Supplemental Data

Downregulation of GATA1 drives impaired hematopoiesis in primary myelofibrosis

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Supplemental Tables 1 and 2

Supplemental Figures 1 through 16

Supplemental Methods

Table S1: PMF patient information

Patient #	Gender	Age	Mutations	Risk category
1	Male	65	TN	Int-1
2	Female	70	JAK2 V617F	Int-2
3	Female	74	JAK2 V617F	Int-2
4	Male	68	JAK2 V617F	Int-2
5	Male	87	JAK2 V617F	Int-2
6	Male	51	*	Low risk
7	Male	66	JAK2 V617F	Int-2
8	Male	74	CALR (type 2)	High risk
9	Female	37	JAK2 V617F	Low risk
10	Male	75	JAK2 V617F	Int-2
11	Male	64	JAK2 V617F	Int-1
12	Male	49	CALR c.1147_1156del/insTGTCGG; p.E383fs*46	Low risk
13	Male	56	JAK2 V617F	Int-2
14	Female	78	MPLW515L	Int-2
15	Male	63	JAK2 V617F	Unknown
16	Female	48	TN	Low risk
17	Male	83	JAK2 V617F	Int-2
18	Male	69	CALR c.1135_1147del; p.E379fs*47	Int-2
19	Female	66	JAK2 V617F	Int-1
20	Male	60	JAK2 V617F	Int-2
21	Male	69	JAK2 V617F	Int-1
22	Male	73	CALR (type 1)	Int-2
23	Male	68	*	Int-1
24	Female	70	JAK2 V617F	Int-2
25	Male	59	CALR (type 1)	Int-1
26	Female	65	JAK2 V617F	Int-1
27	Male	70	TN	High risk
28	Male	72	JAK2 V617F	High risk
29	Female	70	JAK2 V617F	Int-2
30	Female	60	JAK2 V617F	Int-1

TN, triple negative
* JAK2 and MPL WT; CALR not determined

Table S2: PV patient information

Patient #	Gender	Age	Mutation
PV-1	Female	58	JAK2 V617F
PV-2	Male	68	JAK2 exon 12
PV-3	Male	65	JAK2 V617F
PV-4	Male	48	JAK2 V617F
PV-5	Female	79	JAK2 V617F
PV-6	Male	60	JAK2 V617F
PV-7	Male	74	JAK2 V617F
PV-8	Male	54	JAK2 V617F
PV-9	Male	69	JAK2 V617F
PV-10	Male	38	JAK2 V617F

A

**B**

C

D

Hoechst
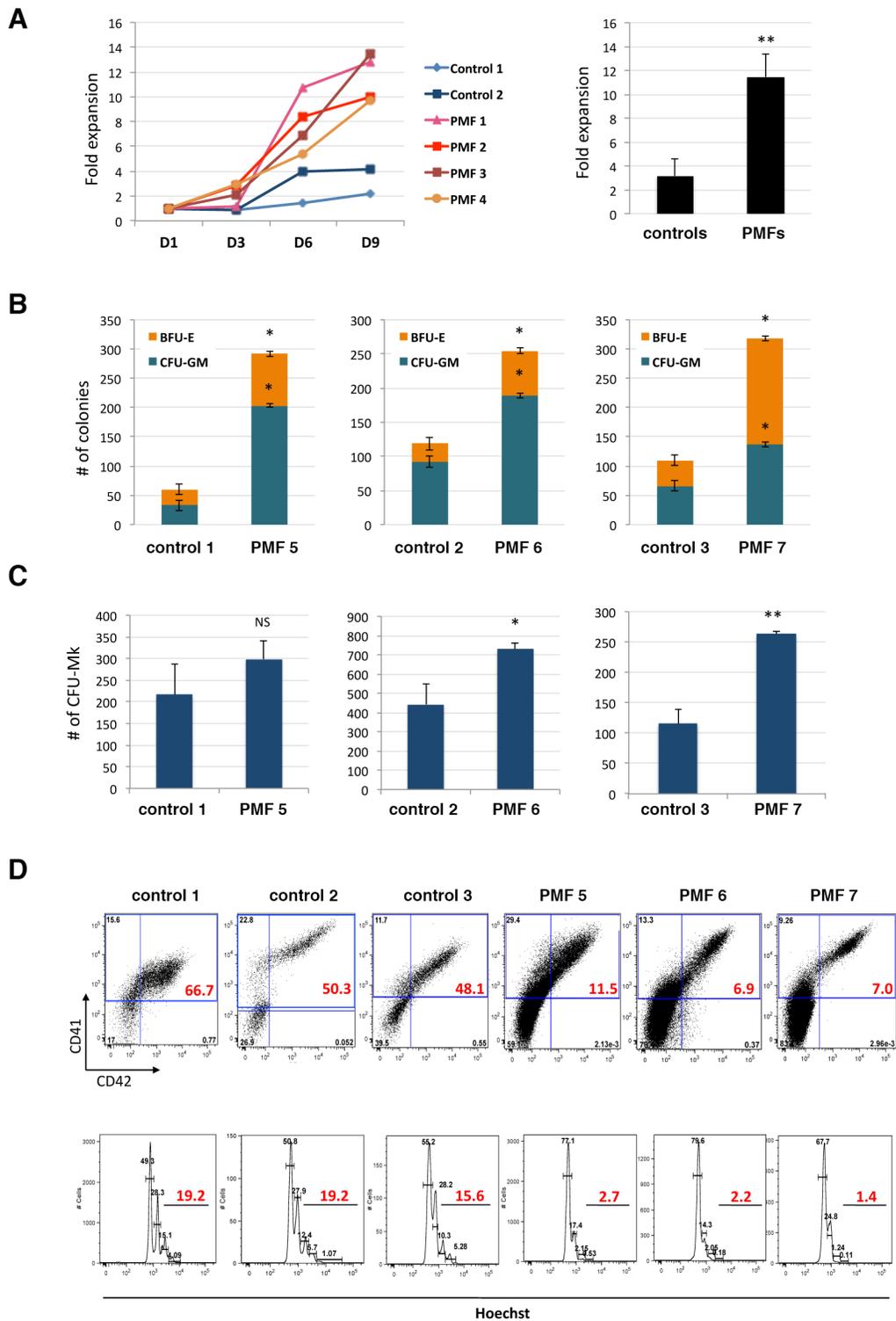
Supplemental Figure 1

Supplementary figure 1: PMF megakaryocytes show impaired differentiation.

(a) The numbers of cells in culture were enumerated at days 1, 3, 6 and 9. CD34$^+$ cells from healthy donors were amplified 3.2 fold, while cells from PMF patients expanded 11.45 fold. The bar graph depicts mean ± SD (n=2 controls and 4 PMF samples). ** p<0.01 by Student's t test. **(b)** CD34$^+$ cells from healthy donors or PMF patients were plated in methylcellulose. After 12 days, the total numbers of colonies were counted. Data are shown as mean ± SD (n=3). * p<0.05 by Student's t test. **(c)** CD34$^+$ cells from healthy donors or PMF patients were plated in MegaCult. After 12 days, slides were stained and CFU-Mks were enumerated. Bar graphs depict mean ± SD (n=3). * P<0.05, ** p<0.01 by Student's t test. **(d)** CD34$^+$ from healthy donors or PMF patients were cultivated in media without serum and supplemented with 10ng/ml THPO. After 8 days, differentiation was assessed by flow cytometry using CD41 and CD42 surface marker staining. On average, a 6.5-fold reduction in megakaryocyte differentiation was observed (n=3, p=0.008). Hoechst staining combined with CD41 staining was used to determine the ploidy level. In control megakaryocytes, we observed an average of 18 ± 2% of cells with a ploidy ≥8N compared to 2.08 ± 0.67% in PMF culture (n=3, p=0.002). The numbers in red refer to the percentage of CD41$^+$CD42$^+$ cells (top) or the percentage of cells with DNA content ≥8N (bottom).
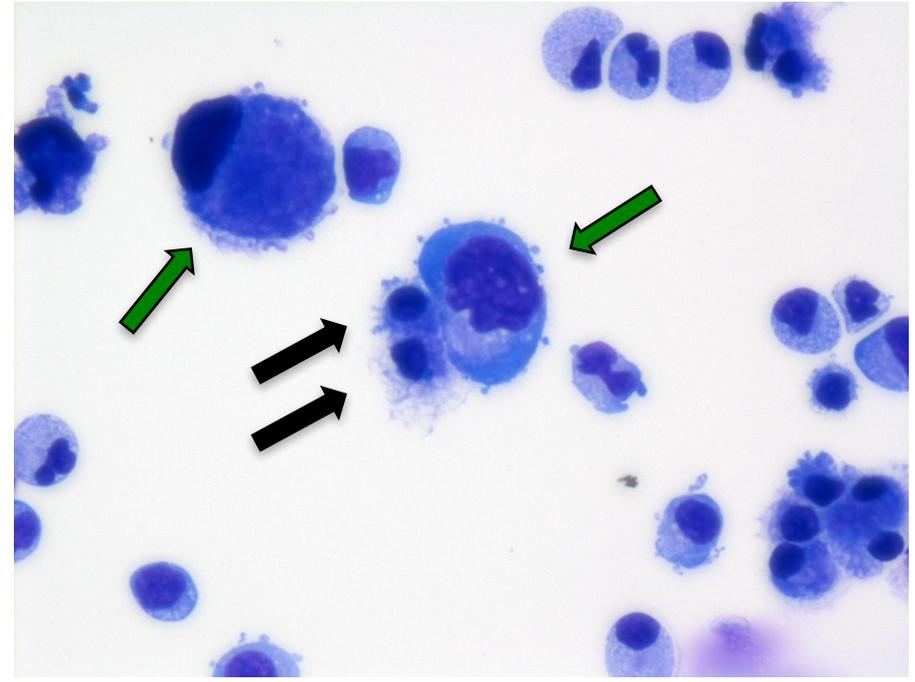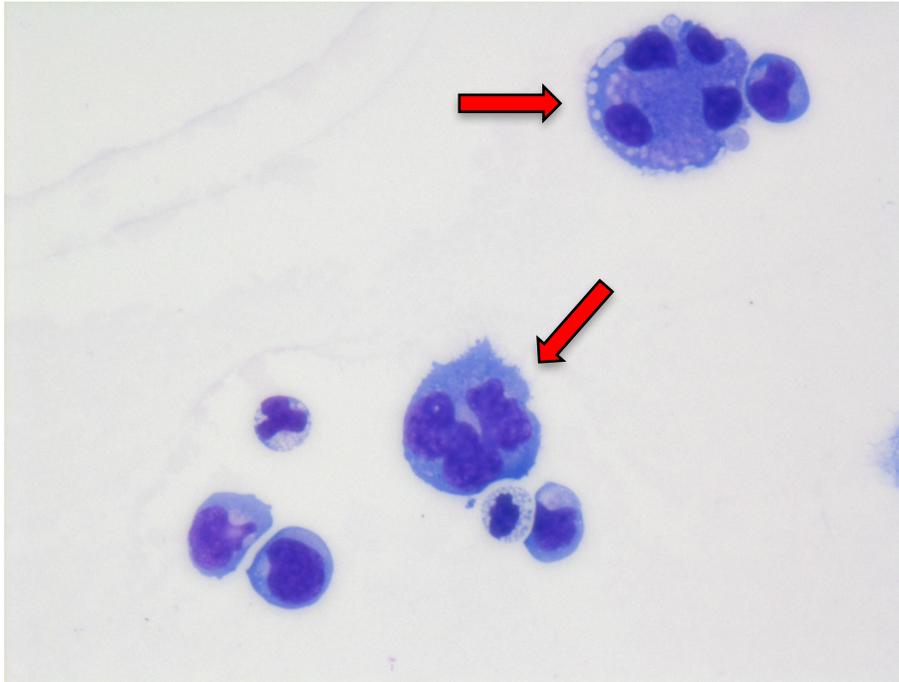
Supplementary Figure 2: Abnormal morphology of megakaryocytes derived from PMF CD34+ cells. Cells in cultures of control (left) or PMF (right) CD34+ cells were cytospun onto slides and stained with Giemsa. Red arrows point to highly polyploid, well differentiated megakaryocytes. Green arrows point to large megakaryocytes that are unilobular or have an abnormal, bulbous nucleus. Black arrows point to small megakaryocytes with membrane blebbing. Original magnification 600X.
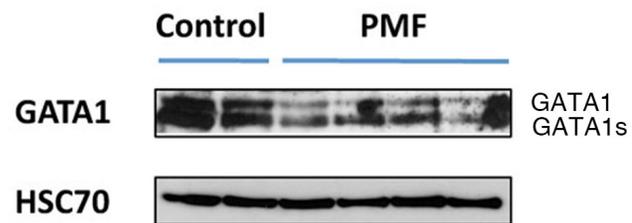
Supplementary Figure 3: GATA1 protein levels are reduced in PMF megakaryocytes. Western blot assay for GATA1 and the loading control HSC70 in control or PMF megakaryocytes. CD34$^+$ cells from 2 healthy donors and 4 PMF patients (PMF 17-20) were cultured ex vivo in 10ng/mL THPO and sorted for CD41$^+$ cells after 8 days in culture
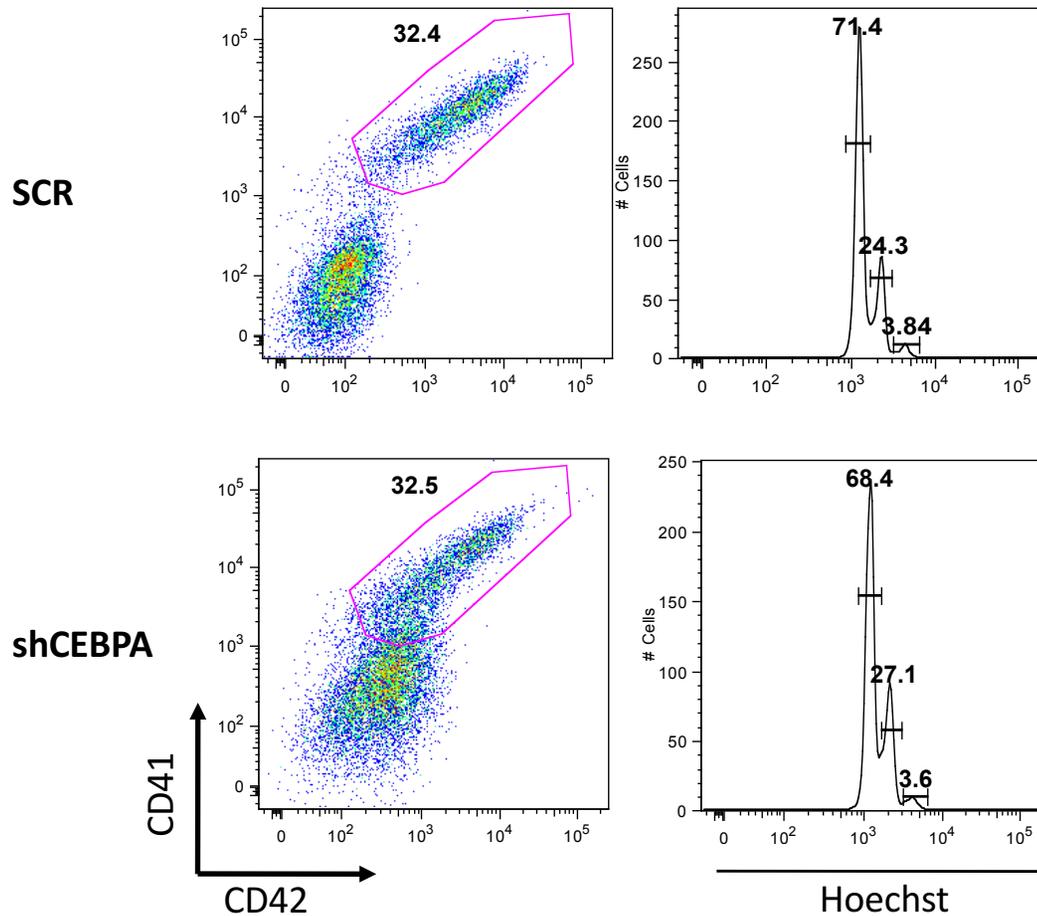
Supplementary Figure 4: Reducing the expression of CEBPA cannot rescue differentiation defect of PMF megakaryocytes. CD34+ from PMF patients were transduced with viruses harboring the shCEBPA or a control hairpin. After 8 days in 10ng/ml THPO, differentiation was assessed by flow cytometry using CD41 and CD42 surface marker staining. Ploidy was determined by Hoechst staining and was gated on the CD41+ population. One of two representative experiments, with sample PMF 23, is shown.
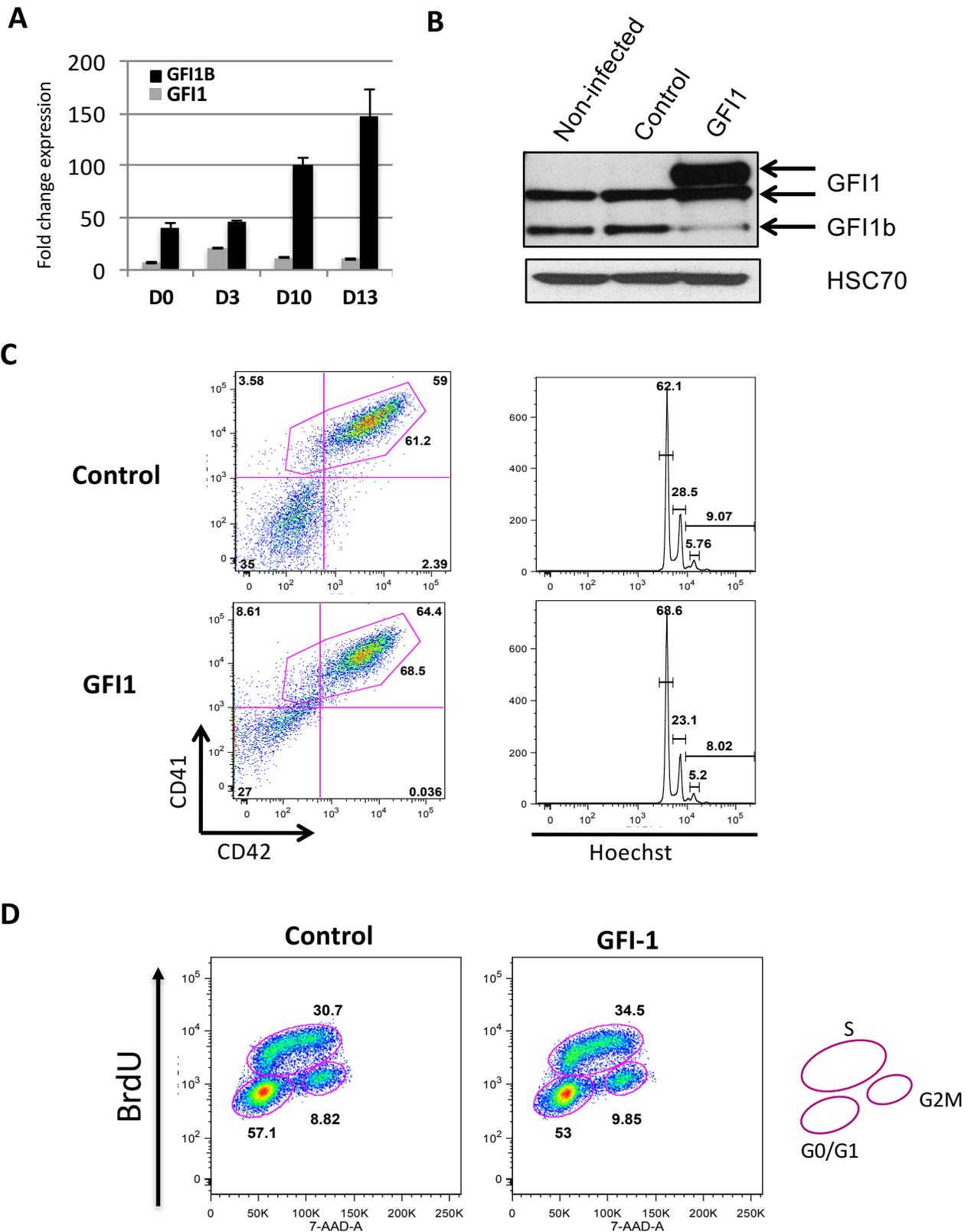
Supplementary Figure 5: Overexpression of GFI1 does not impair megakaryopoiesis.
A) Primary CD34+ cells from healthy donors were cultivated in presence of TPO and collected at different time point of culture. qRT-PCR (normalized to HPRT) was used to determine the expression level of *GFI1* and *GFI1B* during normal megakaryopoiesis. Bars depict means ± SD of three technical replicates. Data are representative of two experiments B) Overexpression of GFI1 was confirmed by western blotting of extracts from the SET2 cell line transduced with control of the GFI1 lentiviral constructs. C) CD34+ cells were transduced with a control lentivirus or GFI1 overexpressing vector and differentiation was assessed at day 8 of culture in 10ng/ml THPO by flow cytometry. One representative experiment of two is shown. D) The effect of GFI1 overexpression on cell proliferation was tested in SET2 cells. No significant difference was observed. One representative experiment of three is shown.
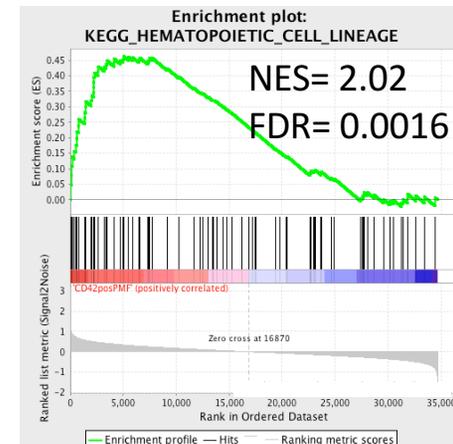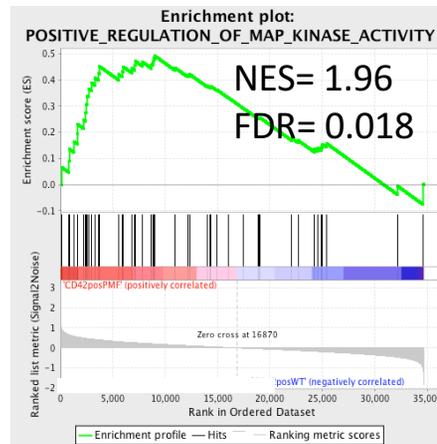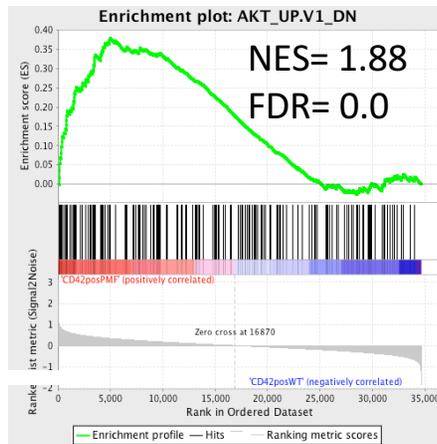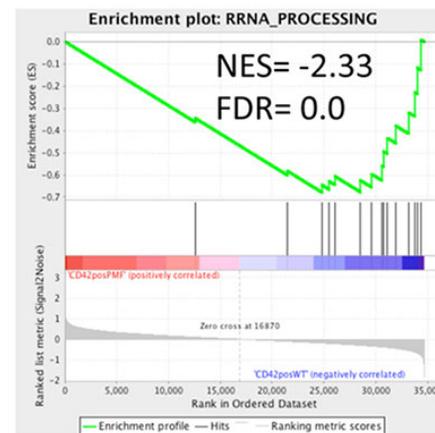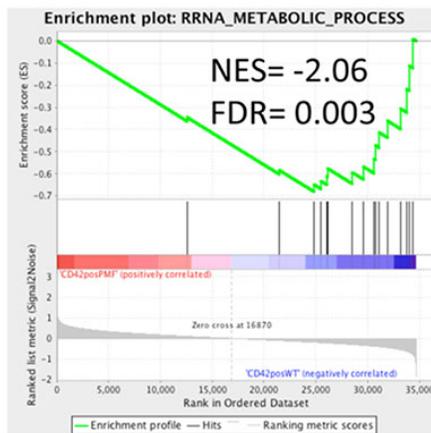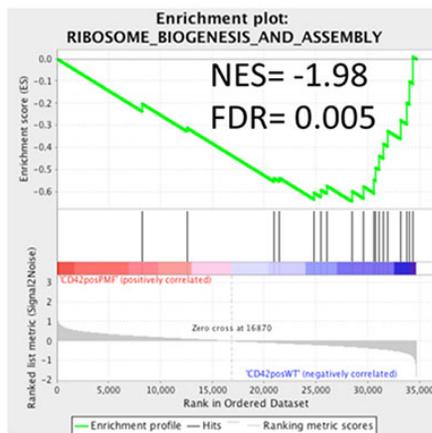
Supplementary Figure 6: GSEA analysis of PMF gene expression data. A) GSEA analysis revealed that AKT, MAP Kinase and hematopoietic cell lineage are enriched in PMF megakaryocytes. B) Multiple ribosome pathways are negatively enriched in PMF specimens.
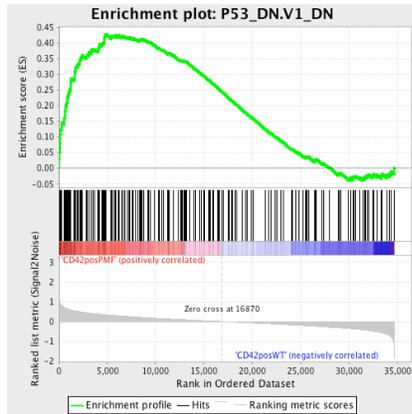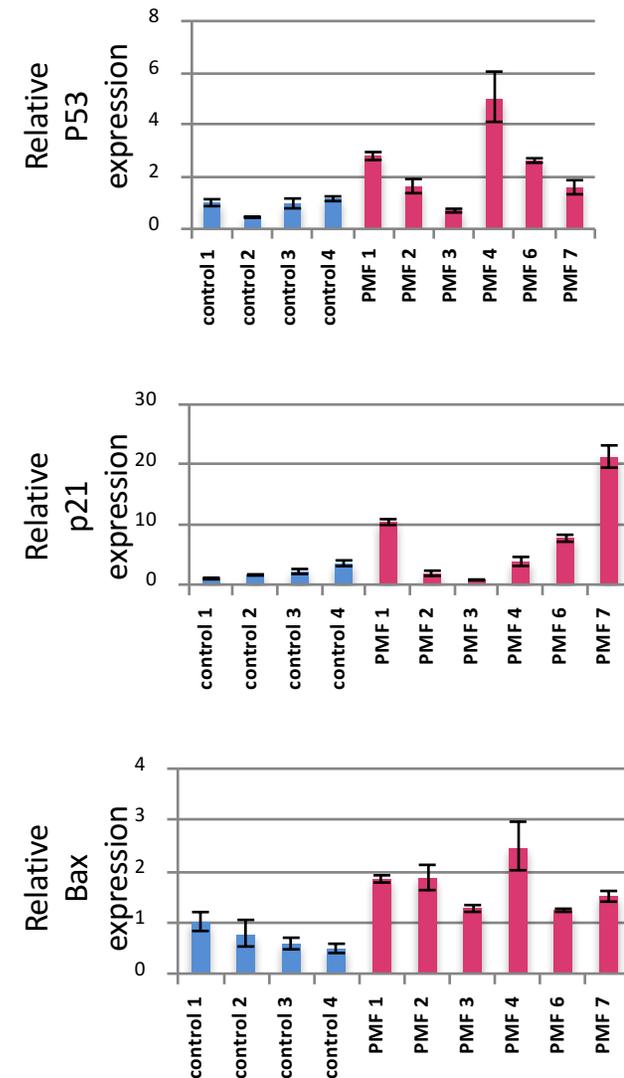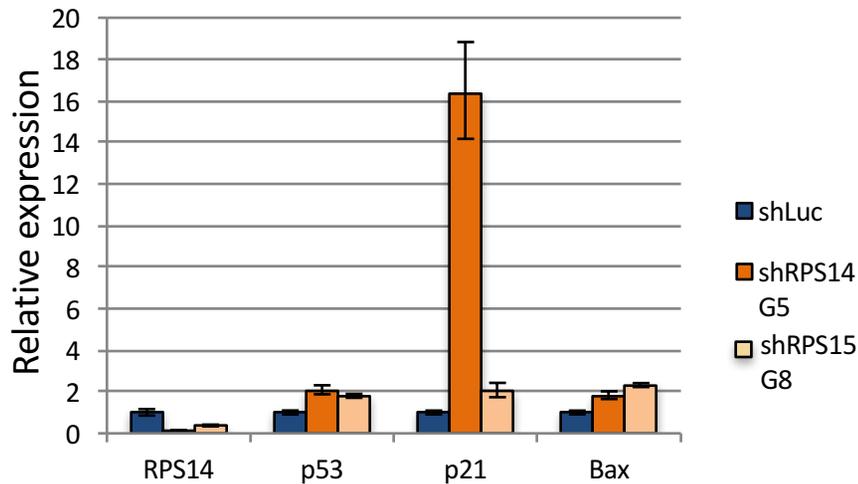
Supplemental Figure 7: The p53 pathway is upregulated in PMF megakaryocytes. a) GSEA analysis revealed that the p53 pathway is up-regulated in PMF megakaryocytes. b) qRT-PCR (normalized to HPRT) determination of *p53*, *p21* and *BAX* expression in CD41⁺CD42⁺ cells derived from normal and PMF samples. Bars depict means ± SD of three technical replicates. c) Expression of *RPS14*, *p53*, *p21* and *Bax* genes in SET2 cells after over-expression of a control shLuc, shRPS14G5 or shRPS14G8. qRT-PCR results are expressed relative to *HPRT*. Bars depict means ± SD of three technical replicates. Data are representative of two experiments.
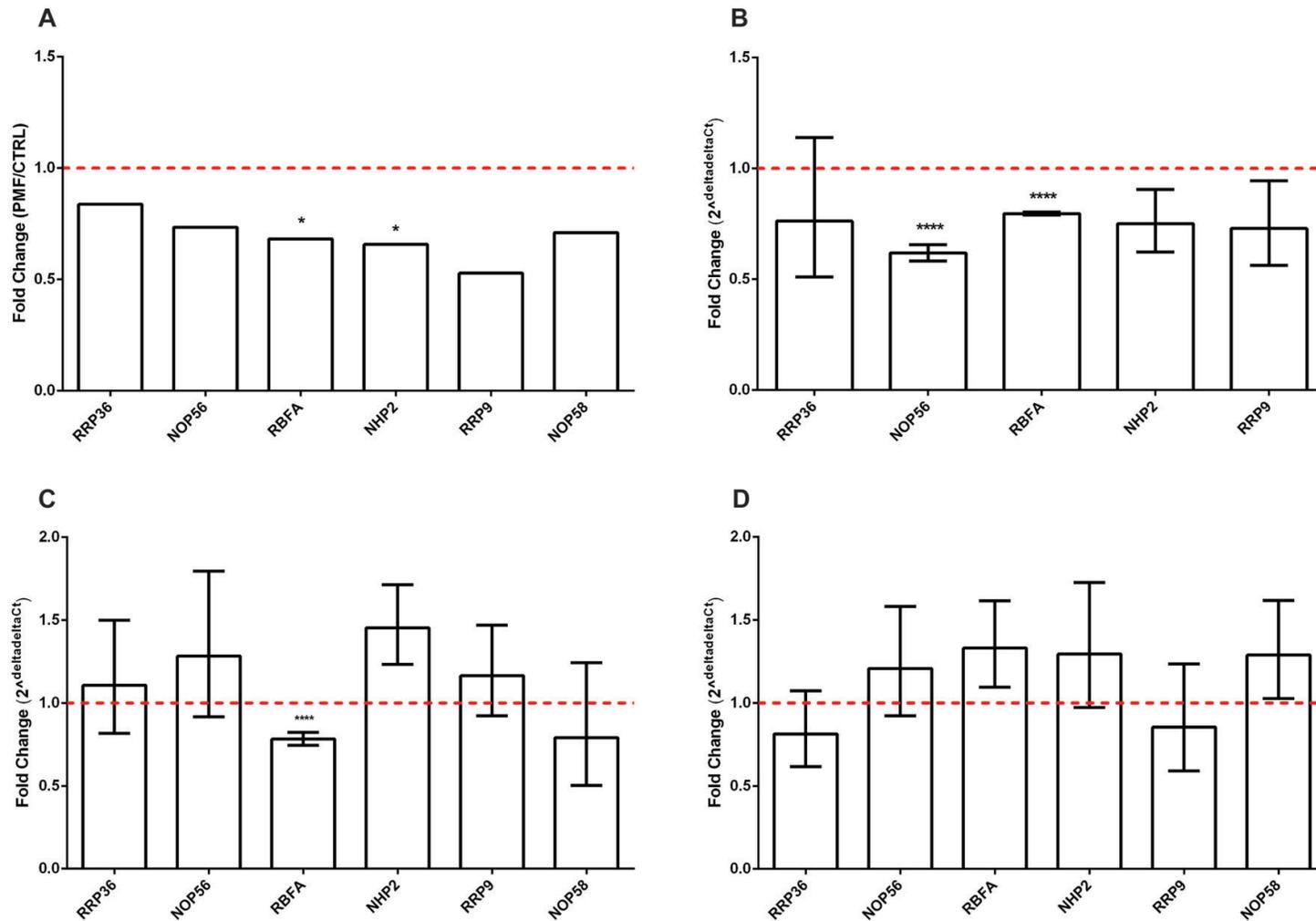
Supplementary Figure 8: Expression of ribosome associated genes is specifically decreased in megakaryocytes. A) Microarray data of expression of the different genes in megakaryocytes derived from PMF CD34+ cells relative to control cells. B,C,D) qRT-PCR for various ribosome associated genes in megakaryocytes (PMF 8,9) (B) and erythroid cells (C) derived from PMF CD34+ cells (PMF 26,27) relative to control cells, and in mononuclear cells (D) from PMF patients (PMF 28,29) or healthy individuals. Bars represent means +/- SEM, n=2. * p<0.05, **** p<0.0001 by Student's t-test.
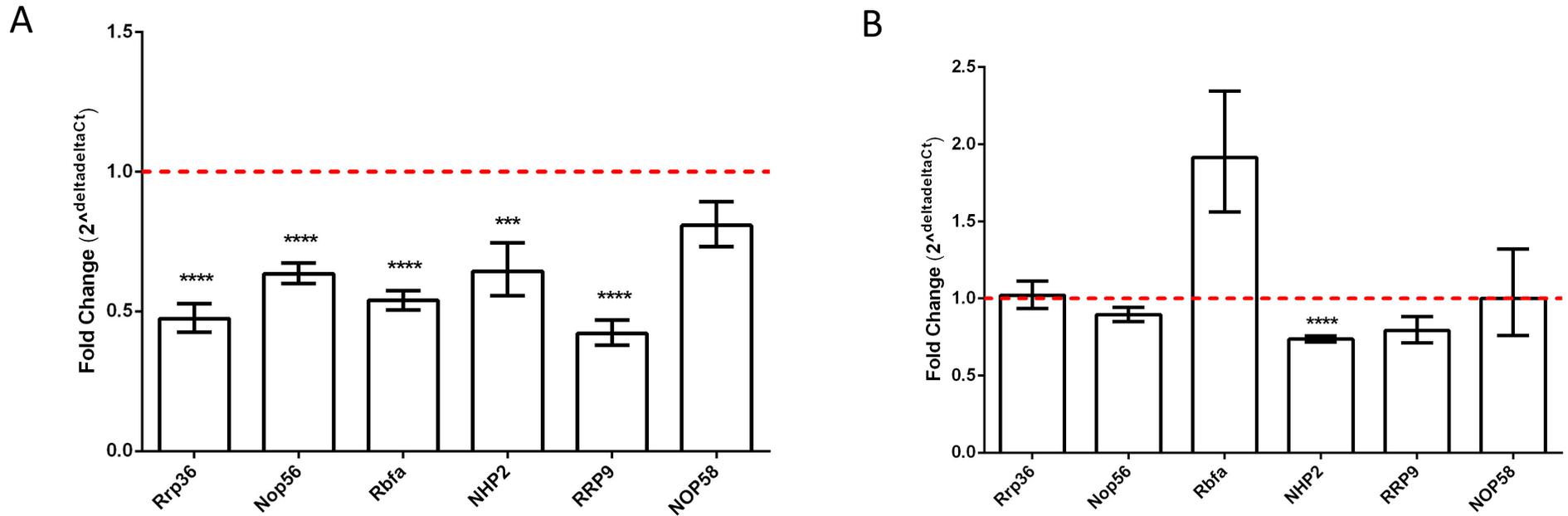
Supplementary Figure 9: Defects in ribosomal gene expression in PV is restricted to megakaryocytes. A) qRT-PCR analysis of expression of various ribosome associated genes in megakaryocytes derived from PV or control CD34$^+$ cells. Patients PV1-5 (Table S2) are included here. (B) qRT-PCR analysis of expression of various ribosome associated genes in erythroid cells derived from PV or control CD34$^+$ cells. Patients PV6-8 (Table S2) are included here. Bars represent means +/- SEM, n=5 (A) and n=3 (B). **** p<0.0001 by Student's t-test.
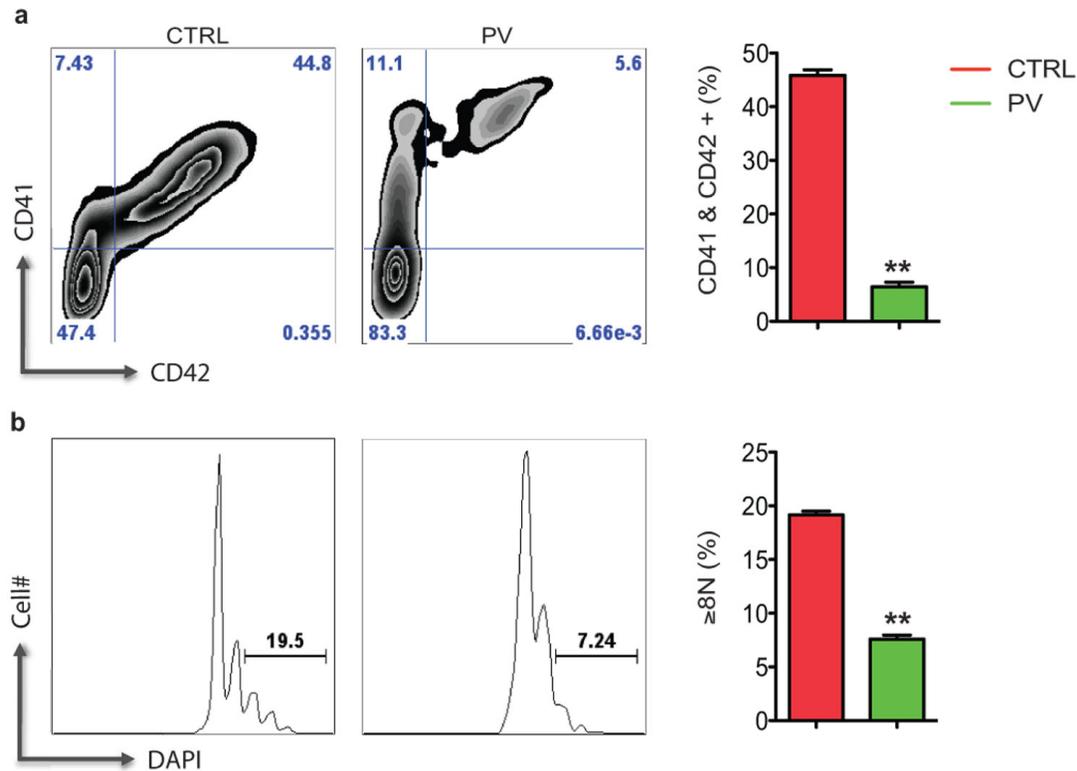
Supplementary Figure 10: Differentiation of PV CD34+ cells reveals impaired megakaryopoiesis. CD34+ cells were purified from frozen mononuclear cells of healthy controls or PV patients and expanded in the presence of human IL-6, SCF and FLT3L for 10 days. The CD34+ cells were then cultured in the presence of 50ng/ml human THPO for 8 days. The expression of CD41 and CD42 (a), and DNA content (b) were analyzed by flow cytometry. Results are representative of 2 samples in each group. Bars represent means +/-SEM ** p<0.01, compared to control group by Student's t-test. Patients PV9,10 are included here.
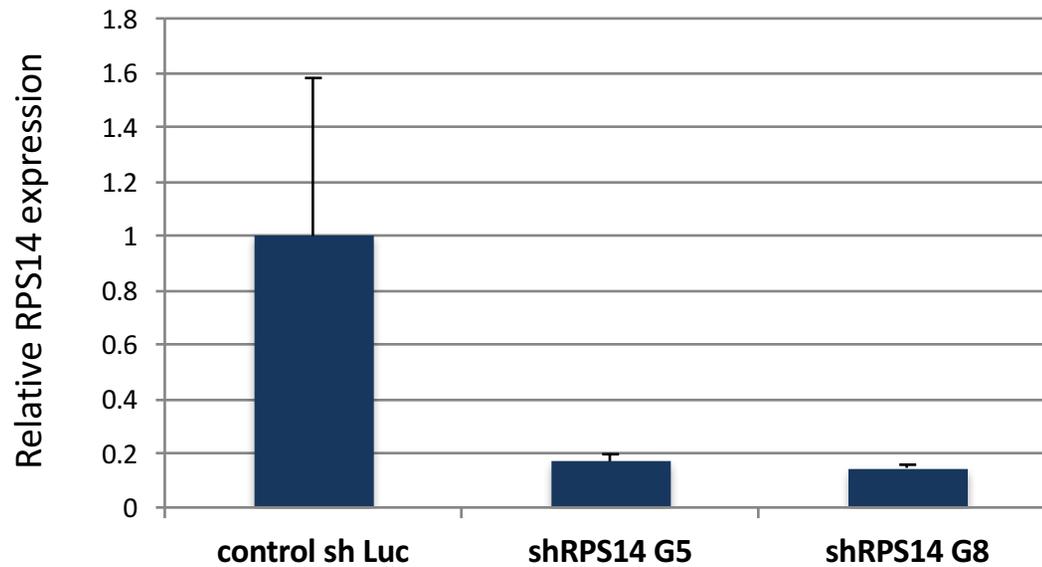
Supplementary Figure 11: shRPS14 efficiency in primary CD34+ cultures.

CD34+ cells were transduced with shRPS14 G5, shRPS14 G8 or control vector shLuciferase (sh Luc). After 8 days of culture, qRT-PCR analysis (normalized to HPRT) revealed a 6.3±0.7 fold reduction in *RPS14* expression in both populations. Bars depict mean ± SD. Results are representative of two experiments.
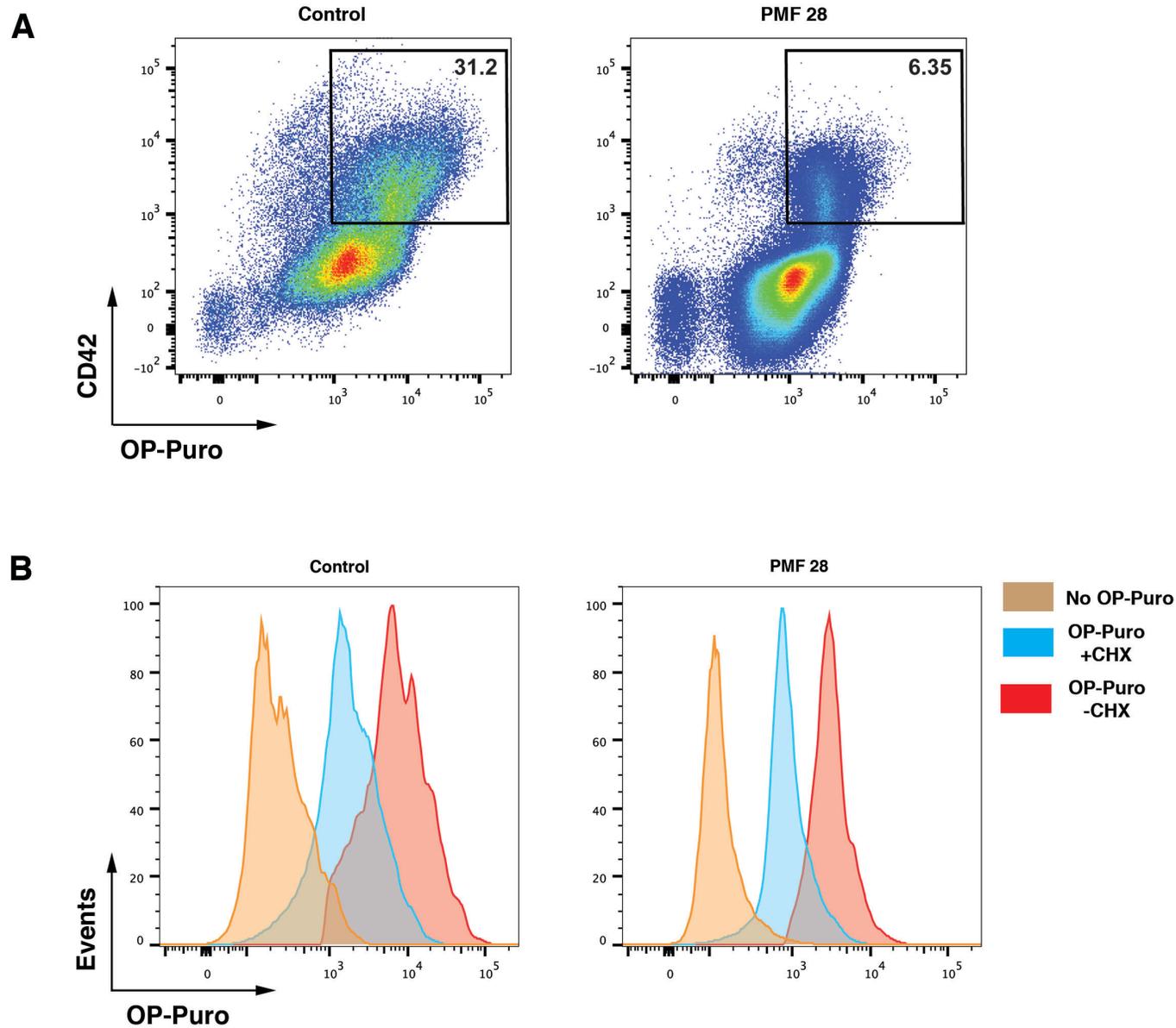
Supplementary Figure 12: Gating strategy and controls for protein synthesis assay. A) Percentage of CD42+ cells within cultures of control or specimen PMF-28. The prominent decline in CD42+ cells in the PMF sample is consistent with Figure S1. B) Flow cytometry of populations without OP-Puro (brown), OP-Puro and cycloheximide (blue), and OP-Puro without cycloheximide (red) illustrate that the assay for protein synthesis is robust.
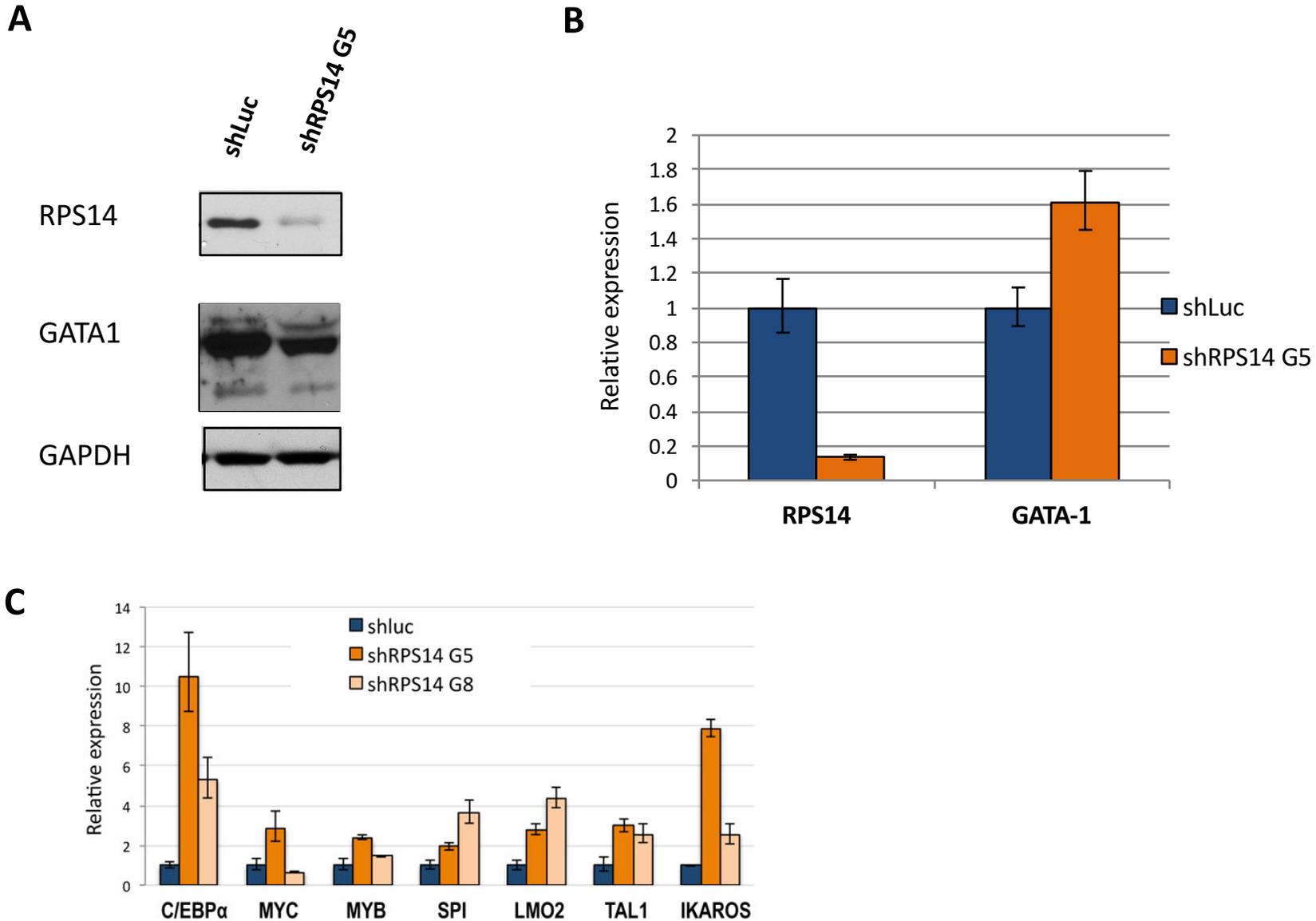
Supplementary Figure 13: The reduction of RPS14 leads to a decrease in GATA1 protein and aberrant myeloid gene expression. a) SET2 cells were transduced with shLuc and shRPS14 G5. Western blotting confirmed a decrease in RPS14 and a decrease in GATA1. b) qRT-PCR (normalized to HPRT) further confirmed the reduction in RPS14, but demonstrated an increase in GATA1 transcript. The bar graph depicts mean ± SD of three technical replicates. One of two representative experiments is shown. c) qRT-PCR (normalized to HPRT) for a number of myeloid genes. The bar graph depicts mean ± SD of three technical replicates. One of two representative experiments is shown.
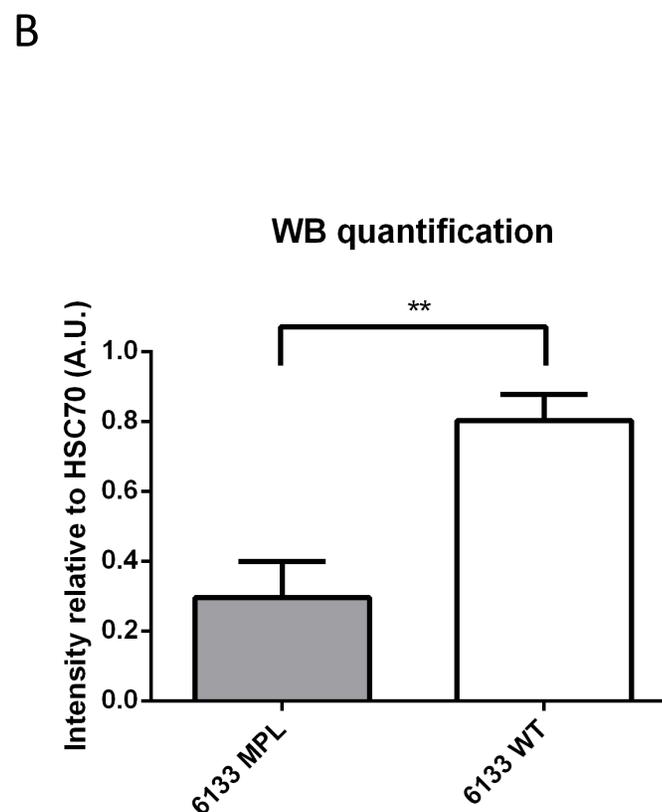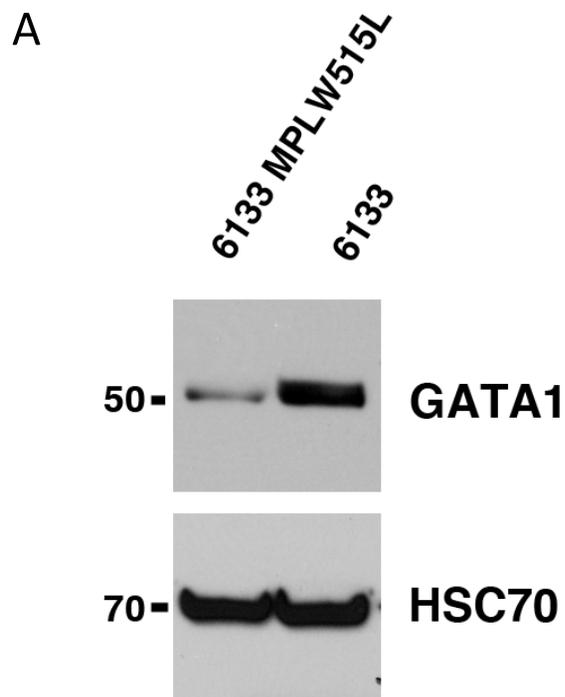
**Supplementary Figure 14: Activation of JAK/STAT signaling is sufficient to reduce expression of GATA1.** a) Western blot for GATA1 and HSC70 in extracts from 6133 cells and those cells stably expressing MPLW515L. b) Relative levels of GATA1 in 6133 (6133 WT) versus 6133 MPLW515L (6133 MPL) cells. The graph depicts the averages +/- SEM for quantitation of three independent western blots. ** p<0.01 by Student's t-test
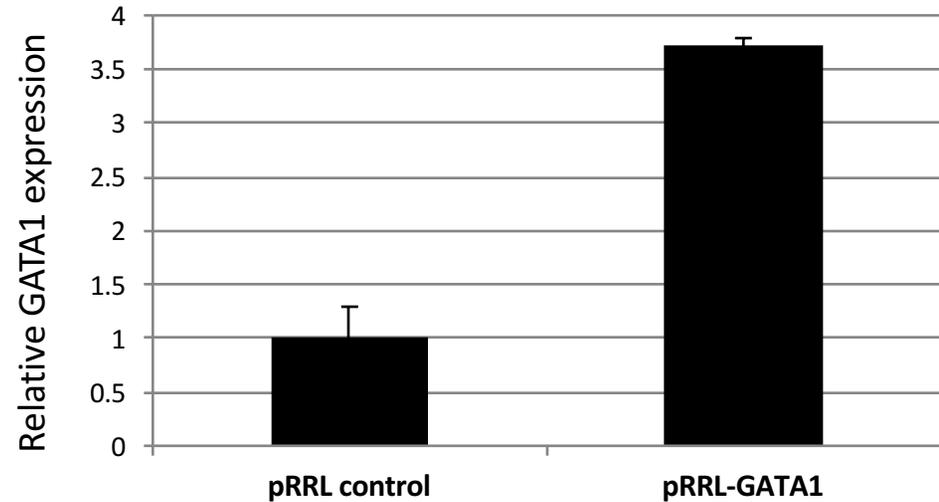
Supplementary Figure 15: Validation of GATA1 overexpression upon transduction with a GATA1 expression construct. a) Depiction of the lentiviral construct. GATA-1 cDNA was subcloned into the pRRL-EF1α-PGK-GFP vector, in which GATA1 expression is controlled by the EFIα promoter, while GFP is expressed under the PGK promoter. b) CD34+ cells were transduced by pRRL-GATA1 or control pRRL. After 8 days of culture GFP+ cells were sorted and qRT-PCR (normalized to HPRT) was used to verify the efficiency of GATA1 over-expression. Bars depict mean ± SD of three technical replicates. One of two representative experiments is shown.
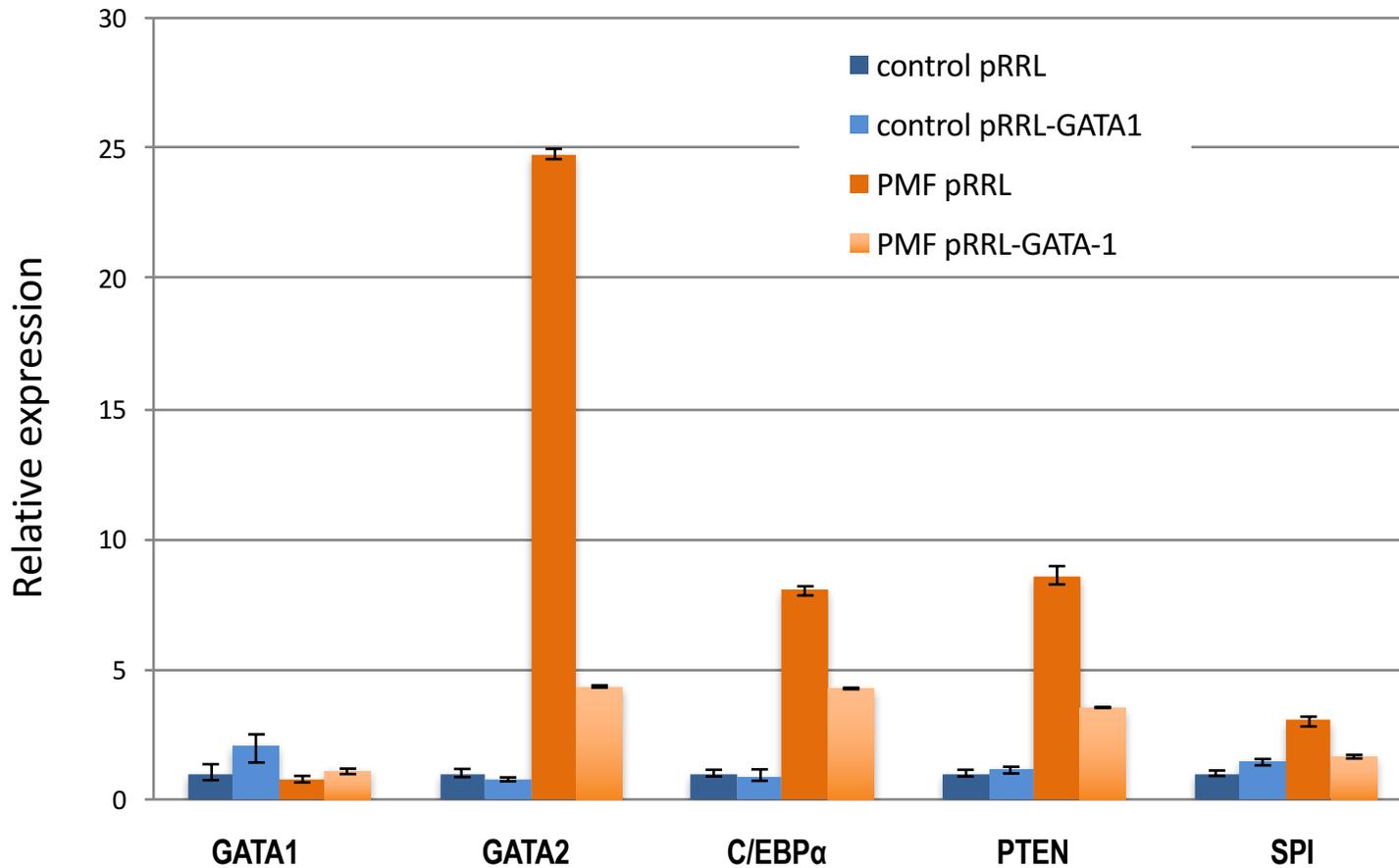
Supplementary Figure 16: Restoration of GATA1 normalizes myeloid gene expression in PMF megakaryocytes. CD34^{+} cells transduced with pRRL control or pRRL-GATA1. GFP^{+} cells were sorted on GFP after 9 days of culture in 10ng/ml THPO. qRT-PCR was performed and the results are normalized to HPRT. Bars depict mean $\pm$ SD of three technical replicates. One of two representative experiments is shown.

Supplemental Methods

***In vitro* growth of megakaryocytes from CD34$^+$ cells**

After isolation of mononuclear cells from the peripheral blood of PMF patients by ficoll gradient, CD34$^+$ cells were positively selected with magnetic beads (Miltenyi). For liquid culture, CD34$^+$ cells were plated in IMDM supplemented with 100U/ml penicillin/streptomycin, 2mM l-glutamine, 76nM α-monothioglycerol, 1.5% BSA, 20μg/ml lipids (Sigma), insulin transferrin selenium (Gibco) and 10ng/mL recombinant human thrombopoietin, and cultivated at 37˚C with 5% CO$_2$. For the progenitor assays, 1500 cells were plated in methylcellulose-based colony assays (Methocult H4435, Stem Cell Technologies) and CFU-GM and BFU-E colonies were counted on day 12. For the CFU-Mk assay, 5000 cells were plated in MegaCult (#04901, Stem Cell Technologies). After 12 days, megakaryocyte colonies were stained with anti-CD41 antibody and enumerated.

Cell lines

The human *JAK2*V617F mutant megakaryocytic SET2 cell line, obtained from Dr. Ross Levine and confirmed to be mycoplasma free, were cultured in RPMI1640 (Life Technology), 20% FBS, 100 U/ml penicillin-streptomycin, and 2mM l-glutamine. 293T cells were maintained in DMEM (Life Technology) 10% FBS, 100 U/ml penicillin-streptomycin, and 2mM l-glutamine. The megakaryocytic leukemia cell line 6133 was obtained from Dr. Thomas Mercher (Institut Gustave Roussy, Villejuif, France) (1). Cells were transduced with MIGR1-*MPL*W515L and sorted for GFP to derive the 6133/MPLW515L line.

Cell sorting and flow cytometry

For the differentiation assay and for sorting of megakaryocytes, cells were incubated for 30 min at 4°C with an APC conjugated anti-CD41a (#559777, BD Pharmingen) and a PE conjugated anti-CD42a (# 558819, BD Pharmingen). For the ploidy analysis, Hoechst 33342 (Life Technology) was directly added to the cell culture at 0.01mM and cells were incubated for 2 hours at 37˚C with 5% CO_2. Proliferation was assessed using the FITC BrdU Flow kit (BD Pharmingen), following the manufacturer's protocol. Acquisitions were performed on a LSRII (BD Bioscience) and were analyzed with FlowJo. Cells were sorted on a FACS ARIAII (BD Bioscience).

Real Time quantitative RT-PCR

Isolation of RNA was performed using the Qiagen AllPrep RNA Mini Kit or the Qiagen RNeasy Micro Kit, following the manufacturer's protocol, and the concentration was determined on a nanodrop. RT-PCR was performed with the DNA engine Opticon 2 (MJ research) or the StepOnePlus Real-Time PCR system (Fisher Scientific) using PerfecTa SYBR Green SuperMix (Quanta Bioscience). Gene levels were calculated using the ΔΔct method. Normalization was performed using HPRT or GAPDH.

Microarray analysis

RNA was isolated from healthy and PMF $CD41^+CD42^+$ megakaryocytes isolated by flow cytometry using the RNeasy Micro Kit following the manufacturer's protocol (Qiagen). Hybridizations were performed on the Illumina HT12v4 Expression BeadChip. Gene Set

Enrichment Analysis was used to identify pathways that were altered in PMF megakaryocytes as described (2).

Lentiviral vector construction and production

For GATA1 overexpression, the GATA1 cDNA was amplified by PCR and then inserted in the pRRL-EF1α-MCS-PGK-GFP vector (3). pGhU6 Scramble and pGhU6 shCEBPα were provided by Meritxell Alberich-Jorda and Dan Tenen (Harvard Medical School, Boston, MA). GFI1 overexpression was performed with the vector pHAGE2- EF1α-GFI1-IRES-GFP provided by Hanno Hock (Harvard Medical School and Massachusetts General Hospital, Boston, MA). The lentiviral vectors pLenti-shLuc, pLenti-shRPS14-G5, pLenti-shRPS14-G8, pLentiDestV5, pLentiUBC-RPS14 and pLentiCMV-RPS14 were provided by Benjamin Ebert (Harvard Medical School, Boston, MA). Lentiviral particles were produced with 293T cells and concentrated by ultracentrifugation for 2hrs at 20,000rpm. Viral stocks were stored at -80°C and concentrations of viral particles were normalized according to the p24 (HIV-1 capsid protein) content in supernatants.

Cell transduction

CD34$^+$ cells (10^5/mL) were stimulated for 24 hrs with THPO (100ng/mL), IL-3 (1,000U/mL), IL-6 (100ng/mL), SCF (250ng/mL) and FLT3-L (100ng/mL). Lentiviral particles were added at a concentration corresponding to 125ng of viral p24/100 μl for 12 hours followed by a second transduction, and then were cultured in the presence of THPO alone. SET2 cells were transduced in the same condition without addition of cytokines.

Western Blot analysis

The following antibodies were used for western blot assays: anti-RPS14 (A01; Abnova), anti-GATA1 (M20; Santa Cruz Biotechnology, Figure S3, S11), anti-GATA1 (3535, Cell Signaling, Figure 1A-D and S13), anti-GFI1 (D3G2, Cell Signaling), anti-GAPDH (0411; Santa Cruz Biotechnology) and anti-HSC70 (B-6; Santa Cruz Biotechnology).

Polysome profiling and fractionation

SET2 cells with RPS14 or control shRNAs were washed twice with PBS with 100μg/ml cycloheximide and then resuspended in 450 μl of hypotonic lysis buffer (5mM Tris, pH 7.5, 2.5mM MgCl2, 1.5mM KCl 100μg/ml cycloheximide, 2mM DTT, protease inhibitor and 1U/μl RNase inhibitor) and vortexed for 4 seconds. Then 25 μl of 10% Triton X 100 and 25μl of 10% Sodium deoxycholate was added and cells were vortexed again for 4 seconds and then centrifuged at 12000g for 5min at 4°C. The lysates were collected and snap frozen in liquid nitrogen. The cell lysate was layered on sucrose gradient (5 to 50%) to isolate ribosomal fractions, as previously described (4). Briefly, after 110min of centrifugation at 35000 rpm in a Beckman SW41-Ti rotor at 4C, the absorbance at 254 nm was measured continuously in an ISCO density gradient fractionator with the following settings: pump speed, 0.80 mL/min; fraction size, 10 drops/fraction; chart speed, 300 cm/h; sensitivity, 1; peak separator, off; noise filter, 0.5 sec. 5% sucrose solution was used to set the baseline in an UA-6 detector for all experiments.

Protein synthesis assay

In vitro analysis of protein synthesis rate was performed with a Protein Synthesis Assay Kit (Cayman Chemicals, USA) using 2×10^6 cells harvested after 6 days of culture in THPO to drive megakaryocyte differentiation. Briefly, cells were resuspended in 500 µl of culture media containing O-Propargyl-Puromycin (OP-Puro) and incubated for 1 hour at 37 °C in 5% CO_2 to allow labeling of translating peptides. Cells were fixed in 4% formaldehyde solution in TBS for 5 minutes and then washed in TBS containing 0.1% polysorbate 20. The FAM-azide staining solution for the azide-alkyne cycloaddition was prepared according to the kit instructions. Cells were incubated with the FAM-azide staining solution for 30 minutes in the dark at room temperature and then washed again. Cell surface staining for CD42 to identify megakaryocytes was performed as described above after the azide-alkyne cycloaddition reaction and samples analyzed by flow cytometry. All the experiments included single color controls, unstained controls, and protein synthesis inhibition of the cells by cycloheximide at a final concentration of 50 µg/ml for 30 minutes at 37 °C in 5% CO_2.

Mouse models of myelofibrosis

Mouse bone marrow cells were transduced with *MPL*W515L or *CALR* type 1 mutation and transplanted to irradiated C57Bl/6 recipient mice as described (5, 6). Spleens cells were collected and lysed for protein by standard methods.

Immunohistochemistry

Paraffin embedded bone marrow cells from *Jak2*V617F or control littermates, and spleen tissues collected from *MPL*W515L, *CALR* type 1 (generously provided by Ann Mullally,

Harvard Medical School, Boston, MA), or control transplanted mice were sectioned at 3µm and transferred to poly-L-lysine coated slides. Sections were deparaffinized in xylene and rehydrated in decreasing alcohol scale to water. Endogenous peroxidases were blocked with 3% hydrogen peroxide for 10 minutes and heat induced antigen retrieval was performed with target retrieval solution citrate buffer (DAKO, Agilent Technologies) in a water bath at 96 °C for 30 minutes. Slides were then blocked with background punisher solution (Biocare medical) for 10 minutes and incubated with GATA1 primary antibody (dilution 1:100, Cell signaling 3535) overnight at 4°C. The sections were then incubated with an HRP conjugated polymer (MACH4, Biocare medical) for 30 minutes and stained with diaminobenzidine (Betazoid DAB, Biocare medical). Counterstain was performed using Mayer's Hematoxylin. GATA1 negative megakaryocytes (GATA1^{neg}) were defined as megakaryocytes lacking evident DAB staining or showing a very faint brown staining for GATA1. Relative quantification was performed reporting the percentage of GATA1^{neg} megakaryocytes counted on randomly selected high power fields with a 40X objective.

Supplemental references

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