Supplemental Information

RNA BINDING PROTEIN SRSF3 CONFERS AN ESSENTIAL ROLE IN MEGAKARYOCYTE MATURATION AND PLATELET PRODUCTION

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Supplemental Methods

Isolation of mouse bone marrow megakaryocytes

To harvest bone marrow, iliac crests, femurs and tibiae were collected, cleaned and flushed with phosphate buffered saline (PBS) supplemented with 2% foetal bovine serum (FBS). The flushed cells were filtered (100μm) and spun at 300g for 5min to harvest the total bone marrow cells. For immunological depletion of lineage positive cells, pelleted cells were immuno-labelled with rat anti-mouse B220, rat anti-mouse MAC-1 and rat anti-mouse GR-1 and collected using anti-rat conjugated Dynabeads (Life Technologies). To analyse or isolate bone marrow MK, lineage depleted cells were labelled with 10μM Hoechst 33342 for 45min at 37ºC, washed and then labelled with AF700-conjugated rat anti-mouse CD41 (Biolegend), hamster anti-mouse CD61-PE (BD Biosciences) or goat anti-mouse Mpl-Biotin (R&D) & SAV-AF647 (Biolegend). The cells were sorted using Influx cell sorter or analysed using LSRII cell analyser (Becton Dickinson).

Immunohistochemistry

Femurs were perfusion fixed with 4% paraformaldehyde-Sorensen's phosphate buffer and decalcified by 10% EDTA, then paraffin embedded. Three-μm sections were stained with rabbit anti-SRSF3 antibody (Abcam) or anti-mouse c-Mpl antibody (R&D), biotinylated anti-rabbit antibody and streptavidin-conjugated Alexa Fluor 597 or Alexa Fluor 488 (Thermo Fisher) and imaged with Olympus BX51 microscope.

Isolation of peripheral blood cells and spleen cells

Peripheral blood was collected as a throat or retro-orbital bleed and cells counted (Sysmex KX-21N). For platelets analysis, whole blood or platelets were harvested by centrifugation and counted as above. Platelets were labelled with AF700-conjugated rat anti-mouse CD41

(Biolegend). Spleens were dissected, weighed and mashed through a 40µm cell strainer. The cells were washed and collected by centrifugation. Cells and platelets in the spleen were counted as above.

Platelet activation assays

Blood was obtained by cardiac puncture into 0.1vol of Aster-Jandl anticoagulant (85mM sodium citrate, 69mM citric acid, 20mg/ml glucose, pH 4.6) followed by centrifugation of the supernatant buffy coat. Platelets were washed twice (140mM NaCl, 5mM KCl, 12mM trisodium citrate, 10mM glucose, and 12.5mM sucrose, pH 6.0) and resuspended in 10mM Hepes, 140mM NaCl, 3mM KCl, 0.5mM MgCl2, 10mM glucose, and 0.5mM NaHCO3, pH 7.4. The platelet count was determined by flow cytometry normalising to FACS calibration beads (Spherotech). Platelets were treated with single agonists ADP (12.5-50µM), Convulxin (12.5-50ng/ml), PAR4-AP (0.125-0.25mM) or thrombin (0.0625-0.25U/ml) in the presence of 1mM CaCl2 and activation of the αIIbβ3 integrin (JON/A) or P-selectin (Emfret Analytics) exposure was assessed by flow cytometry. Diluted whole blood was recalcified, rested at room temperature, treated with PAR4-AP and platelet activation was analysed as above.

Analysis of platelet lifespan

Washed platelets were labelled with 5µM Cell Trace Violet (CTV) or 0.5µM Carboxyfluorescein diacetate succinimidyl ester (CFDA SE/CSFE), quenched with 20% FBS and injected *i.v*. into C57/Bl6 mice. Peripheral blood samples were collected and analysed by flow cytometry. Platelets were labelled *in vivo* by 0.15µg/g anti-mouse CD42C-X488 (Emfret Analytics) (*i.v*.) and whole blood was analysed as above. Transient thrombocytopenia was induced by *i.p*. administration of anti-platelet serum (APS, Cedarlane) and blood samples were collected. Whole blood was labelled with 0.1µg/ml Thiazole orange (TO, Biolegend) and

AF700-conjugated rat anti-mouse CD41 antibody (Biolegend), fixed in 1% paraformaldehyde and washed before flow cytometric analysis.

Analysis of platelet apoptosis

Caspase3/7 activity was measured from purified platelets with Caspase Glo-3/7 assay (Promega). To measure phosphatidylserine exposure on the plasma membrane, purified platelets were incubated with AnnexinV-FITC (BD Pharmingen), diluted with Annexin V buffer and directly analysed by flow cytometry[1].

RNA immunoprecipitation in MEG-01 cells

MEG-01 megakaryoblast cells maintained in RMPI 1640 medium with 10% FBS at 37°C 5% CO2 were transduced with lentiviral pCCL-SRSF3-GFP or pCCL-GFP vector and GFP-positive cells isolated by FACS. The GFP-positive cells were lysed [0.5% NP40, 10mM Tris/pH 7.5, 150mM NaCl, 0.5mM EDTA, cOmplete Protease inhibitors (Roche), 1:1,000 RNaseOUT (Thermo Fisher), 10mM beta-glycerol phosphate]. Five-% of the lysate was saved as an input and the remaining lysate incubated with GFP-Trap magnetic beads (Chromotek). The beads were washed (50mM Tris-HCl pH 7.5, 150mM NaCl and 0.05% Nonidet P-40), RNA isolated using TriReagent (Sigma-Aldrich), DNaseI treated (Promega) and reverse transcribed with SuperScript III (Thermo Fisher). Quantitative PCR was performed in QuantStudio6 real-time PCR system using Luminaris HiGreen qPCR Master Mix-low ROX (Thermo Fisher) and 0.3μM primers (Supplemental Table 1). The cycle threshold values (CT) were calculated and immunoprecipitation normalised to input.

Western Blotting

For Western blots, cells or dissected mouse tissues were lysed (50mM Tris-HCl pH 8, 150mM NaCl, 1% Nonidet P40, 0.5% Sodium deoxycholate, 0.1% SDS). NuPAGE 4–12% gradient Bis-Tris gel system (Thermo Scientific) was used. The nitrocellulose membranes were probed with anti-SRSF3 (Sigma-Aldrich), anti-GAPDH (Abcam) and/or anti-β-ACTIN (Abcam), followed by HRP-conjugated secondary antibodies (Biorad). The blots were developed using Amersham ECL Western Blotting Detection Reagents (GE Healthcare) and visualized with the Biorad ChemiDoc MP Imaging System.

Bioinformatics

The raw reads were trimmed using Trimmomatic[2] with default parameters. The reads were aligned with STAR to Ensembl mm10 reference genome. The aligned files were deduplicated using the Picard MarkDuplicates function, [Picard Toolkit, http://broadinstitute.github.io/picard/; Broad Institute]. To obtain levels of gene expression, read counts were generated with the Subread function FeatureCounts[3]. Genes with 1 count per million (CPM) in at least two samples were kept for further analysis. Normalisation and differential gene expression were conducted with EdgeR.

Torrent Suite Software v5.6 (Thermo Fisher) was used for read processing, base calling, alignment to the reference transcriptome (mm10) and gene expression counts. Differential gene expression analysis was performed using DESeq2. Combined wildtype and Cre-negative *Srsf3*$^{fl/fl}$ samples were used as control for the analysis. Gene ontology enrichment analysis were conducted with DAVID.

SinEx DB 2.0 database[4] was used to identify single exon genes and Biomart tool (GRCm39) to identify alternatively spliced transcripts that were affected by SRSF3 depletion.

In the GRCm39 mouse genome annotation, 17,790 out of 21,885 protein coding genes (81.3%) have more than one isoform.

Supplemental References

1. Josefsson EC, James C, Henley KJ, et al. Megakaryocytes possess a functional intrinsic apoptosis pathway that must be restrained to survive and produce platelets. *J Exp Med*. 2011;208(10):2017-2031.
2. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30(15):2114-2120.
3. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930.
4. Jorquera R, González C, Clausen PTLC, Petersen B, Holmes DS. SinEx DB 2.0 update 2020: database for eukaryotic single-exon coding sequences. *Database*. 2021;2021.

Supplemental Tables

Supplemental Table 1. Primers used in this study.

Gene name	Primer sequence
SRSF3	Fwd AACGGGCTTTTGGCTACTATG Rev TTCACCATTCGACAGTTCCAC
HPRT	Fwd CTGAGGATTTGGAAAGGGTGT Rev GTAATCCAGCAGGTCAGCAAA
ITGA2B	Fwd TCCTGGCGGCTATTATTTCTT Rev TCGAAGTACTCTGGGTTGCTG
ITGB3	Fwd CACGTGCTGACGCTAACTGAC Rev CATCATTCCTCCAGCCAATCT

MPL	Fwd CCTCACTTCCAGACCTGCAC Rev GGGTTCCACTTCTTCACAGGT
GP6	Fwd TAGGAGTATCACCGCCAGTCC Rev CCGCCAGGATTATTAGGATCA
GP1BA	Fwd TCCATGGGGCTAGAAGAGAGA Rev CAAGAGGAGGAGAGGCATGAG
TUBB2B	Fwd CATTCGGCCAGATCTTCAGAC Rev CTCCTTCCTCACCACATCCAG
FLNA	Fwd CCTTCGAGGTGTACGTGGAT Rev CTCTACCGTGCCCTTCTGTC
NBEAL2	Fwd TGCACCCTATGGATACAGCAC Rev GGGCAAACTCATGGTCAAATAC
18S rRNA	Fwd TTGTTGGTTTTCGGAACTGAG Rev GCAAATGCTTTCGCTCTGGTC

Supplemental Table 2. Lists of differentially expressed genes in *Pf4-Srsf3*$^{\Delta/\Delta}$ megakaryocytes and platelets. See a separate file.

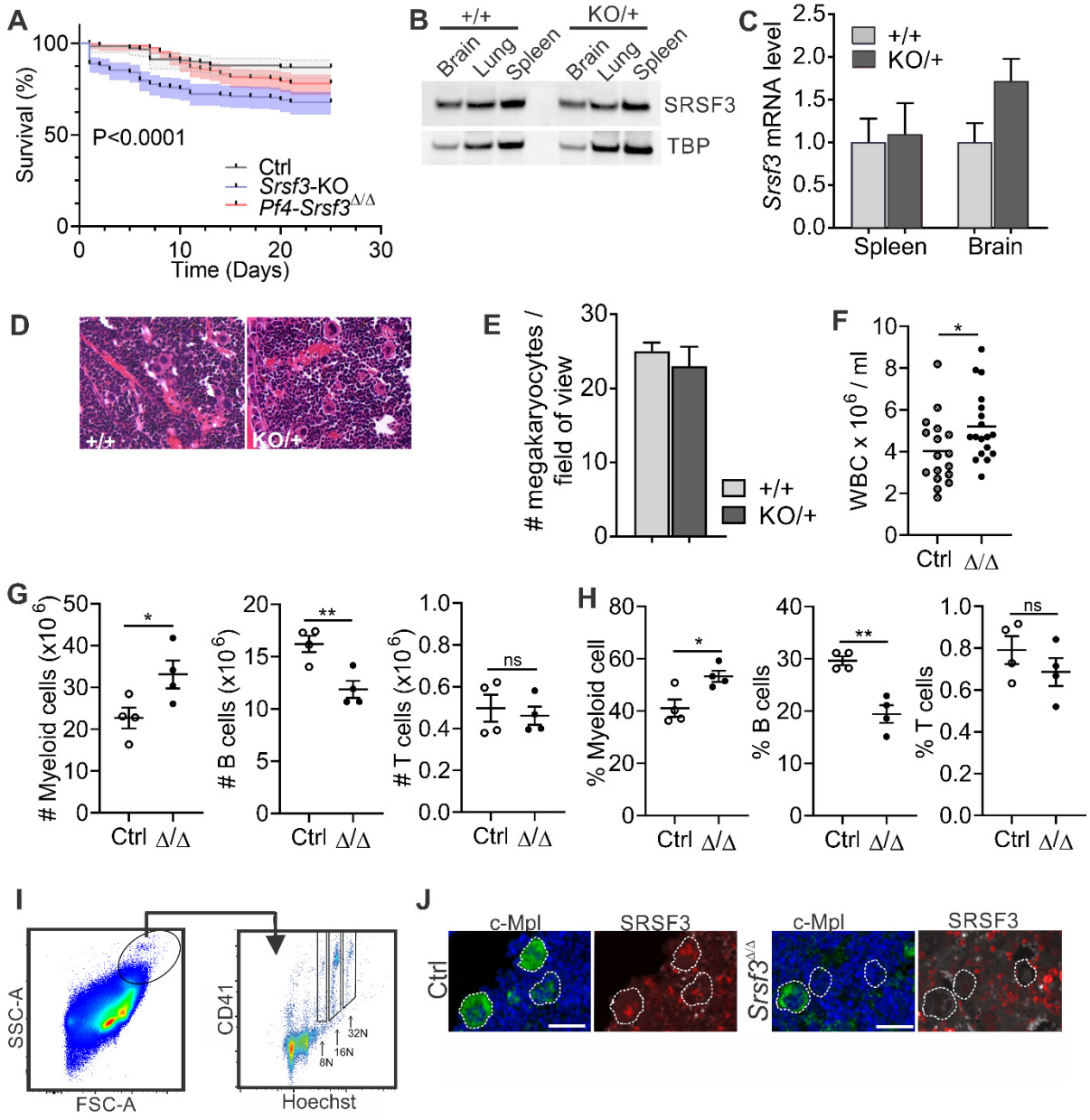
Supplemental Table 3. Comparison of Ctrl and *Pf4-Srsf3*$^{\Delta/\Delta}$ RNA abundancies to the most abundant platelet RNAs in a previous study by Rowley *et al.* 2011.

Rank and gene name in Rowley et al.	Rank in Ctrl	Rank in *Pf4-Srsf3*$^{\Delta/\Delta}$
1. *Nrgn*	11	92
2. *Ppbp*	3	4

3. *Myl9*	39	225
4. *Pf4*	8	7
5. *Gng11*	7	293
6. *Tubb1*	13	490
7. *Itga2b*	18	10
8. *Srgn*	20	35
9. *Gp9*	87	158
10. *Alox12*	152	345
11. *Clec1b*	33	134
12. *Aldh2*	26	36
13. *Plek*	19	38
14. *Lyz2*	23	62
15. *Atp2a3*	66	46
16. *Rgs18*	42	582
17. *Alox5ap*	129	744
18. *Treml1*	219	155
19. *Stx11*	384	na
20. *Rap1b*	35	185
21. *P2ry12*	15	834
22. *Itgb3*	78	99
23. *Arhgdib*	32	9
24. *Fhl1*	71	208
25. *Vwf*	93	95
26. *Laptm5*	27	14

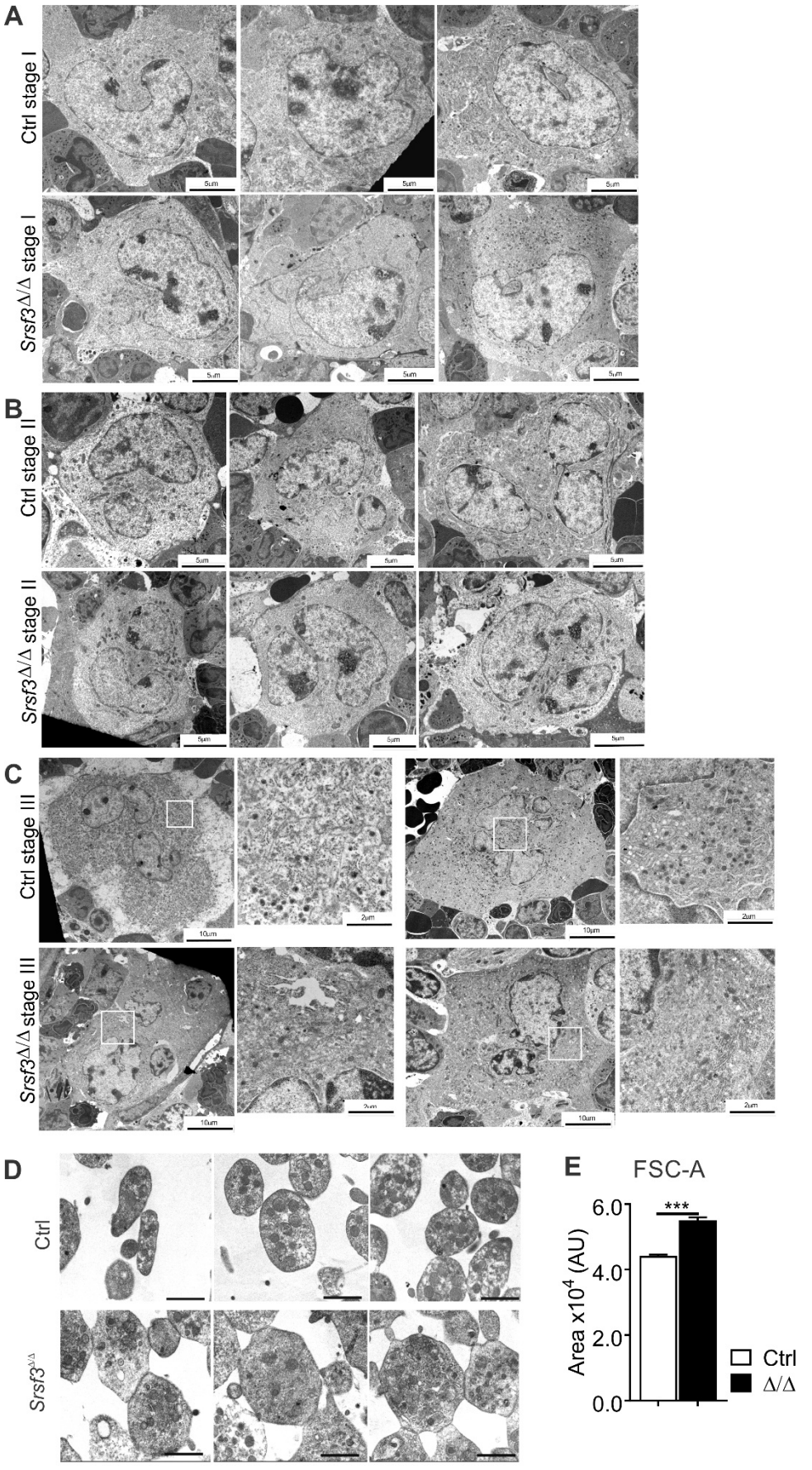
27. *F5*	34	147
28. *Rasgrp2*	155	272
29. *Hist1h2bc*	na	na
30. *Gp5*	na	na
31. *Slc2a3*	50	456
32. *Arhgap10*	41	145
33. *Cxx1c*	365	2476
34. *Sh3bgrl2*	94	1034
35. *Cd226*	29	581
36. *Prkar2b*	532	1183
37. *Treml2*	206	4131
38. *Mmrn1*	106	377
39. *Sdpr*	na	na
40. *F13a1*	96	677

Supplemental Figures



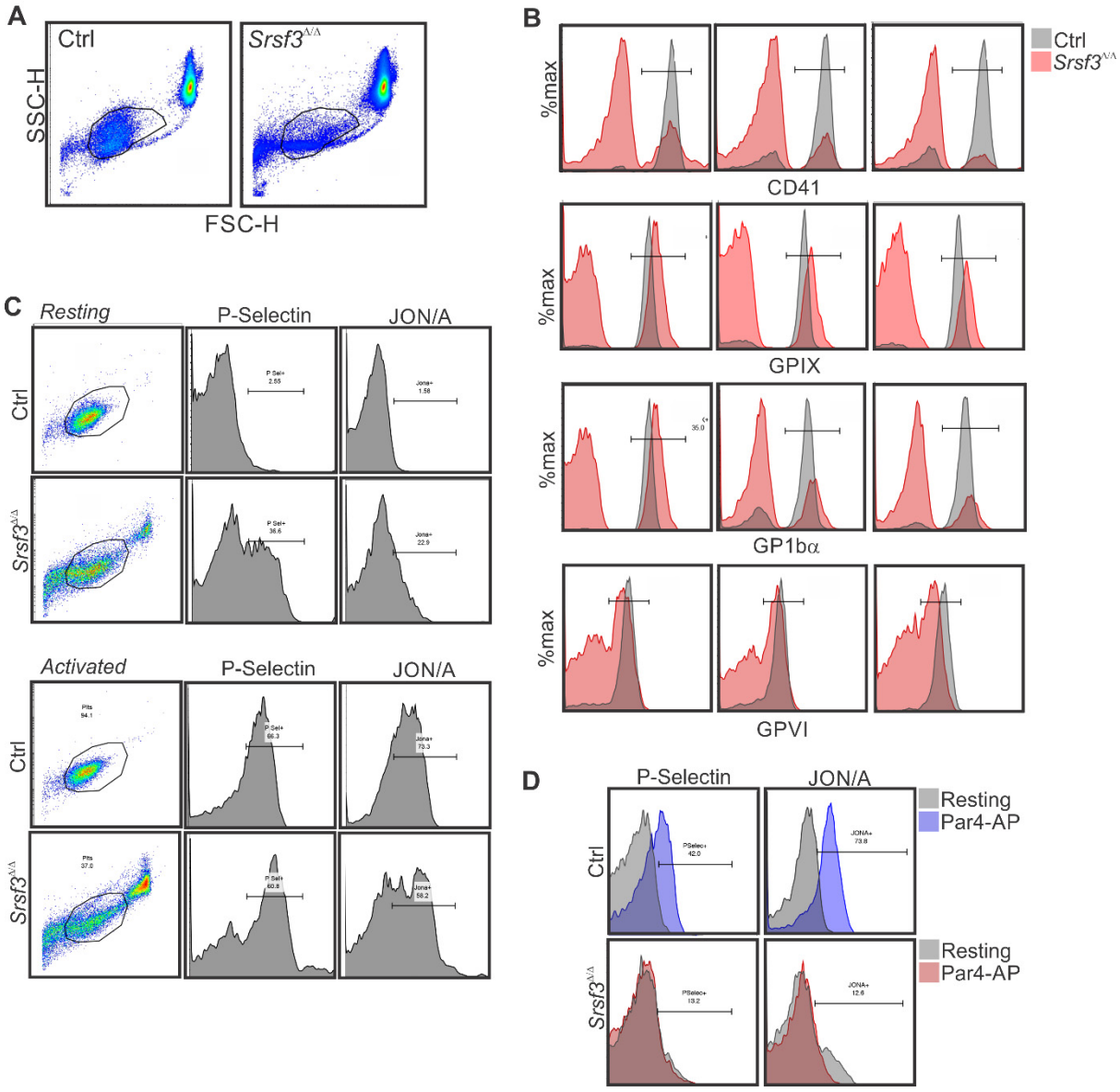
Supplemental Figure 1. (A) Kaplan-Meier plot depicting the survival rate of control, *Srsf3*-KO (KO/+) and *Pf4-Srsf3*$^{\Delta/\Delta}$ mice. *Srsf3*-KO heterozygotes were crossed with wildtypes and Cre-positive *Pf4-Srsf3*$^{\Delta/\Delta}$ homozygotes were crossed with Cre-negative *Pf4-Srsf3*$^{\Delta/\Delta}$ homozygotes. Log-rank statistics, error bars as 95% confidence interval. (B) Western blot depicting no change in SRSF3 protein levels in heterozygous *Srsf3*-KO (KO/+) and control mice. Three representative tissues are shown. (C) RT-qPCR analysis of *Srsf3* mRNA levels in

heterozygous *Srsf3*-KO (KO/+) and control mice. (D) Haemotoxylin and Eosin (H&E) staining of heterozygous *Srsf3*-KO (KO/+) bone marrow and (E) quantification of megakaryocyte numbers from the H&E stained sections. (F) White blood cell (WBC) counts in the peripheral blood of control and *Pf4-Srsf3*$^{\Delta/\Delta}$ mice. (G) The absolute number per one hind limb and (H) incidence of myeloid, B and T cells in control and *Pf4-Srsf3*$^{\Delta/\Delta}$ bone marrow. (I) Gating strategy for the analysis and isolation of megakaryocytes of different ploidy. (J) Immunohistochemistry of control and *Pf4-Srsf3*$^{\Delta/\Delta}$ bone marrow sections depicting the expression of c-MPL (green), DAPI (blue) and SRSF3 (red). Three MK are highlighted with a dashed outline for clarity. Scale bar 100µm. Students two-tailed unpaired t-test $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, ns=not significant. Ctrl=control mice, Δ/Δ=*Pf4-Srsf3*$^{\Delta/\Delta}$ mice and KO=*Srsf3* systemic knockout mice.

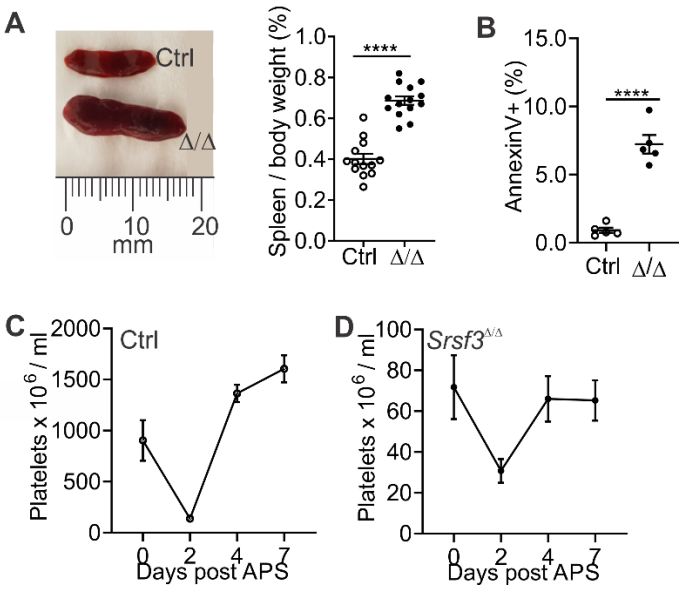


Supplemental Figure 2. (A-C) Representative TEM images of control and *Pf4-Srsf3*$^{\Delta/\Delta}$

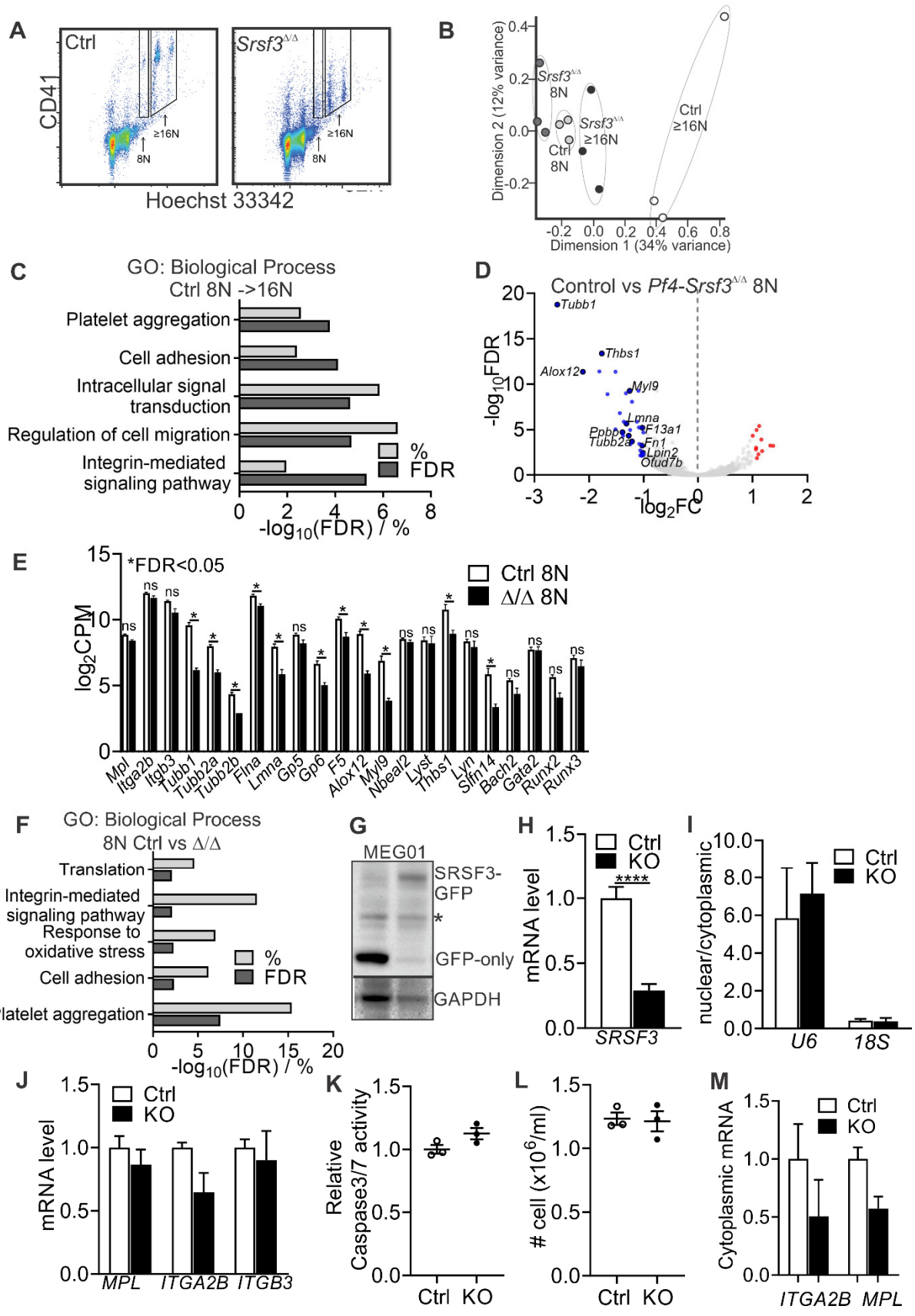
megakaryocytes at different stages of maturation. In C, a higher magnification of the boxed areas are shown. (D) Representative TEM images of control and *Pf4-Srsf3*$^{\Delta/\Delta}$ platelets. (E) Platelet size as measured by forward scatter area (FSC-A) in *Pf4-Srsf3*$^{\Delta/\Delta}$ and control mice. Students two-tailed unpaired t-test ***$P \leq 0.001$, Ctrl=control mice and Δ/Δ=*Pf4-Srsf3*$^{\Delta/\Delta}$ mice.



Supplemental Figure 3. (A) Platelet SSC-H/FSC-H profile in whole blood. (B) Platelet surface receptor expression in whole blood. The platelet gate is shown in A. Three biological replicates are shown in the histograms and the gates indicate marker positive populations. (C) P-selectin and JON/A cell surface levels in resting and activated (Thrombin, 0.25U/ml) control and *Pf4-Srsf3*$^{\Delta/\Delta}$ washed platelets. The platelet gate and a histogram of a representative mouse is shown. (D) P-selectin and JON/A cell surface levels in resting and Par4-AP activated control and *Pf4-Srsf3*$^{\Delta/\Delta}$ platelets in whole blood. A histogram of a representative mouse is shown. The platelet gate is shown in A.

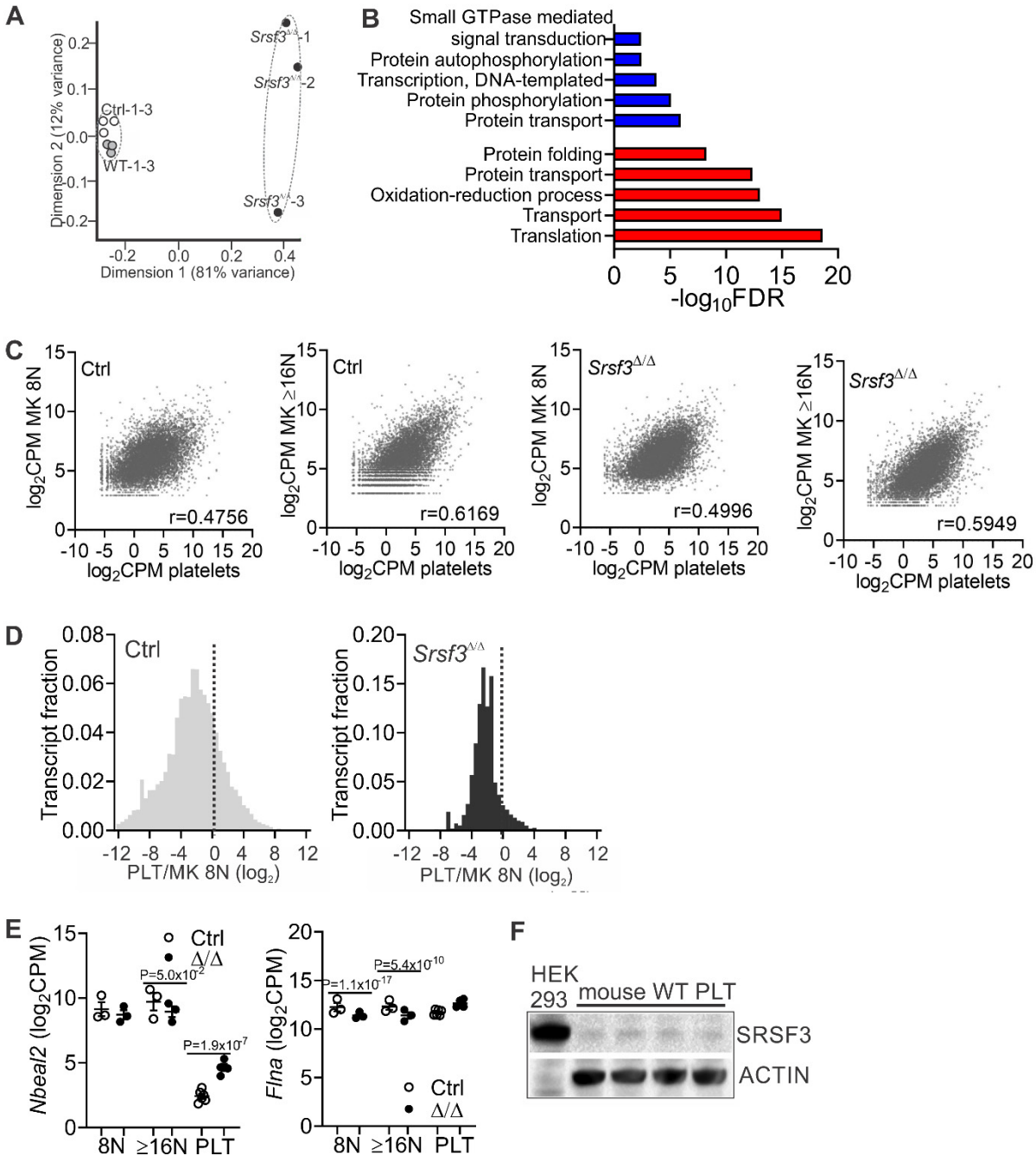


Supplemental Figure 4. (A) Spleen size relative to the total body weight of control and *Pf4-Srsf3*$^{\Delta/\Delta}$ mice. A representative image of control (above) and *Pf4-Srsf3*$^{\Delta/\Delta}$ (below) spleen. (B) The fraction of AnnexinV-positive control and *Pf4-Srsf3*$^{\Delta/\Delta}$ platelets. Platelet counts in (C) control and (D) *Pf4-Srsf3*$^{\Delta/\Delta}$ mice following anti-platelet (APS) challenge. The data is presented as mean ± SEM. Students two-tailed unpaired t-test, ****$P \leq 0.0001$, Ctrl=control mice and Δ/Δ=*Pf4-Srsf3*$^{\Delta/\Delta}$ mice.



Supplemental Figure 5. (A) Gating strategy for the sorting of 8N and ≥16N megakaryocytes for RNA-sequencing. (B) MDS plot depicting the similarity of individual megakaryocyte

RNA-sequencing samples. (C) Significantly enriched GO terms (Biological Process) among DEGs between control 8N and ≥16N megakaryocytes (FDR<0.05, FC>2). The x-axis depicts %genes and -$\log_{10}$(FDR) of each category. (D) Volcano plot depicting DEGs between control and *Pf4-Srsf3*$^{\Delta/\Delta}$ 8N megakaryocytes as in Figure 5A. (E) Expression of genes encoding proteins central for megakaryocyte structure and function in control and *Pf4-Srsf3*$^{\Delta/\Delta}$ 8N megakaryocytes. (F) Significantly enriched GO terms (Biological Process) among DEGs between control and *Pf4-Srsf3*$^{\Delta/\Delta}$ 8N megakaryocytes (FDR<0.05, FC>2). The x-axis depicts %genes and -$\log_{10}$(FDR) of each category. (G) Western blot analysis of SRSF3-GFP or GFP expression in MEG-01 megakaryoblast cell lines, *nonspecific band. (H) Quantification of *SRSF3* mRNA levels in MEG-01 cells following SRSF3 deletion by CRISPR/Cas9 gene editing. (I) Quantification of U6 snRNA and 18S rRNA levels in nuclear and cytoplasmic fractions in MEG-01 cells following SRSF3 deletion by CRISPR/Cas9 gene editing. The data is presented as nuclear/cytoplasmic ratios. (J) Quantification of *MPL, ITGA2B* and *ITGB3* mRNA levels in MEG-01 cells following SRSF3 deletion by CRISPR/Cas9 gene editing. Quantification of (K) Caspase 3/7 activity and (L) cell numbers in MEG-01 cells following SRSF3 deletion by CRISPR/Cas9 gene editing. (M) Quantification of cytoplasmic *MPL* and *ITGA2B* mRNA levels in MEG-01 cells following SRSF3 depletion. The data is normalised to total mRNA levels and presented relative to control. Ctrl=MEG-01 cells targeted with scrambled control guide RNA, KO=MEG-01 cells targeted with SRSF3 guide RNA. The data is presented as mean ± SEM. Students two-tailed unpaired t-test, ****$P \leq 0.0001$.



Supplemental Figure 6. (A) MDS plot depicting the similarity of platelet RNA-sequencing samples. (B) Significantly enriched KEGG pathways among RNAs of different abundance in control and *Pf4-Srsf3*$^{\Delta/\Delta}$ platelets (FDR≤0.05). The blue bars depict downregulated RNAs in *Pf4-Srsf3*$^{\Delta/\Delta}$ platelets and the red bars upregulated RNAs. The x-axis denotes %genes and -log$_{10}$(FDR) of each category. (C) Pearson correlation between 8N or ≥16N megakaryocyte and platelet RNA levels. CPM=counts per million. (D) Frequency distribution histograms of

platelet/megakaryocyte RNA abundance ratio (platelet CPM/MK 8N CPM) in control and *Pf4-Srsf3*$^{\Delta/\Delta}$. MK=megakaryocyte, PLT=platelet. (D) *Nbeal2* and *Flna* mRNA levels in control and *Pf4-Srsf3*$^{\Delta/\Delta}$ 8N and ≥16N megakaryocytes and platelets. (E) Western blot depicting the absence of SRSF3 protein in wildtype platelets. HEK293 cells serve as a positive control for SRSF3 expression.