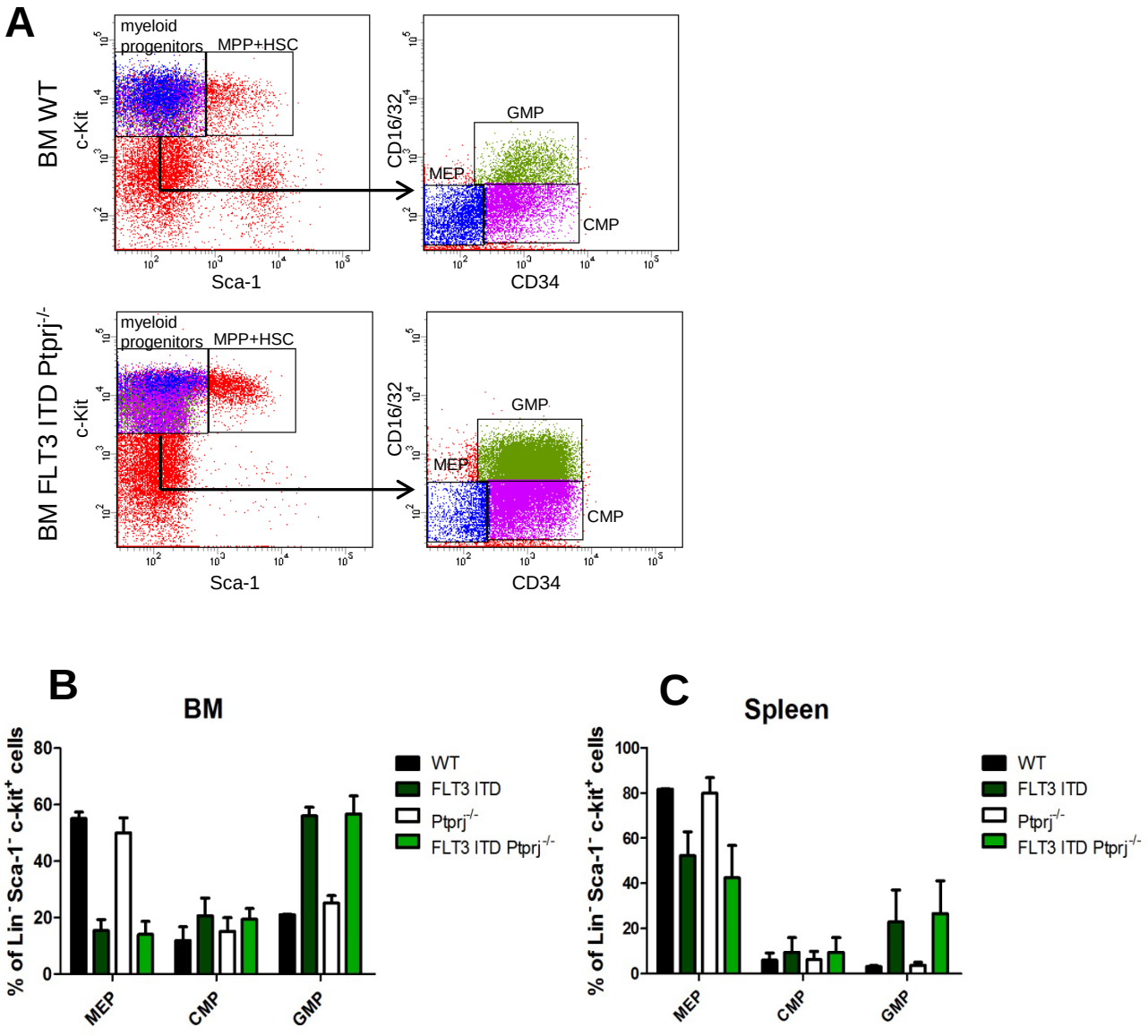

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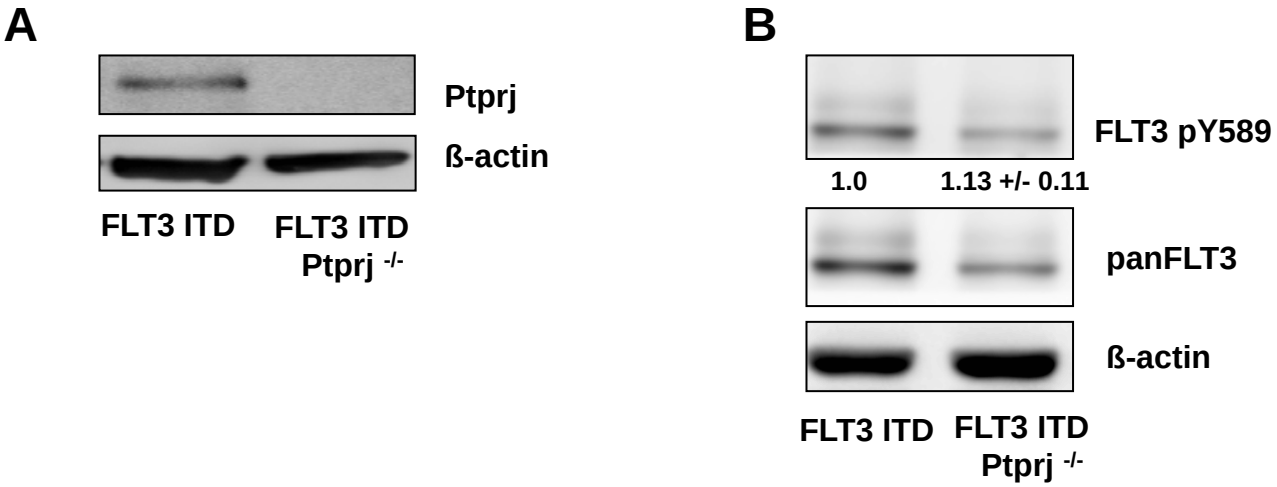

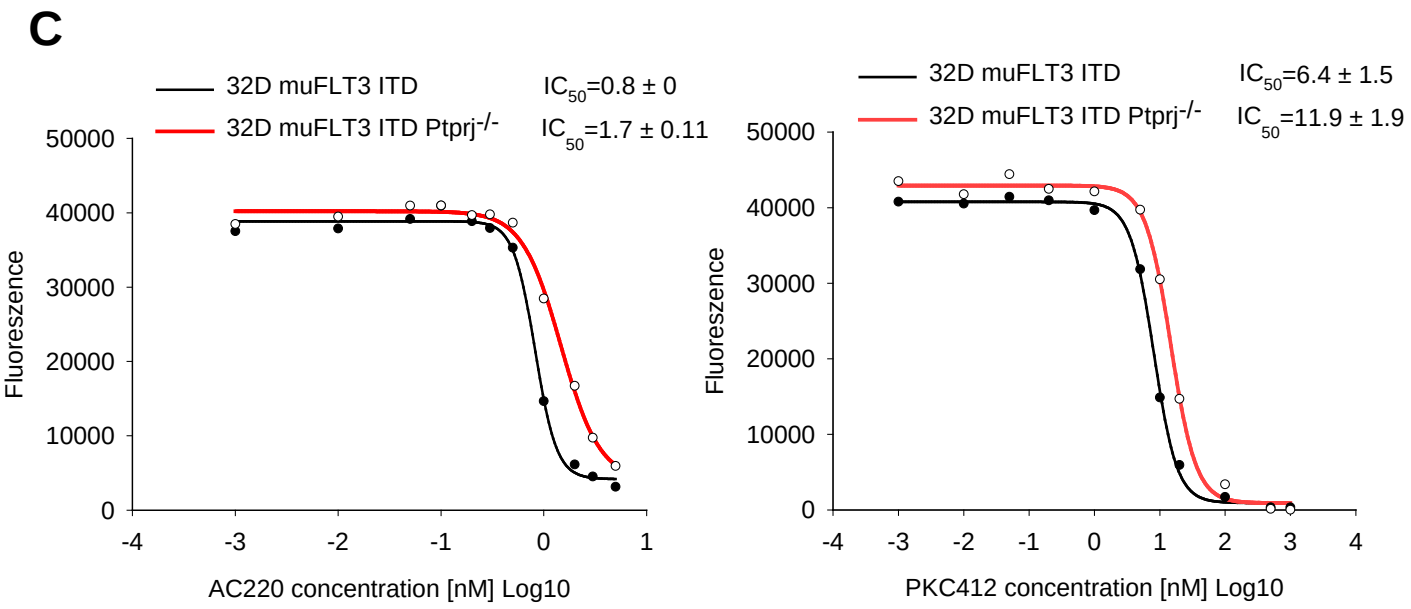

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